LRH-1/NR5a2
in regulation of the immune system

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2 ABSTRACT

The orphan nuclear receptor Liver receptor homolog-1 (LRH-1/NR5a2) is involved in the regulation of development, lipid metabolism and proliferation, and is predominantly expressed in epithelial tissues. However, its expression in immune cells and its function in immune regulation is currently poorly understood.

Here, LRH-1 expression in primary and secondary lymphatic tissues, as well as in mature CD4\(^+\) and CD8\(^+\) T cells, was examined. Based on \textit{in silico} analysis, potential LRH-1 binding sites within the promoter of CD95/Fas ligand (FasL), an apoptosis-inducing ligand and regulator of immune cell homeostasis and cytotoxicity, were identified. LRH-1 directly binds to its binding sites in the \textit{FASLG} promoter and thereby drives \textit{FASLG} transcription. Furthermore, mutations in the LRH-1 binding sites reduce \textit{FASLG} promoter activity. Pharmacological inhibition of LRH-1 decreased activation-induced FasL mRNA expression, as well as FasL-mediated activation-induced T cell apoptosis and cytotoxicity. Moreover, in a mouse model of Concanavalin A-induced and FasL-mediated hepatitis, pharmacological inhibition of LRH-1 resulted in decreased hepatic FasL expression and a significant reduction of associated liver damage. Also in cells of the innate immune system, i.e. macrophages, pharmacological LRH-1 inhibition illustrated a regulatory function on the toll-like receptor-induced expression of pro-inflammatory cytokines, such as TNF\(\alpha\), IL-6, and IL-1\(\beta\), and the respiratory capacity.

In summary, these data describe different regulatory roles of LRH-1 in T cells and macrophages, and reveal important insights into the potential of pharmacological intervention of LRH-1 in immunopathologies.
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3 ZUSAMMENFASSUNG

Der nukleäre Rezeptor LRH-1 (Liver Receptor Homolog-1 / NR5a2) ist ein wichtiger Transkriptionsfaktor in der Embryonalentwicklung, im Metabolismus der Leber und der Zellproliferation. LRH-1 wird neben der Leber hauptsächlich im epithelialen Gewebe exprimiert. Die Expression von LRH-1 in Immunzellen und dessen immunregulatorische Rolle ist bis heute kaum beschrieben.

In dieser Arbeit wurde die LRH-1 Exprimierung in primären und sekundären lymphatischen Geweben, als auch in reifen CD4⁺ und CD8⁺ T Zellen untersucht. Basierend auf in silico Analysen wurden mögliche LRH-1 Bindungsstellen innerhalb des Promotors von CD95/Fas Ligand (FasL) gefunden. FasL ist ein Apoptosis-induzierender Ligand, der wichtige regulatorischen Funktionen in der Immunzellhomeostase und Zytotoxizität hat. Tatsächlich konnte in dieser Arbeit gezeigt werden, dass LRH-1 an spezifische Bindungsstellen im FASLG Promoter bindet und die Aktivierung der FASLG Transkription induziert. Diese Daten unterstützend, reduzierten Mutationen der LRH-1 Bindungsstellen die FASLG Promoter Aktivität nach LRH-1 Überexpression. Zusätzlich resultierte eine pharmakologische Inhibierung von LRH-1 in vermindelter Aktivierungs-induzierten FasL mRNA Expression, in FasL-vermittelten T Zell Apoptosis und Zytotoxizität. In einem Mausmodell mit einer Concanavalin-A-induzierten Hepatitis wurde darüber hinaus eine Reduktion der FasL Expression und der FasL-vermittelten Gewebeschädigung mittels pharmakologischer LRH-1 Inhibierung erreicht. Nicht nur in T Zellen, auch in Makrophagen, welche zum angeborenen Immunsystem gehören, führte eine pharmakologische LRH-1 Inhibierung zu einer starken Reduktion der pro-inflammatorischen Zytokine TNFα, IL-6 und IL-1β, und inhibierte die respiratorische Kapazität der Zellen.

Zusammengefasst beschreiben diese Daten, dass LRH-1 eine unterschiedliche regulatorische Wirkungsweise in T Zellen und Makrophagen aufweist und identifiziert LRH-1 als mögliches therapeutisches Ziel für Immunopathologien.
## 4 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AF-1</td>
<td>activation function-1</td>
</tr>
<tr>
<td>AF-2</td>
<td>activation function-2</td>
</tr>
<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
</tr>
<tr>
<td>ALPS</td>
<td>autoimmune lymphoproliferative syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APR</td>
<td>acute phase response</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B cell lymphoma-2</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>cellular FLICE-like inhibitory protein</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cpd7</td>
<td>compound 7</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DED</td>
<td>death-effector domain</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signalling complex</td>
</tr>
<tr>
<td>DLPC</td>
<td>dilauroyl phosphatidylcholine</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FASLG</td>
<td>human Fas ligand gene</td>
</tr>
<tr>
<td>FTZ-F1</td>
<td>fushi tarazu factor 1</td>
</tr>
<tr>
<td>GalN</td>
<td>N-galactosamine</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>gld</td>
<td>generalized lymphoproliferative disease</td>
</tr>
<tr>
<td>GvHD</td>
<td>Graft-vs-host disease</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylases</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>hLRH-1</td>
<td>human LRH-1</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IECs</td>
<td>intestinal epithelial cells</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

kb  kilobase
kDa  kilodalton
LAT  linker for activation of T cells
LBD  ligand binding domain
LCMV  lymphocytic choriomeningitis virus
lpr  lymphoproliferative disorder
LPS  lipopolysaccharide
LRH-1  liver receptor homolog-1
M-CSF  macrophage colony-stimulating factor
MAPK  mitogen-activated protein kinase
mh  myc-his tagged
MHC  major histocompatibility complex
MOMP  mitochondrial outer membrane permeabilization
mRNA  messenger ribonucleic acid
MS  multiple sclerosis
mut  mutation
NF-κB  nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFAT  nuclear factor of activated T cells
NLS  nuclear localization sequence
NO  nitric oxide
NR  nuclear receptor
PCR  polymerase chain reaction
pH  potential of hydrogen
PIP2  phosphatidylinositol 4,5-bisphosphate
PLC  phospholipase C
PMA  phorbol 12-myristate 13-acetate
RT  room temperature
SF-1  steroidogenic factor 1
SHP  small heterodimer partner
SRC  steroid receptor coactivator
SUMO  small ubiquitin-related modifier
TCA  tricarboxylic acid
TCR  T cell receptor
TGF  transforming growth factor beta
Th  T helper
TLR  toll-like receptor
TNF  tumor necrosis factor
TRAIL  TNF-related apoptosis-inducing ligand
Treg  regulatory T cells
wt  wild type
5 INTRODUCTION

Nuclear receptors are universal regulators for specific gene expression and their dysfunction is linked to numerous severe pathologies such as cancer, infertility, obesity and diabetes (Gronemeyer et al., 2004). Liver receptor homologue 1 (LRH-1, NR5a2), a member of this family, is an orphan nuclear receptor with important effector functions in the regulation of embryogenesis, metabolism, and proliferation, mostly in tissues of endodermal origin (Fayard et al., 2004). Though no endogenous ligand has been identified so far, intensive research of this essential transcriptional regulator accumulates evidence for the existence of endogenous ligands. Similarly, identification of synthetic agonistic and antagonistic chemical compounds suggests LRH-1 as a pharmacological drug target. The analysis of LRH-1 expression in pancreatic tumors revealed LRH-1-positive immune cells infiltrating the tumor tissue (Benod et al., 2011). A recent study conducted in the group of Schoonjans and Coste described for the first time LRH-1 expression and function in macrophages, supporting a novel and so far, unrecognized role of LRH-1 in hematopoietic cells (Lefevre et al., 2015). Although these data open up new exciting perspectives, several questions await deeper understanding. For instance, in which T cell subsets is LRH-1 expressed? What is the physiological role of LRH-1 in the adaptive and innate immune system? Which are the target genes? And most interestingly, is LRH-1 a target for pharmacological intervention in LRH-1-dependent immunopathological diseases?

This introduction will summarize the main features of the family of nuclear receptors and it will highlight the structural components of LRH-1. Given the pivotal role of LRH-1 in (patho)physiological processes, there is high interest in unraveling its physiological ligands, modulators, and targets. The focus of this work will be on the role of LRH-1 in diverse immunological aspects, such as inflammatory conditions and regulated cell death via apoptosis, which is essential for the maintenance of immune homeostasis and immune effector functions. Detailed information about the extrinsic cell death inducer FasL will be discussed, including its transcriptional regulation and its role in immune physiology, as in this study LRH-1 was found to act as a transcriptional regulator of FasL.
5.1 Liver receptor homolog-1

5.1.1 The family of nuclear receptors

All cells in a living organisms contain the same genetic information. However, transcription factors make sure that the distinct cell types can exert their unique function by binding to specific DNA sequences and regulating their expression. In this way, gene expression is regulated by stabilizing or blocking RNA polymerase binding, and by recruiting coactivators or corepressors (Rosenfeld et al., 2006). In addition, transcription factors are extensively studied to understand the molecular mechanisms of a variety of human diseases, including cancer, autoimmunity, neurological disorders, diabetes, cardiovascular disease, and obesity. This research opens up new strategies in order to develop drugs for the successful treatment of these disorders (Lee and Young, 2013).

One of the largest family of transcription factors are the nuclear receptors (NR), with 48 members identified by sequencing of the human genome (Germain et al., 2006). The NR superfamily involves an evolutionary related but diverse set of ligand-regulated transcription factors that are very important for intercellular signalling. According to their sequence alignment and phylogenetic tree construction, NR are classified into seven subfamilies (NR0–NR6) (Nuclear Receptors Nomenclature, 1999). NR exhibit a conserved modular structure consisting of five to six domains with two highly conserved domains, the DNA-binding domain (DBD or C domain) and the ligand-binding domain (LBD or E domain). The N-terminal A/B domain often contains a ligand-independent transcriptional activation function-1 (AF-1), the D domain serves as a hinge between the DBD and the LBD allowing rotation of the DBD and the C-terminal F domain is highly variable in sequence (Burris et al., 2013). The different domains of NR as well as the mechanism of action are schematically depicted in Figure 1.

Figure 1: NR structure and mechanism of action
A) NR domains with an N-terminal AF-1 region (A/B region), followed by zinc-finger DBD (C region), a hinge domain (D region), an LBD containing the AF-2 region (E region), and some receptors have a C-terminal F domain. B) Mechanistically, NR are regulated by small molecule ligands, which generally stabilize the receptor into a conformation suitable to bind co-regulator proteins. Ligands can also modulate receptor post-translational modifications. Ultimately, these events have an impact on the expression of receptor-specific target genes by modulating co-regulator recruitment at specific DNA-response element sites in the target gene promoter (Burris et al., 2013).

### 5.1.2 Liver receptor homolog-1 structural components

The liver receptor homolog-1 (LRH-1) belongs to the family of NR and is termed NR5A2 (nuclear receptor subfamily 5, group A, member 2). Initially, LRH-1 was identified due to its homology with the firstly cloned Drosophila Fushi tarazu factor 1 (Ftz-F1; NR5A3) (Ueda et al., 1990, Ikeda et al., 1993). Afterwards, LRH-1 orthologs have been found in various species including humans (Galarneau et al., 1996, Boerboom et al., 2000, Kudo and Sutou, 1997, Liu et al., 1997). Several groups were able to independently isolate LRH-1 and therefore different names were provided: Fushi tarazu factor 1 (Ikeda et al., 1993), α-fetoprotein transcription factor (FTF) (Galarneau et al., 1998), pancreas homolog receptor 1 (PHR-1) (Becker-Andre et al., 1993), human B1-binding factor (hB1F) (Li et al., 1998) and Cyp7A promoter-binding factor (CPF) (Nitta et al., 1999). LRH-1 shows high sequence homology with steroidogenic factor-1 (SF-1, NR5A1) (Fayard et al., 2004) and shares an identical DNA consensus sequence (Ueda et al., 1992).

The human gene encoding LRH-1 was mapped to chromosome 1 q32.11 (Galarneau et al., 1998) and the determination of the gene structure revealed eight exons spanning a 150 kb region (Zhang et al., 2001). Due to alternative splicing, at least three human LRH-1 isoforms exist (Fayard et al., 2004). The most abundant isoform is simply termed hLRH-1 (transcript variant 2, NM_003822.4, 4956 bp) and it does differ from the slightly larger hLRH-1v1 (transcript variant 1, NM_205860.2, 5094 bp) due to a deletion in the A/B domain caused by the splicing of exon 2. Both isoforms are identical in their DNA binding properties and transactivation capacities. The transcriptionally inactive isoform, hLRH-1v2 (transcript variant 3, NM_001276464.1, 4787 bp), results from an additional deletion in exon 5, which codes for the hinge region and LBD domains of LRH-1 (Nitta et al., 1999).

The N-terminal region A/B is the least conserved domain and shows a high variability in sequence and length. The following DBD or C domain is the most conserved domain of NR, which enables the recognition and binding of specific DNA sites. It is a highly structured domain composed of two α-helices, which are stabilized by two zinc-finger motifs (Kumar and
Thompson, 1999, Bain et al., 2007). One major structural difference of LRH-1 to other NR is the presence of the Ftz-F1 box located in the C-terminus of the DBD, which together with the zinc finger motif, allows it to bind to the specific target site with high-affinity. This highly conserved stretch of 26 amino acids with the Ftz-F1-consensus binding sequence 5’-YCA AGG YCR-3’ (where Y is any pyrimidine and R is any purine) is the hallmark feature of the NR5A subfamily and therefore also termed Ftz-F1 (Ueda et al., 1992). LRH-1 has two functional nuclear localization signals (NLS) mediating its nuclear import (Yang et al., 2011). NLS 1 overlaps with the second zinc finger in the DBD while NLS 2 is located in the Ftz-F1 box. Subsequently, mutation of both NLS 1 and NLS 2 results in the cytoplasmic accumulation of LRH-1. The hinge region or D domain is the variable and flexible link between the DBD and the C-terminal LBD allowing different conformations after ligand binding. A characteristic element of NR is the hydrophobic, highly structured and multi-functional LBD or E domain. It acts as a molecular switch by translating ligand binding into conformational changes that convert the receptor in an activator or repressor of transcription. The LBD of LRH-1 is organized as a compact structure consisting of 12 α-helices which shape a highly variable ligand binding pocket, allowing the potential binding of many molecules (Bain et al., 2007) (Figure 2).

Figure 2: LRH-1 LBD structure

(A and B) Ribbon representation of LRH-1 LBD structure. The view on B) is rotated ~90° relative to that shown in A) (Sablin et al., 2003).

Especially important in this context is the stabilization of helix 12 against the LBD core. It facilitates the interaction with the short conserved NR box or LXXLL motif (where L is leucine and X is any amino acid) found in NR coactivator proteins (Renaud et al., 1995, Heery et al., 1997). However, LRH-1 exhibits constitutive activity, which can be explained by crystal structure analysis of the LRH-1 LBD. In contrast to other LBDs, LRH-1 revealed an extended helix 2 supporting the constitutive stabilization of helix 12 against the LBD core as seen in Figure 2 (Sablin et al., 2003). Additional to its specific ligand binding function, the LBD harbors
the ligand-dependent activation function 2 (AF-2) domain, which influences recruitment of coactivators and corepressors (Nolte et al., 1998, Tsai and O'Malley, 1994, Moras and Gronemeyer, 1998).

5.2 LRH-1 regulation

5.2.1 Orphan NR

Most NR are regulated by specific small hydrophobic ligands, such as steroid hormones, thyroid hormones, certain vitamins and fatty acids (Mangelsdorf et al., 1995, Sever and Glass, 2013). However, for the NR5A subfamily members no obvious endogenous ligand has been found and they are therefore termed orphan NR. Interestingly, still half of the total number of all NR (24 of a total of 48 different genes in human) are considered as orphan NR (Benoit et al., 2006). Other receptors previously classified as orphan became “adopted” as soon as their endogenous ligands were identified. This is the case for the peroxisome proliferator-activated receptor (PPAR) (Berger and Moller, 2002), liver X receptor (LXR) (Zelcer and Tontonoz, 2006) and pregnane X receptor (PXR) (Orans et al., 2005). Given the fact that adopted orphans have large binding pockets allowing the low-affinity binding of diverse metabolites, it is speculated that this may represent a special hallmark of these receptors (Germain et al., 2006). Among the NR, LRH-1 still remains orphan. Early crystallographic x-ray structures showed that both the murine and human LRH-1 ligand binding domains (LBDs) contain a large hydrophobic ligand binding cavity (800–1200 Å) that can easily accommodate ligands (Li et al., 2003).

5.2.2 Phospholipids as LRH-1 activators

In 2005, three research groups resolved the crystal structures of human SF-1 and human LRH-1 and revealed that phospholipid molecules can bind to both proteins (Wang et al., 2005, Ortlund et al., 2005, Krylova et al., 2005). Mass spectrometry and in vitro experiments showed that mutations of phospholipid-binding residues reduced the binding of phospholipids and the transcriptional activity of the receptor (Wang et al., 2005, Ortlund et al., 2005). A comparison of LBD from murine and human SF-1 and LRH-1 showed the presence of phospholipids, but with a much lower concentration in murine LRH-1, where only 10% was bound by ligand (Krylova et al., 2005). Furthermore, structural and biochemical data for human LRH-1 revealed binding of the second messenger, phosphatidyl inositol, and the importance of ligand-binding to reach maximal LRH-1 activity (Krylova et al., 2005). Interestingly, these studies demonstrate species-related differences due to sequence variations among LRH-1 orthologs, which may
INTRODUCTION

affect phospholipid binding and regulation. However, both human and mouse LRH-1 responded to a newly discovered medium chain phospholipid agonist; dilauroyl phosphatidylcholine or DLPC (Musille et al., 2013). This unusual phosphatidylcholine species with two saturated 12-carbon fatty acid acyl side chains was shown to act as LRH-1 agonistic ligand (Lee et al., 2011). Its binding resulted in a more dynamic DLPC-ligand binding domain complex, which was suggested to affect the ability of LRH-1 to interact with co-regulators (Musille et al., 2012). A recent study explained how the absence of phospholipids shows a bigger conformational space for the binding of co-repressors such as small heterodimer partner (SHP), while the binding of DLPC favors the interaction of LRH-1 with the transcriptional co-activator transcriptional intermediary factor 2 (TIF2) due to allosteric changes (Figure 3) (Musille et al., 2016).

Figure 3: Allosteric paths in LRH-1 binding pocket contribute to co-regulator selectivity

Schematic loop view of LRH-1 (yellow), TIF2 (green) and SHP (red) in the A) apo-LRH-1·TIF2, B) LRH-1·DLPC·TIF2, C) apo-LRH-1·SHP, D) LRH-1·DLPC·SHP complexes (Musille et al., 2016).

Taken together, these structural and functional studies suggest that LRH-1 can be active in the absence of a ligand, but its binding to a specific phospholipid can further enhance its transcriptional activity by recruiting co-activators.
5.2.3 Endogenous LRH-1 modulators

Co-activators are recruited to DNA-binding transcription factors thereby increasing the transcriptional activity. Several of these are described to modulate also LRH-1 activity. For instance, binding of PGC-1α (peroxisome proliferator-activated receptor gamma co-activator 1α) to the AF-2 domain of LRH-1 stimulates target gene expression in hepatoma cells, breast adipocytes and ovarian granulosa cells (Shin and Osborne, 2008, Safi et al., 2005, Yazawa et al., 2010). Another mechanism of activation was shown by steroid receptor coactivators (SRC), mainly SRC-1, SRC-2 and SRC-3, which possess a histone acetyltransferase activity and therefore increase the transcriptional activity of LRH-1 after direct interaction with the LBD (Xu et al., 2004). LRH-1 directly interacts with β-catenin, which leads to a reciprocal activation of both proteins and an increased expression of their target genes cyclin D1 and E1 (Yumoto et al., 2012, Botrugno et al., 2004). The multiprotein bridging factor (MBF-1) was shown to link nuclear receptors that are implicated in lipid metabolism including LRH-1 to the transcriptional machinery through its interaction with the transcription factor IID complex (Brendel et al., 2002). Moreover, also other NR are known to interact with LRH-1. The combined activity of LRH-1 with the NR hepatic farnesoid X receptor (FXR) or liver X receptor (LXR) enhances the expression of their target genes (Chong et al., 2010, Luo et al., 2001, Matsukuma et al., 2007, Back et al., 2013).

Co-repressors, which in contrast to activators inhibit LRH-1 activity, have also been described to regulate LRH-1 activity. The atypical and closely related NR SHP (NR0B2) and DAX-1 (DSS-AHC critical region on the X chromosome, gene 1, NR0B1) both lack a DBD. SHP interacts with several nuclear hormone receptors, such as the thyroid hormone receptor, the retinoic acid receptor, the peroxisome proliferator-activated receptor-α, the estrogen receptor-α and the retinoid-X-receptor (RXR). Of interest, it binds to the AF-2 domain of LRH-1 and reduces its transcriptional activity (Ortlund et al., 2005, Lee and Moore, 2002). Notably, LRH-1 itself induces SHP expression and therefore can influence its own inhibition, representing a negative feedback loop (Goodwin et al., 2000, Lu et al., 2000). Dax-1 acts a repressor, which inhibits SF-1 and estrogen receptor and was further described to represses LRH-1 activity by binding to its LBD (Suzuki et al., 2003). Prospero homeobox protein 1 (Prox-1) is an additional potent inhibitor, which interacts with the DBD and the LBD of LRH-1, and represses LRH-1 target genes involved in reverse cholesterol transport (Qin et al., 2004, Stein and Schoonjans, 2015, Stein et al., 2014). LRH-1 targeted genes can furthermore be decreased by the interaction of LRH-1 with the nuclear receptor co-repressor 1 (NCoR1) and the silencing mediator for retinoid or thyroid-hormone receptors (SMRT) co-repressor complexes, which are associated
with histone deacetylases and lead to gene silencing (Fayard et al., 2004, Venteclef et al., 2010).

In addition to binding of co-activators or co-repressors, LRH-1 is subject to different posttranslational modifications, which are able to modulate its activity, subcellular localization and protein turnover. Like most NR, the NR5A subfamily presents a distinct large hinge domain that can be modified by posttranslational modifications. Stimulation of cells by phorbol 12-myristate 13-acetate (PMA) has been reported to trigger LRH-1 activation. The transactivation of LRH-1 was induced by phosphorylation of the serine residues S238 and S243 located within the LRH-1 hinge region by the MAP kinase ERK 1/2 (extracellular signal–regulated kinase 1/2) (Desclozeaux et al., 2002, Lee et al., 2006). Furthermore, the covalent attachment of small ubiquitin-like modifier (SUMO) proteins, termed SUMOylation and also occurring in the hinge region of LRH-1, leads to a change in the intranuclear localization of the transcription factor LRH-1 away from active chromatin to transcriptionally inactive nuclear bodies (Venteclef et al., 2010, Chalkiadaki and Talianidis, 2005, Yang et al., 2009). In addition, SUMOylation of LRH-1 stabilizes the interaction with co-repressors, such as Prox-1 and NCoR1 (Stein et al., 2014, Venteclef et al., 2010).

5.2.4 Pharmacological LRH-1 modulators

As can be inferred from the state-of-art research described until now, there is a diverse collection of endogenous regulators, which can positively or negatively modulate LRH-1 activity. NR in general are attractive targets for drug discovery, and interestingly they represent 13% of all current FDA-approved drugs (Overington et al., 2006). Pharmacological modulators could represent useful tools to gain a better understanding of the LRH-1 biology and could be potential therapeutic agents in LRH-1-associated pathological conditions, such as cancer or metabolic diseases (Mamrosh et al., 2014, Benod et al., 2013), which will be described in detail in paragraph 1.3.3 of this work.

There are several synthetic ligands that can increase the expression of LRH-1 target genes, such as the already discussed DLPC. GSK8470, a substituted cis-bicyclo[3.3.0]oct-2-ene, was identified as a high-affinity ligand for LRH-1 in a high throughput screening assay using coactivator TIF2 recruitment as readout (Whitby et al., 2006). With the aim to develop new compounds that were more stable and easily to use, Whitby et al. after extensive modification of GSK8470, established the compound RJW100 (Whitby et al., 2011). This new compound was successfully tested in cellular assays and in in vivo settings (Zhang et al., 2011, Mamrosh
et al., 2014). The development of pharmaceutical compounds continues. In 2016, de Jesus Cortez and coworkers identified new compounds with a new approach based on the compounds ability to form a disulfide bond with a naturally occurring cysteine residue in the ligand binding pocket of LRH-1, which they named PME8 and PME9 (de Jesus Cortez et al., 2016). Both compounds are able to bind with remarkably high affinity to the LBD of human LRH-1 in a reversibly manner, thereby increasing its activity. In comparison with the existing LRH-1 synthetic agonist RJW100, PME8 showed comparable induction of the LRH-1-dependent target gene CYP24A1. Of interest, very recently publication by Mays et al. 2016 revealed by crystal structure analysis the complexity of the interactions of small molecule agonists with LRH-1. They demonstrated that unlike phospholipid LRH-1 ligands, GSK8470 and RJW100 bind deep into the LBD pocket and do not interact with residues near the mouth of the binding pocket like phospholipids do (Mays et al., 2016).

As discussed above, all available crystal structures of the LRH-1 LBD represent the receptor in its active state and the identified ligands are stabilizing its active conformation. By virtual screening and molecular modeling novel antagonists of LRH-1 were identified. The hit compounds were synthesized and biologically assayed. With such an approach, preliminary results suggested analogues, which might generate an inactive protein conformation through binding and thus antagonize this NR (Rey et al., 2012). Similarly, Benod et al. generated a model of a transcriptionally inactive state of the LRH-1 receptor LBD, which is based on the structural similarity between LRH-1 and estrogen receptor α (ERα) LBD (Figure 4), and hypothesized that these receptors could be antagonized in a comparable manner (Benod et al., 2013). After verification of the top-ranked molecules by direct binding and transcriptional assays, they found a specific small molecule, named 3d2, which stabilizes the inactive conformational state of the LBD of LRH-1, and inhibits LRH-1-targeted gene expression in vitro and in vivo (Nissim et al., 2016).
5.3 LRH-1 physiology

5.3.1 LRH-1 in embryonic development and female reproduction

LRH-1 transcriptional activity fulfills important regulatory functions in early differentiation and embryogenesis (Rausa et al., 1999). Mouse embryonic stem cells express LRH-1 mRNA abundantly (Galarneau et al., 1996) and early enterohepatic development target genes are activated by LRH-1 (Pare et al., 2001). Recently, the role of LRH-1 in the regulation of liver and...
pancreas embryonic development was defined in zebrafish using the pharmacological antagonist 3d2 (Nissim et al., 2016). The importance of LRH-1 in development is underlined by the fact that genomic deletion of LRH-1 results in embryonic lethality (Pare et al., 2004). Further, LRH-1 is a critical factor in maintaining the undifferentiated state of embryonic stem cells by promoting gene expression of the pluripotency and self-renewal transcription factors octamer-binding transcription factor 4 (Oct-4) and Nanog (Gu et al., 2005a, Wagner et al., 2010).

LRH-1 is highly expressed in the ovaries and was suggested to play a role in the regulation of estrogen biosynthesis (Sirianni et al., 2002, Hinshelwood et al., 2003). The ovaries synthesize steroid hormones from cholesterol, which are delivered to steroidogenic tissues by the LRH-1-regulated target scavenger receptor class B type I (SR-BI) (Schoonjans et al., 2002). LRH-1 influences critical steps in steroid synthesis (Labelle-Dumais et al., 2007) as well as in ovulation (Duggavathi et al., 2008), and is crucial during pregnancy (Zhang et al., 2013). In addition, LRH-1 reveals a prominent role in the hypothalamus in regulating the female reproductive axis (Atkin et al., 2013).

Based on these facts, LRH-1 can be described as a master regulator in the network of transcription factors and signalling molecules for stemness, embryogenesis, and reproduction. In addition to the role of LRH-1 in the ovaries, it is also expressed in other endodermal-derived organs, such as the liver, pancreas, and intestine and presents a relevant role in different metabolic processes (Bookout et al., 2006).

5.3.2 LRH-1 in metabolic processes

LRH-1 targets key molecules of metabolic processes, such as cholesterol homeostasis. Cholesterol is of great importance for a functional organism. It represents an essential structural component of the cell membrane, functions as a precursor of bile acids, what impacts the intestinal absorption of fat molecules and fat-soluble vitamins, and is the starting molecule for steroid hormone synthesis (see paragraph 1.3.1.). However, accumulation of cholesterol in macrophages and immune cells is an important hallmark of chronic metabolic inflammation disorders, like atherosclerosis and obesity (Tall and Yvan-Charvet, 2015). Therefore, the cholesterol homeostasis has to be tightly regulated.

The LRH-1 target gene SR-BI mediates selective cellular cholesterol uptake from high-density lipoproteins (HDLs) in the liver (Schoonjans et al., 2002). LRH-1 functions as positive
transcription factor for the ATP-binding cassette (ABC) transporters ABCG5 and ABCG8, which are both involved in sterol and bile acid secretion from the liver and the intestine (Freeman et al., 2004). Other cholesterol homeostasis regulating proteins such as apolipoprotein A1 (Delerive et al., 2004) and cholesteryl ester transfer protein-1 (Luo et al., 2001) are also transcriptionally regulated by LRH-1. As already stated, bile acid synthesis also depends on cholesterol. Amongst the first LRH-1 target genes identified are the cytochrome P450 (CYP) family members, CYP7A1 and CYP8B1. CYP7A1, encoding for 7α-hydroxylase, is the first rate-limiting enzyme in the metabolism of cholesterol to bile acid and is expressed exclusively in the liver (Nitta et al., 1999, Goodwin et al., 2000). Downstream in this enzymatic cascade follows sterol 12α-hydroxylase which is encoded by CYP8B1 (del Castillo-Olivares and Gil, 2000). In a liver pathological scenario, the cytokine tumor necrosis factor α (TNFα) was shown to up-regulate LRH-1 expression and to induce the expression of a hepatocellular ABC transport protein, the multidrug resistance-associated protein 3 (MRP3), which plays an important role in protecting hepatocytes by excreting toxic organic anion conjugates, including bile salts and therefore represents a typical hepatoprotective response in pathologic liver conditions (Bohan et al., 2003). In the intestine, LRH-1 further influences the reclamation of bile salts mediated by apical sodium-dependent bile acid transporter (ASBT) (Chen et al., 2003). Besides in the liver and intestine, also in the pancreas important LRH-1-regulated enzymes are present regulating lipid metabolism. For example, the pancreatic carboxyl ester lipase catalyzes the hydrolysis of cholesteryl esters in the intestine and contributes to lipoprotein assembly (Fayard et al., 2003). Holmstrom et al. showed a general role of LRH-1 on pancreatic target genes (Holmstrom et al., 2011). They found in a pancreatic-restricted Lrh-1 knockdown in adult mice reduced concentrations of several lipases and proteases in pancreatic fluid and impaired pancreatic fluid secretion.

Beside the established role of LRH-1 as a regulator of cholesterol and bile acid homeostasis, this NR has key functions in the carbohydrate metabolism. Liver-specific Lrh-1 knockout mice display reduced level of the hepatic glucose sensor glucokinase and consequently reduced glycogen synthase fluxes (Oosterveer et al., 2012). Given the fact that the phospholipid DLPC induces LRH-1 transcriptional activity, this molecule was tested in obese and diabetic mouse models, and impressively improved glucose tolerance and insulin resistance without affecting the body weight in diet-induced or genetically obese and diabetic mice (Lee et al., 2011). Testing a genetic model of heterozygous Lrh-1 mice revealed a mild but significant increase in body weight when exposed to a high-fat diet, but conversely did not exhibit changes in glucose or insulin tolerance (Hattori et al., 2014).
In summary, LRH-1 plays a key role in metabolic processes, which can influence not only liver physiology but also could have profound implications in tumorigenesis. Recently, a microarray analysis of liver-specific Lrh-1 knockout mice revealed a striking reduction of enzymes involved in glutamine metabolism and directly regulates the expression of mitochondrial glutaminase 2 (GLS2) (Xu et al., 2016). Cancer cells usually switch from oxidative metabolism to a highly glycolytic metabolic status, which relies on glutamine (Vander Heiden et al., 2009). LRH-1 was shown to coordinate this glutamine-induced metabolism and signaling in the liver and to promote tumorigenesis.

5.3.3 LRH-1 in pathological conditions

Beside its metabolic role, there is increasing evidence that LRH-1 influences cancer development in different tissues. Botrugno et al. discovered that the retroviral expression of LRH-1 promotes cell proliferation (Botrugno et al., 2004). Whereas β-catenin co-activates LRH-1 on the cyclin E1 promoter, LRH-1 acts as a potent tissue-restricted coactivator of β-catenin on the cyclin D1 promoter. Furthermore, they found a correlation of LRH-1 expression with cell proliferation in the intestinal crypts and suggested a role of LRH-1 in intestinal cell renewal. Of interest, β-catenin is one of the key molecules in the development of colon cancer and indeed it was shown to also contribute to intestinal tumor formation (Schoonjans et al., 2005). In pancreatic cancer, LRH-1 controls MYC gene expression and promotes cell proliferation (Benod et al., 2011). Based on its implication on tumorigenesis, pharmacological antagonists of LRH-1 were tested in vitro in human pancreatic, breast and colon cancer cell lines, showing a promising inhibition of proliferation (Benod et al., 2013).

Not just in cancer, but also in other pathological scenarios LRH-1 was described as important regulator. Based on the association of type 2 diabetes and fatty liver disease with the accumulation of misfolded proteins, resulting in chronic endoplasmatic reticulum (ER) stress, and on the fact that these metabolic diseases can be attenuated by activation of LRH-1 (Ozcan et al., 2006, Lee et al., 2011), Mamrosh and coworkers discovered that LRH-1 can relieve ER stress (Mamrosh et al., 2014). Interestingly, they showed that hepatocyte treatment with the LRH-1 agonist RJW100 resolved high levels of ER stress, suggesting a new target for drug treatment of metabolic diseases (Mamrosh et al., 2014).
5.3.4 LRH-1 in inflammatory conditions

Extensive studies of Venteclef et al. provided more evidences for the important role of LRH-1 in pathological scenarios and suggested pharmacological strategies for the treatment of inflammatory diseases (Venteclef et al., 2006, Venteclef and Delerive, 2007, Venteclef et al., 2010). They report LRH-1 as negative regulator of the hepatic acute-phase response (APR), which is a complex systemic early defense system activated by trauma, infection or inflammation. A major component of APR is a cytokine-mediated altered hepatic synthesis of proteins involved in coagulation, lipid metabolism and the complement system activation (Cray et al., 2009). Anti-inflammatory properties of LRH-1 were shown since its synthetic agonist GR8470 significantly inhibited hepatic APR gene expression. Interestingly, this was induced by SUMOylation-dependent recruitment of LRH-1 to hepatic APR promoters (Venteclef et al., 2010, Venteclef et al., 2006). Besides trans-repressing acute phase proteins, LRH-1 mediated the expression of interleukin-1 (IL-1) receptor antagonist, an inhibitor of the pro-inflammatory IL-1 signalling (Venteclef and Delerive, 2007). Therefore, LRH-1 has a dual anti-inflammatory role in APR in the liver, which can be pharmacologically regulated by LRH-1 agonists, such as GR8470.

Moreover, LRH-1 plays a crucial role in the regulation of inflammation and immune responses in the intestinal mucosa. Besides its contribution to the intestinal epithelial barrier integrity by regulating the stem cell proliferation, LRH-1 has also been shown to indirectly regulate inflammatory processes via the synthesis of immunoregulatory glucocorticoids (GC) in the intestinal crypts, as extensively reviewed by Kostadinova et al. (Kostadinova et al., 2014). Upon psychological and immunological stress, the adrenal glands have long been considered as the major and only source of GCs. However, extra-adrenal GC synthesis was also discovered in the gut, which contributes to the maintenance of local immune homeostasis and the regulation of inflammatory processes. The murine intestinal crypts were shown to express steroidogenic enzymes and to release the GC corticosterone in response to T cell activation (Cima et al., 2004). In a parallel study, LRH-1 has been reported to be expressed in intestinal crypt cells (Botrugno et al., 2004) and to promote the local GC synthesis in vitro and in vivo (Mueller et al., 2006). Supporting this observation, mice lacking LRH-1 expression in the intestinal epithelium showed a reduced T cell-mediated GC synthesis and resulted in the development of intestinal inflammation (Coste et al., 2007).

TNFα was identified as an important sensor of immunological stress as well as an inducer of intestinal GC synthesis and the therapeutic administration of TNF suppressed the pathogenesis of intestinal inflammation (Noti et al., 2010a). Further research revealed that
direct stimulation of macrophages by lipopolysaccharides (LPS) induced the GC production in a TNFα-mediated manner, however, this specific activation of the GC pathway seemed to be independent of LRH-1 (Noti et al., 2010b). Surprisingly, high levels of pro-inflammatory factors were shown to correlate with a decreased LRH-1 gene expression in intestinal tumors, which is in line with the report of an inverse correlation of inflammation markers with LRH-1 in patients suffering from inflammatory bowel disease (Schoonjans et al., 2005, Coste et al., 2007). Whether the damage of intestinal crypts or specific negative regulators during the inflammatory stage of the disease lead to this inverse correlation is at present not clear.

When discussing the role of LRH-1 in inflammatory scenarios, it is interesting to note that the first “accidental” description of LRH-1 expression in hematopoietic cells was reported while analyzing LRH-1 expression in pancreatic tumors, revealing an LRH-1-positive staining of immune cells infiltrating the tumor tissue (Benod et al., 2011). More recently, LRH-1 expression was reported in macrophages, where the NR contributed to the anti-inflammatory response of alternatively activated macrophages (Lefevre et al., 2015). Pro-inflammatory challenges like LPS or interferon gamma (INFγ) lead to a reduced LRH-1 expression, while IL-13 alternatively induced LRH-1 expression in macrophages.

Although these data open up new exciting perspectives of the role of LRH-1 in the regulation of inflammation, a deeper understanding is needed regarding how LRH-1 directly impacts immune cells regulation. Since several of the above described small molecules are able to act as specific LRH-1 antagonists or agonists, one could speculate that LRH-1 is also a target for pharmacological intervention in immunopathologies. In order to gain a better understanding of the complex interplay of different immune cells and their regulation, the next chapter will describe the different defense lines of the immune system with their specialized cell types and their physiological characteristics during health a disease.

5.4 Immune system

5.4.1 General context

The immune system is a complex host-defense system comprising different lines of protection against pathogens (Janeway, 2001). It hereby strongly depends on the ability to distinguish between self and non-self molecules. In the immunology field, the class of non-self molecules is called antigens (short for antibody generators) and these molecules are defined as substances, which bind specific immune receptors and cause an immune response. The first
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The line of defense is formed by the skin and the mucosa and is composed of physical, chemical and biological barrier mechanisms. If pathogens disrupt this barrier, the so-called innate immune system provides an immediate, but non-specific protection. In addition, the innate response activates a very specific protection of the body through the adaptive immune system for further support. After the recognition and elimination of the pathogen, the immune system establishes an immunological memory in order to respond more rapidly and effectively to pathogens that have been encountered previously. The central lymphoid tissues are the bone marrow and thymus, since here immune cells develop and mature. The peripheral lymphoid organs including the lymph nodes, the spleen and mucosal lymphoid tissues are found in the gut, nasal, respiratory and urogenital tract and contain mature naïve lymphocytes. In these sites, lymphocytes are getting activated by antigens, which are presented by specialized cells, called antigen-presenting cells (APC). Dendritic cells, macrophages and B cells are regarded as the classical APC of the immune system (Kambayashi and Laufer, 2014). The focus of the next paragraph will be on the interesting role of macrophages in the innate immune system.

5.4.2 Macrophages

The innate immune system as the first cellular line of defense includes phagocytic cells, such as macrophages, neutrophils, and dendritic cells, as well as natural killer cells and mast cells (Janeway, 2001). Macrophages represent a multifunctional heterogeneous cell type, which are present in most tissues of vertebrates (Sasmono et al., 2003). In the bone marrow, the common myeloid precursor cell gives rise to monocyte-progenitors, which differentiate into monocytes. Subsequently, monocytes exit from the bone marrow into the blood and, after recruitment to the peripheral tissues, they further differentiate into macrophages (Ginhoux and Jung, 2014). The differentiation of macrophages from bone marrow progenitors to mature functional cells is controlled by a lineage-specific growth factor, the macrophage colony-stimulating factor (M-CSF) (Stanley et al., 1997). Interestingly, monocytes do not exclusively contribute to the tissue macrophage populations. In most murine tissue, macrophages are derived from embryonic precursors and persist in adult tissue due to self-renewal (Ginhoux and Jung, 2014).

Macrophages were first characterized by Eli Metchnikoff who described the role of phagocytes in immunity against bacteria, fungi and viruses (Tauber, 2003). Beside their phagocytic activity, macrophages are essential for tissue development and homeostasis. They represent an important source of growth factors, are able to sense tissue damage and to orchestrate tissue...
repair responses (Lavin and Merad, 2013). The tissue-resident macrophages are a very heterogeneous population due to adaptation to their tissue environment (Okabe and Medzhitov, 2016). Therefore, their effector functions are ranging from bone tissue resorption by osteoclasts, production of pulmonary surfactant by lung alveolar macrophages, synaptic pruning of microglia or the recycling of red blood cells in the red pulp of the spleen. Such transitions need the induction of appropriate transcriptional programs, which are initiated by chemokines, cytokines, growth factor or microorganism-associated molecular pattern (Italiani and Boraschi, 2014).

Depending on the released cytokine, macrophages can either stimulate the immune system or play an anti-inflammatory role by decreasing immune reactions. The M1 and M2 nomenclature of macrophages mimics the T helper 1 (Th1) and Th2 responses of lymphocytes (Mills et al., 2000). Inflammatory stimuli such as LPS, IFNγ or TNFα induce a pro-inflammatory phenotype of classical activated M1 macrophages, which produce TNFα, IL-1, IL-6 and IL-12 (Martinez and Gordon, 2014). Pathogen-derived factors, such as LPS, are known as pathogen-associated molecular patterns (PAMP) and bind to pathogen-recognition receptors (PRR) on macrophages, e.g. toll-like receptors (TLR), triggering an adequate innate immune response. In more detail, the inflammatory response to LPS is mediated mainly by TLR4 which builds a complex with the LPS binding protein, CD14 (cluster of differentiation) and MD-2 (Lu et al., 2008). After activation of the TLR4, the cytoplasmatic domains recruit the adaptor molecules myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (Trif) and trigger subsequently the activation of mitogen-activated protein kinases (MAPK) including c-Jun N-terminal kinase (JNK), p38 and ERK 1/2 mediating the activation of activator protein 1 (AP-1). In addition, the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is activated too. In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by the inhibitor of κB (IκB). TLR4 stimulation induces the phosphorylation of the IκB kinase (IKK) protein complex, which in turn phosphorylates the IκB proteins. The phosphorylation of IκB leads to its ubiquitination and degradation followed by NF-κB release to the nucleus and activation (Kawai and Akira, 2006). The transcriptional regulators, AP-1 and NF-κB, activated by the LPS signaling pathway are essential for the induction of the pro-inflammatory cytokine transcription.

The pro-inflammatory role of macrophages has been implicated in various diseases. Especially macrophage-derived cytokines, such as TNFα, are implicated in rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, atherosclerosis and sepsis (Parameswaran and
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Immune system suppression of these diseases is clinically achieved by FDA-approved TNFα blocking agents, such as the monoclonal antibodies infliximab, adalimumab, certolizumab pegol and golimumab, or the circulating receptor fusion protein etanercept (FDA, 2015). Nevertheless, one-third of the patients treated with anti-TNFα drugs do not respond (Vincent et al., 2013). Therefore, further investigations of the pro-inflammatory signalling process in macrophages are necessary to find other targets for successful pharmacological investigation.

So far macrophages were described with their stimulating role of the immune system. However, macrophages have also an important anti-inflammatory role by decreasing immune reactions depending on the release of cytokines. The phenotypic switch of M2 macrophages is induced upon IL-4, IL-13 or IL-10 stimulation, and leads to high levels of IL-10 and transforming growth factor β (TGFβ), which in turn induces lymphocytes proliferation and tissue repair (Martinez and Gordon, 2014). Interestingly, reciprocal interactions between macrophages and activated lymphocytes demonstrate an important link to the adaptive immunity. For instance, IFNγ secreted by Th1 cells as well as IL-4 from Th2 cells influence macrophage activation and polarization. The other way round, macrophages present antigens to T lymphocytes and thus, activate the adapted immune system for specialized immune responses (Underhill et al., 1999).

5.4.3 Adapted immune system

The adaptive immune system is carried out by different lymphocyte subsets, such as B cells and T cells. The responses are classified into antibody-mediated immune responses executed by activated B cells secreting immunoglobulins in the bloodstream. Thus, the specific binding of antibodies to the corresponding antigen inactivates viruses and microbial toxins by blocking their ability to bind to host cell receptors and marking invaded pathogens for destruction (Casadevall and Pirofski, 2004, Lodish).

Conversely, T cells exert their part in an immune response in a cell-dependent manner, which depends on specific antigen recognition (Malissen et al., 2014). Naïve T cells resting in the peripheral lymphoid organs get activated upon formation of a so-called immunological synapse with an APC or a target cell, which leads to their activation, proliferation and differentiation. Thereby, the T cell receptor (TCR) recognizes a complex consisting of an antigen-derived peptide bound to major histocompatibility complex (MHC) molecules (Brownlie and Zamoyska, 2013). The process of T cell activation induces multiple pathways
and is summarized in Figure 5 (Chakraborty and Weiss, 2014). First, the SRC family kinase member lymphocyte-specific protein tyrosine kinase (LCK), which is associated with the intracellular tail of the CD4 and CD8 co-receptors, phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) after their recruitment to the TCR-CD3 complex. This phosphorylation enables the recruitment of the ζ-chain associated protein kinase of 70 kDa (ZAP70) and its subsequent phosphorylation and activation. Following, active ZAP70 phosphorylates the linker for activation of T cells (LAT) and together with the adaptor molecule SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) form a multiprotein complex, termed the LAT signalosome (Roncagalli et al., 2014). The LAT signalosome activates multiple downstream pathways. One of the activated pathways leads to the phosphorylation of phospholipase C γ1 (PLCγ1), which results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3). The second messenger IP3 triggers the release of calcium (Ca^{2+}) from the ER, which promotes entry of extracellular Ca^{2+} into the cytosol. Calcium-bound calmodulin activates the phosphatase calcineurin, which induces the transcription factor nuclear factor of activated T cells (NFAT). In parallel, the other second messenger DAG activates protein kinase C (PKC), which in turn promotes transcription factor NFκB activation. Moreover, DAG activates the MAPK kinase pathway, which results in the activation of the transcription factor AP-1. These transcription factors promote transcription of genes involved in the function, survival, and homeostasis of T cells. For example, NFAT and AP-1 regulate IL-2 production and control differentiation (Smith-Garvin et al., 2009).
Figure 5: T cell activation pathway

The activation of the TCR leads to a signalling cascade that involves the phosphorylation of proximal TCR components (blue), signalling by the Ras-Erk pathway (green), activation of the transcription factor NFκB (red) by PKC-θ, and Ca2+ flux-mediated signalling (yellow) (Morris and Allen, 2012).

After activation, mature T cells can be differentiated into several subsets, which have distinct functions and can be distinguished according to their surface expressed glycoprotein co-receptors. T lymphocytes are called CD4⁺ T cells (helper T cells) or CD8⁺ T cells (cytotoxic T cells). CD4⁺ lymphocytes differentiate into different subsets. The Th1 subset is important for cellular immune responses and secretes TNFα and IFNγ, while the Th2 cells mediate humoral immune responses due to IL-4, IL-5 and IL-13 secretion (Raphael et al., 2015). Moreover, additional Th subsets were discovered with a unique cytokine profile and functional properties, such as Th9, Th17, Th22, regulatory T cells (Treg) suppressing immune responses, and follicular helper T cells (Tfh). The different subsets of CD8⁺ lymphocytes depend strongly on their differentiation status, whereby the cytotoxic effector function is increased upon differentiation, the memory function and proliferation are decreased (Golubovskaya and Wu, 2016).

For the development of a functional immune system a controlled way of cell death, termed apoptosis, is of great importance and will be discussed in the following chapter in detail.

5.5 Fas ligand-mediated apoptosis for immune regulation

5.5.1 Immune cells and apoptosis

Multicellular organisms are dependent on cellular homeostasis. In this regard, proliferating cells such as immune cells have to be tightly regulated by programmed cell death. Besides the best-studied form of cell death, apoptosis that requires caspase proteases, various non-apoptotic various forms of cell death exist, such as necroptosis or pyroptosis (Tait et al., 2014). Necroptosis resembles morphologically necrosis, but it is an active form of cell death induced by apoptotic stimuli under conditions of caspase inhibition with receptor-interacting serine/threonine-protein kinases (RIPK) as key molecules (Galluzzi et al., 2012). Pyroptosis is a caspase-dependent form of programmed cell death and it requires the activation of caspase 1 and caspase 11 (caspase 5 in humans) (Kayagaki et al., 2011). In the following, the focus will be on the mechanism of apoptosis cell death, since it plays an important role in immune homeostasis.
Kerr et al. introduced 1972 the term apoptosis for a defined form of cell death with distinct morphological characteristics, such as nuclear fragmentation, chromatin condensation, cytoplasmic shrinkage and an intact membrane (Kerr et al., 1972). Apoptosis has long been regarded as a non-inflammatory process, but this aspect is nowadays under debate (Rock and Kono, 2008, Davidovich et al., 2014). Apoptosis induces daily the programmed cell death of around 50 billion cells in a human adult (Alberts, 2015) and consequently, abnormalities in this form of cell death are associated with a wide variety of diseases, including immunological and developmental disorders, neurodegeneration and cancer (Fuchs and Steller, 2011).

Various processes in the immune system depend on proper induction of programmed cell death, such as T cell development in the thymus, where neglected or autoreactive thymocytes are eliminated by apoptosis (Germain, 2002). In the bone marrow, developed lymphoid progenitor cells migrate to the thymus where they develop their TCR as well as co-receptors. In the process of maturation, they interact with the cortical epithelial cells, which express MHC class molecules associated with self-peptides. Those thymocytes that do not recognize the peptide-loaded self-MHC molecules and therefore lack a TCR signal undergo apoptosis also termed death-by-neglect (Szondy et al., 2012). However, too much signalling induces apoptosis as well (termed negative selection) and helps to avoid autoimmunity (Ohashi, 2003). The appropriate, intermediate level of TCR signalling initiates the positive selection of thymocytes. Thymocytes expressing TCR that binds self-peptide-MHC-class-I complexes are CD8+ T lymphocytes, whereas those that express TCRs that bind self-peptide-MHC-class-II ligands are CD4+ T lymphocytes (Swain, 1983). As described, intrathymic clonal deletion of self-reactive cells is an important mechanism of T cell tolerance. Similarly, apoptosis of mature T cells in the periphery further helps to maintain immune homeostasis by shutting down cellular and humoral immune responses (Kawabe and Ochi, 1991, Xing and Hogquist, 2012). During an acute immune response, T and B lymphocytes respond to antigens and expand upon activation. After several days, the inflammation is resolved and more than 90% of the effector lymphocytes undergo apoptosis, often below the original levels (Kawabe and Ochi, 1991, Zhang et al., 2005, Badovinac et al., 2002, Sprent and Tough, 2001). Another scenario of apoptosis in the immune system occurs when cytotoxic lymphocytes (CTLs) kill virus-infected or altered target cells (Andersen et al., 2006).

5.5.2 Apoptosis pathways

In all types of apoptotic cell death, the proteolytic enzymes caspases (cysteiny1 aspartate-specific proteinases) play a central role. All caspases exist as inactive pro-enzymes (zymogens)
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and undergo proteolytic activation (Thornberry and Lazebnik, 1998). Caspases can be divided into the initiator caspase group, including caspases 2, 8, 9 and 10, (Parrish et al., 2013) and the effector caspase group, including caspases 3, 6 and 7 (Slee et al., 2001). Following dimerization and/or cross-autoproteolytic cleavage in the initiation complex, initiator caspases are activated and cleave effector caspases resulting in the cleavage of important cellular proteins. As a result of this cascade, typical apoptotic biochemical and morphologic characteristics are induced. For example, actin and nuclear lamin A and C cleavage results in chromatin condensation and nuclear shrinkage (Igney and Krammer, 2002). In order to ensure destruction of potential genome-integrated viral genes, cleavage of the inhibitor of the caspase-activated DNase (ICAD) leads to the release of the endonuclease fragmenting DNA in the nucleus (Wyllie, 1980, Bortner et al., 1995) and inactivates DNA topoisomerases and DNA repair enzymes, such as the poly-ADP ribose polymerase (PARP) (Lazebnik et al., 1994). In dying cells, changes in the plasma membrane like phosphatidylserine externalization occur to attract phagocytes (Martin et al., 1995). Caspases participate also in various non-apoptotic functions including the activation, proliferation, and survival (Lamkanfi et al., 2007).

Inflammatory caspases, such as caspase 1, 4 and 5 in humans and caspase 1, 11 and 12 in rodents mediate innate immune responses by inducing pyroptosis (Jimenez Fernandez and Lamkanfi, 2015). In this pathway, caspase 1 is activated by a multiple adaptor complex, termed the inflammasome, and leads for instance to cytokine maturation by the proteolytic cleavage of pro-IL-1β to IL-1β (Mariathasan et al., 2004, Martinon et al., 2002).

There are two caspase-dependent pathways leading to apoptosis induction: the extrinsic pathway initiated after death receptor activation and the intrinsic or mitochondrial cell death pathway as schematically depicted in Figure 6 (Krammer et al., 2007). The extrinsic pathway is stimulated by death ligands binding to their corresponding death receptors. Members of the TNF superfamily bind to members of the TNF receptor superfamily, e.g. TNF binds to the tumor necrosis factor receptor 1 (TNFR1), Fas (CD95/Apo-1) ligand (FasL) to Fas, and the TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L to death receptor 4 and 5 (Ashkenazi, 2002). Death receptors are type 1 membrane proteins and contain a death domain (DD) in their cytoplasmic tail which is essential for apoptosis induction (Boldin et al., 1995). The sequential events of the extrinsic apoptosis induction are described for the FasL/Fas pathway. Since Fas was reported to pre-exist in a trimeric but inactive form binding of FasL to its receptor was thus considered to cause a higher-order aggregation of the receptor molecules (Siegel et al., 2000). This is followed by the recruitment of signalling molecules and the formation of a large protein complex at the cell membrane, termed death-inducing signaling complex (DISC) (Krammer, 2000). The DISC complex consists of oligomerized Fas, the DD-containing adaptor
molecule Fas-associated death domain (FADD), pro-caspase 8, pro-caspase 10 and the cellular FLIP (cFLIP) proteins. Several mechanisms are reported which finally regulate the activation of the Fas/FasL pathway, including the formation of Fas microclusters and actin reorganization after ligand binding (Algeciras-Schimnich et al., 2002). Interestingly, in activated T cells, TCR re-engagement results in a redistribution of Fas receptor into lipid rafts and this accumulation increases the sensitivity of restimulated T cells to Fas-mediated apoptosis even before its engagement with FasL (Muppidi and Siegel, 2004). In detail, the binding of FasL to Fas causes the recruitment of the adaptor protein FADD due to homotypic interactions between the DD of FADD and Fas. The death-effector domain (DED) of FADD in turn, interacts with the N-terminal tandem DEDs of pro-caspase 8, pro-caspase 10 and cFLIP, and results in the assembly of the DISC complex (Kischkel et al., 1995). Interestingly, these DED proteins exceed that of FADD by several-fold and form DED chains at the DISC, enabling the formation of dimers and efficient activation of caspase 8 (Dickens et al., 2012, Schleich et al., 2012). According to the ‘induced proximity’ model, the high local concentrations and the favorable mutual orientation of pro-caspase 8 molecules at the DISC leads to auto-proteolytic processing to the active form caspase 8 that allows the enzyme to leave the DISC and to activate downstream effector caspases, such as caspase 3 and caspase 7 (Boatright et al., 2003). Active caspase 8 further proteolytically cleaves RIPK1, which gets thereby inactivated and prevents necroptosis (Lin et al., 1999). The intrinsic pathway can also be engaged by caspase 8 by cleavage of the Bcl-2 family member Bid (BH3 interacting-domain death agonist), the central molecule in the crosstalk between the two pathways, to truncated Bid (tBid) (Locksley et al., 2001). This fragment inhibits and activates other Bcl-2 family members, thereby triggering mitochondrial outer membrane permeabilization (MOMP), a hallmark of the intrinsic apoptosis pathway (Schug et al., 2011).

The intrinsic or mitochondrial pathway can further be induced upon multiple non-receptor-mediated stimuli, like toxins, growth factor deprivation, cytokine withdrawal, glucocorticoids as well as (chemo)genotoxic- or radiation-induced DNA damage (Kujoth et al., 2005). Key molecules in this pathway are the 25 members of the Bcl-2 family, including pro-survival Bcl-2 proteins (e.g. A1, Mcl-1 Bcl-w, Bcl-xL and Bcl-2), which inhibit apoptosis by direct interaction with pro-apoptotic molecules. Furthermore, the BH3-only proteins (e.g. Bim, Bid, Bad, Puma, Noxa, Bfm, Hrk and Bik) sensitize the cell to apoptosis by inactivating pro-survival Bcl-2 proteins and support the MOMP induction, which is executed by Bax and Bak (Volkmann et al., 2014, Youle and Strasser, 2008). Nevertheless, the exact mechanism of the Bcl-2 family members in apoptosis regulation is still under debate. The BH3-only proteins are upregulated or activated upon apoptosis stimulation (Guadagno et al., 2013) and antagonize the binding
of the pro-survival Bcl2 members to Bax and Bak (Willis et al., 2007, Letai et al., 2002). However, the BH3-only proteins Bim and Bid have also been described to directly activate Bax and Bak (Korsmeyer et al., 2000, Kim et al., 2006). Activated Bax and Bak initiate pore formation within the mitochondrial membrane and lead to the release of proteins from the inter-membranous space of the mitochondrion, which is termed mitochondrial outer membrane permeabilization or shortly MOMP (Goldstein et al., 2000). The released cytosolic cytochrome c catalyzes the formation of the apoptosome, a complex that consists of apoptotic protease activating factor 1 (APAF1), cytochrome c and adenosine triphosphate (ATP). The apoptosome forms a platform that is able to rapidly recruit and activate pro-caspase 9 via its caspase activation and recruitment domain (CARD) by motif-dependent homotypic interactions (Riedl and Salvesen, 2007, Hill et al., 2004). The apoptosis initiator caspase 9 in turn activates effector caspases, such as caspase 3, 6 or 7 (Schafer and Kornbluth, 2006). Next to cytochrome c, the mitochondrion releases the second mitochondria-derived activator of caspase (SMAC, also known as DIABOLO (direct IAP-binding protein with low pl)) and the serine protease OMI (also known as HTRA2) to the cytosol. These factors block the X-linked inhibitor of apoptosis protein (XIAP)-mediated inhibition of caspase activity (Tait and Green, 2010).
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Figure 6: Intrinsic and extrinsic apoptotic pathway

The intrinsic pathway (left) triggered by DNA damage and various genotoxic agents activates/stabilizes the family of BH3-only proteins, which results in Bax and Bak activation. This promotes mitochondrial MOMP and leads to the release of cytochrome c and subsequent recruitment to the apoptosome. This complex leads to the activation of caspase 9 that in turn activates caspase 3 and 7, initiating the apoptosis cascade. XIAP, which inhibits caspase 9 and 3 activation, is inactivated after MOMP-induced cytosolic translocation of SMAC and OMI. The extrinsic pathway (right) is activated by death ligand/death receptor interaction and activates via FADD caspase 8 followed by caspase 3 activation. BID cleavage is a communication between the two pathways. Bcl: B cell lymphoma; BH3: Bcl-2 homology domain 3; Cyt c: cytochrome c; FADD: Fas-associated protein with death domain; MOMP: mitochondrial outer membrane permeabilization; SMAC: second mitochondria-derived activator of caspases; tBID: truncated BID; XIAP: X-linked inhibitor of apoptosis protein.

5.5.3 FasL and immune homeostasis

The extrinsic apoptosis inducer FasL will be described in more detail because of its essential role in the immune system by regulating T cell homeostasis and cytotoxicity. Suda et al. identified FasL in 1993 to be expressed on activated splenocytes and thymocytes. Through cloning expression, it was demonstrated that FasL belongs to the TNF family of proteins. FasL
is a 40 kDa type II transmembrane protein with its N-terminus in the cytoplasm and its C-terminal region extended into the extracellular space (Figure 7) (Suda et al., 1993).

Figure 7: Human FasL structure

FasL is synthesized as a 281-amino-acid protein. FasL contains a proline-rich domain (Pro-Rich) a single transmembrane domain (TM) and a self-assembly domain. Receptor binding occurs at the very end of the COOH-terminus. Cleavage of FasL occurs at site 129/130 (Kavurma and Khachigian, 2003).

FasL is expressed by various types of cells and tissues, but in particular by activated T lymphocytes and natural killer cells. It is involved in different effector functions ranging from the killing of pathogen-infected cells to the induction of cell death of no longer needed or autoreactive lymphocytes (Brunner et al., 2003). In in vitro studies, after restimulation of previously activated so-called primed T cells, FasL expression is rapidly induced and the cell-autonomous interaction with the Fas receptor causes apoptosis (cell-autonomous suicide), which is termed activation-induced cell death (AICD) (Brunner et al., 1995, Dhein et al., 1995, Alderson et al., 1995, Ju et al., 1995). Likewise, the interaction of FasL with Fas on neighboring cells induces fratricide killing.

The Fas/FasL pathway shows a crucial role in regulating the balance between cell survival and cell death. Mutant generalized lymphoproliferative disease (gld) mice, which present non-functional FasL due to a naturally occurring single point mutation in the Fasl gene, are neither able to bind Fas receptor nor to induce apoptosis resulting in lymphoproliferative disorders (Takahashi et al., 1994, Cohen and Eisenberg, 1991). Also, lymphoproliferative disorder (lpr) mice, containing a natural mutation due to insertion of a retroviral transposon in the Fas gene show abnormal Fas expression and subsequently, these mice suffer from lymphadenopathies and autoimmune diseases (Watanabe-Fukunaga et al., 1992). In patients with the autoimmune lymphoproliferative syndrome (ALPS) a similar phenotype can be observed. ALPS is caused by mutations in FasL, Fas or Fas-associated signalling molecules like FADD, suffering from lymphadenopathy, splenomegaly, and various autoimmune diseases (Fischer et al., 2000). The severe autoimmune phenotype resulting from Fas/Fasl mutations suggested a role of the Fas/FasL pathway in the negative selection of autoreactive T cells in the thymus.
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Although there are data supporting the contribution of Fas in thymocytes deletion (Kishimoto et al., 1998), other studies show the opposite (Singer and Abbas, 1994, Villunger et al., 2004). Important for T cell homeostasis is the peripheral deletion. After successfully fighting off pathogens, when the effector cells are no longer needed, the effector cells undergo FasL-mediated apoptosis to restore normal cellularity in the peripheral lymphoid organs and to limit immune cell-induced tissue damage, for example by inflammatory cytokines (Strasser and Pellegrini, 2004). In the case of chronic infections, the elimination of activated lymphocytes is of great importance in order to achieve a state of co-habitation between the persistent pathogen and the host, with an acceptable antigen titer and limited immune cell activation to minimize the harm for the healthy tissue. Interestingly, both gld and lpr T lymphocytes show defective peripheral deletion and AICD, what underlines the importance of Fas/FasL interaction in T cell homeostasis (Nagata and Suda, 1995).

Of interest, besides their defective T cell homeostasis, gld and lpr mice show also accumulations of autoreactive antibodies (Cohen and Eisenberg, 1991). Although FasL is not expressed on B cells, they express its receptor Fas (Ogasawara et al., 1995). B lymphocytes and CD4⁺ T lymphocytes are co-localized within germinal centers and are able to recognize the same antigenic compounds, which leads to a reciprocal activation. It was illustrated that Fas-deficient B cells were not eliminated by CD4⁺ T cells, and therefore suggested that a Fas-mediated mechanism is important for the elimination of autoreactive B cells (Rathmell et al., 1995). To further elucidate the role of the Fas/FasL pathway in B cell homeostasis, Fukuyama et al. restored B cell Fas expression in lpr mice, and with this prevented the autoimmune disease (Fukuyama et al., 2002). Interestingly, in the germinal center Fas-mediated apoptosis of B lymphocytes was also demonstrated to be essential for the T cell homeostasis, since blocking of T cell - B cell interaction completely prevented the fatal lymphoproliferation (Hao et al., 2008). The T cell - B cell interaction promotes the activation, differentiation, and effector functions of T cells and influences apoptosis resistance.

T lymphocytes do not always show the same resistance towards apoptosis (Krammer, 2000). On one hand, in the IL-2-dependent clonal expansion and effector phase after antigen challenge, activated T cells are resistant to apoptosis. The same behavior is observed in surviving memory T cells. On the other hand, after the effector phase most antigen-specific T cells are eliminated by AICD as described before. The apoptosis resistance can be explained by co-stimulatory signals. CD28 is a major co-stimulating co-receptor in T cells that functions to increase cytokine production. T cell survival can be induced by TCR activation with additional co-receptor CD28 stimulation by its ligands CD80 and CD86, which are expressed
on APC, such as B cells (Krammer, 2000). Such a co-stimulatory signal leads to an upregulation of the anti-apoptotic short form of c-FLIP (Kirchhoff et al., 2000), the downregulation of FasL and induces the expression of the survival factor Bcl-xL (B-cell lymphoma-extra large), another member of the Bcl2 family (Boise et al., 1995). Moreover, the survival of both naïve and memory CD4+ and CD8+ T cells is dependent on IL-7 signaling (Zhang et al., 2005). The IL-7 signaling pathway activates the pro-survival Bcl-2 proteins Bcl-2 and Mcl-1 and inhibits the pro-apoptotic BH3-only proteins Bad and Bim to promote T cell survival.

In order to gain a better understanding how different immune cell subsets are involved in the Fas/FasL-mediated controlling of immune cell expansion and autoimmunity, Mabrouk and co-workers deleted the FasL gene through Cre-mediated recombination in T, B, and myeloid cells (Mabrouk et al., 2008). They found on one hand that the loss of FasL in either T or B cells regulates autoimmunity, but not sufficiently to reproduce the dramatic phenotype of gld mice. On the other hand, the lack of FasL on neutrophils or macrophages did not lead to the development of autoimmune pathologies. Interestingly, a recent publication reported FasL expression on the immunosuppressive T cell subset Treg and their direct control of CD8+ T cells via the Fas/FasL system (Akane et al., 2016). These findings show that T cells themselves, but also the interaction with B cells and Tregs are essential in controlling T cell homeostasis by FasL/Fas-mediated apoptosis.

Not exclusively FasL-mediated cell death, also the withdrawal of growth-promoting cytokines, which is dependent on the pro-apoptotic BH3 protein Bim (Bcl-2-like protein 11), was reported to be involved in T cell homeostasis (Bouillet et al., 1999). There are controversial data describing the different importance of the extrinsic signalling via Fas receptor or the intrinsic pathway via Bim. Studies in staphylococcal enterotoxin B-activated T lymphocytes found that the termination of an acute immune response was only in part Fas/FasL-dependent (Mogil et al., 1995). Another publication described that Fas deficiency had no impact but that loss of the intrinsic apoptosis mediator Bim provided profound protection (Hildeman et al., 2002). Of interest, further studies demonstrated important but independent roles for both Bim and FasL in maintaining T cell homeostasis depending on the type of infection (Hughes et al., 2008, Kassahn et al., 2008). During high antigen loads, which mimic a chronic pathogen infection, both Fas/FasL and Bim are required, while the killing of antigen-activated T lymphocytes after single low doses like in an acute immune response was found to be a FasL-independent but Bim-dependent process.
However, there is strong evidence that T cell homeostasis is not just regulated by T lymphocytes themselves. In bone marrow chimera experiments irradiated wild type (wt) mice reconstituted with gld bone marrow showed peripheral deletion, while wt T cells strikingly failed to undergo peripheral deletion in gld recipients (Bonfoco et al., 1998). This indicates that immune responses can induce FasL in non-lymphoid tissues, leading to apoptosis in activated lymphocytes. Further, TNF-mediated activation of lymphocytes in the small intestine was also reported to trigger FasL expression on non-lymphoid tissue, such as intestinal epithelial cells, which in turn controlled lymphocyte infiltration and inflammation by apoptosis induction (Pinkoski et al., 2002). Indeed, FasL is constitutively expressed in specialized tissues such as the eye, testis, ovary and the central nervous system (CNS). There, the Fas/FasL interaction displays an important mechanism among others leading to immune privilege through the induction of apoptosis in infiltrating inflammatory cells (Green and Ferguson, 2001, Choi and Benveniste, 2004, Griffith et al., 1995). In summary, FasL-mediated cell death leads to T and B cell homeostasis and is a regulator of immune privilege. Nevertheless, activated FasL-expressing T lymphocytes can induce cell death not only in immune cells but also in any Fas-positive tissue.

5.5.4 FasL and cell-mediated cytotoxicity

Another effector function of FasL is the cell-mediated cytotoxicity executed by primed CD8$^+$ CTLs, but also CD4$^+$ T helper cells or NK cells. They rapidly express FasL upon reactivation of the TCR and the interaction with the Fas receptor on target cells induces apoptosis (Brunner et al., 1995, Lin et al., 1998). CTLs are crucial components of the adaptive immune system that function to eliminate intracellular pathogens and tumor cells. Besides FasL-mediated apoptosis, CTLs use also other cell death inducer, such as perforin in combination with granzyme B (Andersen et al., 2006). CTLs store perforin, a pore-forming protein, and granzyme B, which is a serine protease that cleaves substrates in the cytoplasm of target cells, in cytolytic granules. After interaction with the target cell an immune synapse is formed and CTLs release their stored perforin and granzyme molecules into the intercellular space (de la Roche et al., 2016). Comparable to Fas/FasL, granzyme B activates caspase 3 and 8, and was also reported to amplify the cell death through Bid cleavage and activation of the mitochondrial pathway (Voskoboinik et al., 2015). Perforin and FasL act synergistically in CTLs, while perforin is fast-acting and FasL shows a slower response (Hassin et al., 2011). Thus, perforin- as well as FasL-deficient CTL have an impaired lytic activity and the killing of target cells is completely suppressed when both pathways are inactivated (Lowin et al., 1994). FasL- as well as perforin-based mechanisms have been shown to be critical for the antigen-specific
elimination of virus-infected target cells. Thus, cell death of non-infected bystander cells was mainly induced by FasL in order to restrict the virus spreading (Ando et al., 1997). FasL-induced target cell killing appears to be prominent in tissue destruction due to apoptosis of endothelial cells where it is involved in the induction of immunopathological disorders (Janin et al., 2002).

Such noxious FasL-mediated immune responses were extensively studied in hepatitis B virus- and LPS-induced hepatitis mouse models (Kondo et al., 1997). Furthermore, in patients infected with the hepatitis B virus, it was shown that the Fas/FasL system is one of the important pathways regulating the response to the infection, which leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma (Zaki Mel et al., 2008). Another important example for FasL-mediated tissue destruction is Graft-vs-host disease (GvHD), which is a dramatic result that often occurs after allogeneic bone marrow transplantsations (Lin et al., 1998, Wasem et al., 2001). Moreover, FasL is relevant in autoimmune diseases as shown in ALPS patients, but also in type I diabetes and multiple sclerosis (MS). Knowing the major role of the perforin-granzyme system in the destruction of β cells in type I diabetes (Kagi et al., 1997), it is of interest that also FasL was described to play a contributory role in β cell destruction during the development of this autoimmune disease (Su et al., 2000). Interestingly, expression of Fas and FasL are elevated in in MS patients, which suffer from demyelination and axon damage caused by infiltration of autoreactive lymphocytes and macrophages. FasL contributes to the progression of this disease via its interaction with Fas on CNS elements and infiltrating lymphocytes (Sabelko-Downes et al., 1999). However, FasL may have antagonistic roles in MS depending on the FasL expression of the T cell subset, where Th17 cells have a severe pathogenic and Tregs a protective role (Volpe et al., 2016).

In addition to the function of FasL in T cell-mediated cytotoxicity of virus-infected cells or its immunopathological effect, there is a controversial discussion about the role of FasL in cancer. On one hand, FasL could be an important factor in the elimination of cancer cells, which constitutively express functional Fas receptor. However, many cancer cells are resistant to FasL-mediated apoptosis (Kornmann et al., 2000). On the other hand, FasL expression on apoptosis-resistant cancer cells may enable tumor cells to eliminate anti-tumor lymphocytes (Igney and Krammer, 2005). Furthermore, FasL was described to be critical for cancer cell survival and to promote cancer stem cells (Peter et al., 2015). Describing the critical role of FasL in maintaining immune cell homeostasis and T cell-mediated cytotoxicity, it is of great importance to understand the mechanisms of FasL regulation.
5.5.5 Posttranslational modifications of FasL

To fulfill its cytotoxic activity and to prevent non-specific killing by T cells, FasL cell surface expression has to be tightly regulated and rapidly induced after TCR activation. This fast mobilization is dependent on posttranslational modifications (Voss et al., 2008). In CTLs, FasL is transported to storage vesicles, due to selective tracking. This sorting process is controlled by SRC family tyrosine kinases which bind to the proline-rich domain in the cytoplasmic tail of FasL at the trans-Golgi network and then facilitate the transport of FasL-containing vesicles to the secretory lysosome. Upon stimulation, FasL is secreted into the cytotoxic immunological synapse (Blott et al., 2001, Zuccato et al., 2007). Interestingly, in a disease-related situation in animals during experimental GvHD T cells accumulate FasL protein and released it in a protein synthesis-independent manner (Wasem et al., 2001). Moreover, with the help of the proline-rich domains FasL clusters in lipid rafts to reach its full apoptosis-inducing capacity (Nachbur et al., 2006). However, once at the cell surface, FasL surface expression is rapidly downregulated by a metalloprotease to limit the apoptosis-inducing activity. ADAM 10 (a disintegrin and metalloprotease) sheds membrane-bound FasL into a soluble form and counteracts the cytotoxic activity of full-length FasL (Schulte et al., 2007).

5.5.6 Transcriptional FasL regulation

In T cells, the de novo synthesis of FasL is regulated by several well-described transcription factors, which interact with the Fasl promoter to control FasL transcription (Kavurma and Khachigian, 2003). A prominent regulator of TCR-mediated FasL expression is NFAT, binding to two consensus sites within the activated T cells promoter. As described in chapter 1.4.4, the activation of PLCγ1 after TCR stimulation is critical in the activation of several transcription factors, including NFAT. Since IP3 increases the intracellular Ca\(^{2+}\) levels, this subsequently leads to the activation of the calcium- and calmodulin-dependent serine-threonine protein phosphatase calcineurin. Calcineurin activates NFAT and consequently upregulates FasL expression. This activation can efficiently be blocked by immunosuppressive drugs, such as cyclosporin A (CsA) and FK506 (Tacrolimus), which target calcineurin (Brunner et al., 1996). The FasL transcription factors early growth response protein-2 and -3 (EGR-2 and -3) share the same activation pathway with NFAT, and are therefore inhibited with the same drugs (Mittelstadt and Ashwell, 1999). FasL transcription can further be inhibited via NFAT blockage by MHC class II transactivator (CIITA) transcription factor as well as retinoic acid (Gourley and Chang, 2001). A second important pathway is initiated by the second messenger DAG which promotes the activation of PKC, and the transcription factors NFkB and activator protein-1 (AP-1) (Ramaswamy et al., 2009). The phorbol ester PMA mimics DAG and together with the
Ca^{2+} ionophore ionomycin can be used to activate T cells and to induce FasL up-regulation (Villalba et al., 1999). Another transcriptional regulator is interferon regulatory factor-1 (IRF-1), which induces FasL transcription upon TCR stimulation, and was shown to interfere with human herpesvirus 8, which leads to the downregulation of FasL and may, therefore, protect virus-infected T cells from FasL-mediated apoptosis (Kirchhoff et al., 2002).

Not just TCR activation, also DNA damaging agents can induce FasL-mediated apoptosis. DNA damaging agents activate AP-1 and NFkB via the Jun kinase pathway, which in turn up-regulates FasL expression (Kasibhatla et al., 1998). Nitric oxide (NO) represents another factor which can modulate apoptosis by interfering with AP-1 and in turn enhances FasL expression (Melino et al., 2000). Furthermore, the activation of the heat shock transcription factor-1 (HSF-1) at fever-like temperatures resembles a stress-inducible transcription factor that positively regulates FasL expression and maintains the immune homeostasis (Bouchier-Hayes et al., 2010).

In naïve and resting T cells, all these transcription factors become readily activated upon stress or TCR activation but result in little Fasl transcription. In contrast, robust and rapid FasL expression is induced upon reactivation of primed T lymphocytes. Primed proliferating T lymphocytes express c-Myc, which not only regulates cell proliferation but also binds to specific binding sites in the human FasL (FASLG) promoter and thereby promotes FASLG transcription and controls apoptotic cell death (Brunner et al., 2000, Kasibhatla et al., 2000). Of interest, also TGFβ can negatively influence TCR-induced Fasl expression through down-regulation of c-Myc expression (Genestier et al., 1999). Thus, activation-induced, as well as cell cycle-regulated transcription factors, are critically involved in the regulation of FasL expression in effector T cells.

In summary, FasL-mediated apoptosis is essential for immune homeostasis and is also involved in a variety of diseases, such as hepatitis, GvHD or MS. Taking this into account, pharmacological intervention in the Fas/FasL signalling may be of interest for therapeutic approaches in the treatment of T cell-dependent and FasL-mediated immunopathologies. Unfortunately, first trials revealed complications, such as the development of fatal hepatitis in mice after administration of agonistic Fas- or FasL-specific antibodies (Ogasawara et al., 1993, Shiraishi et al., 2004). Nevertheless, a better understanding of FasL regulators like transcription factors might be another option in order to modulate FasL expression for the treatment of immunopathologies.


6 AIMS OF THE STUDY

The NR LRH-1 has an essential transcriptional role for multiple functions during embryogenesis, metabolism and proliferation in tissues of endodermal origin, such as the liver, pancreas, intestine and the ovaries. In addition to its role in physiological processes, LRH-1 is also involved in many pathological processes, such as cancer, diabetes or fatty liver disease, and has been shown to indirectly modulate the immune system and associated inflammatory processes via the regulation of the acute phase response in the liver or the synthesis of immunoregulatory glucocorticoids in the intestinal crypts. With extensive research, the structural characteristics of the orphan NR LRH-1 are under investigation, especially in order to discover endogenous as well as pharmacological ligands to modulate its activity.

This study aims to elucidate the so far almost unknown role of LRH-1 in the immune system and includes characterizing the expression of LRH-1 in cells of hematopoietic origin, as well as examining its possible target genes and its influence on their effector functions. The goal of this work is to gain a better understanding of LRH-1 as a potential pharmacological target in order to treat LRH-1-mediated diseases. To address these questions recently published pharmacological LRH-1 inhibitors were tested in different immune cell subsets, as well as in in vivo disease models.
7 MATERIALS AND METHODS

7.1 Materials

Table 1: Reagents for immune cell stimulation and treatment

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<tr>
<th>Product</th>
<th>Supplier</th>
<th>Notes</th>
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<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>Enzo Life Sciences, Lörrach, Germany</td>
<td></td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Enzo Life Sciences, Lörrach, Germany</td>
<td></td>
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<tr>
<td>3d2</td>
<td>ChemBridge Corp, San Diego, CA, USA</td>
<td>(Benod et al., 2013)</td>
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<tr>
<td>Cpd7</td>
<td>ChemBridge Corp, San Diego, CA, USA</td>
<td>(Benod et al., 2013)</td>
</tr>
<tr>
<td>SR1848</td>
<td>Sigma-Aldrich, Steinheim, Germany</td>
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<td>Interleukin-2 (IL-2), Proleukin</td>
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<td>LPS (S. minnesota)</td>
<td>Sigma-Aldrich, Steinheim, Germany</td>
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<td>Hamster anti-mouse FasL antibody (MFL3)</td>
<td>BD Bioscience, Heidelberg, Germany</td>
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<tr>
<td>Hamster anti-mouse CD3ε antibody (clone 145-2C11)</td>
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Table 2: Plasmids

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7.2 Methods

7.2.1 Mice

For \textit{ex vivo} and \textit{in vivo} experiments wild type C57BL/6 mice, \textit{gld} (generalized lymphoproliferative disease) mice with a natural mutation in the \textit{FasL} gene and specific conditional knockout mice were used in an age between 8–12 weeks. The conditional knockout mice are LRH-1 floxed mice, which were crossed with CD4-Cre transgenic mice to generate T cell-specific LRH-1 knockout on a C57BL/6J background. Such T cell-specific LRH-1 knock-out mice are referred to as LRH-1\textsuperscript{cko}. Control mice with floxed LRH-1 but lacking Cre recombinase expression are termed LRH-1\textsuperscript{L2L2}. Mice were bred in the animal facility of the University of Konstanz and were accustomed to a 12 h light/dark cycle with free access to food and water. All animal experiments complied with animal experimentation regulations of Germany and were approved by the Ethics Review Committee of the regional council.

7.2.2 Cell culture

The human embryonic kidney fibroblast cell line HEK 293T cells was obtained from American Type Culture Collection (ATCC) and was maintained in Dulbecco’s Modified Eagle’s Medium. The murine T cell hybridoma A1.1 cells (Fotedar et al., 1985) and the human leukemic T lymphocyte Jurkat IT cells (Kasibhatla et al., 1998) were cultured in RPMI-1640 Medium. The mouse Abelson murine leukemia virus-transformed macrophage cell line RAW 264.7 was cultured in DMEM. All cell culture media were supplemented with 10% FCS and 30 μg/ml gentamycin (Sigma-Aldrich, Steinheim, Germany) and all cells were maintained at 37 °C under 5% CO\textsubscript{2}. Aliquots of cells at early passage were stored in liquid nitrogen in freezing medium (complete growth medium, 10% DMSO). Frozen cells were rapidly thawed and cultured for at least 3 days before use in experiments.

7.2.3 Isolation of murine splenocytes

Mouse spleens were removed and mechanically dissociated between frosted glass object slides in PBS. Cells were filtered through a cell strainer (100 μm, VWR). After centrifugation (500 x g, 5 min, RT) the cell pellet was shortly resuspended in the remaining liquid by agitation. Subsequently, erythrocytes were removed by hypotonic lysis. To do so, 900 μL distilled sterile water was added and the cell suspension was shaken for 15 sec before the solution was
Complemented with 100 μL 10x PBS. The Falcon tube was filled up to the top with PBS, again filtered through a cell strainer and centrifuged. The cell pellet was resuspended in T cell full medium containing RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 30 μg/ml gentamycin. All reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

7.2.4 Culture of ConA blasts

Concanavalin A (ConA) is a lectin, which stimulates unprimed mouse T cells, such as splenocytes, and results in activation, proliferation and differentiation of both CD4⁺ and CD8⁺ T cells. Further IL-2 signals optimize the effector T cell generation. Therefore, murine T cell blasts were generated by stimulating 3 x 10⁶ cells/ml freshly isolated splenocytes (see 3.2.3. Isolation of murine splenocytes) with 1 μg/ml ConA for 1 day keeping the flask upright at 37 °C with 5% CO₂. Afterwards, cells were washed to remove the lectin and were cultured with 100 units/ml recombinant IL-2 (Proleukin, Prometheus, Vevey, Switzerland) for additional 4 days to generate T cell blasts.

7.2.5 Gradient centrifugation

Four days after ConA stimulation and IL-2-induced expansion (see 3.2.4. Culture of ConA blasts), murine T cell blasts were purified using a Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) gradient to remove dead cells. ConA blasts (3 x 10⁶ cells) were resuspended in 2 ml of medium and transferred to a 15 ml Falcon tube. Cells were carefully underlaid with 2 ml Histopaque-1077 at room temperature (RT) by using a glass pipette. The tubes were centrifuged at 400 x g for 20 min at RT without break. The interphase containing viable cells (400 μl) was carefully harvested with a Pasteur pipette, washed and and resuspended in 9 ml full medium for further experiments.

7.2.6 Cell sorting

For cell sorting isolated splenocytes (see 3.2.3. Isolation of murine splenocytes) were stained with anti-CD4-FITC (1:16'000) and anti-CD8-PE (1:8'000) antibodies diluted in PBS for 20 min at 4°C in the dark. Afterwards, cells were washed once with PBS and sorted on a FACSAria III using FACSDiva software (BD Heidelberg, Germany).
7.2.7 Culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were isolated and differentiated according to the protocol of Weischenfeldt and Porse (Weischenfeldt and Porse, 2008). In this protocol, bone marrow cells are cultured in the presence of M-CSF, which is secreted by L929 cells and is used as L929-conditioned medium. Under these conditions, the bone marrow macrophage progenitors will proliferate and differentiate into a homogeneous population of mature BMDM.

For the preparation L929-conditioned medium, mouse fibroblasts L929 cells were cultured in RPMI medium supplemented with 10% FCS and 1% penicillin/streptomycin (PAA laboratories, Pasching, Austria). In total \(4.7 \times 10^5\) L929 cells were plated in a 75-cm\(^2\) flask containing 55 ml medium. The day after the medium was changed with fresh full medium. One day later the cells were harvested, divided into three 75-cm\(^2\) flasks with 55 ml medium each and grown for 7 days. The supernatant was collected, sterile filtered and stored at -20°C in aliquots. The bone marrow-derived macrophage full medium contained DMEM, supplemented with 10% FCS, 10% of the L929 supernatant and 30 µg/ml gentamycin.

Bone marrow cells were isolated from the marrow of the femurs and tibias of C57/BL6 mice. Both edges of the femurs and tibia were cut, the marrow was flushed with a 25-G needle with medium and cells were filtered through a cell strainer (70 µm, VWR). After centrifugation (500 x g, 5 min, RT) erythrocyte lysis with distilled sterile water was followed (see 3.2.3. Culture of ConA blasts). After centrifugation, cells were resuspended in full medium and \(2 \times 10^6\) cells/ml were seeded in 10 cm tissue culture dishes in 12 ml medium. Every two days the medium was discarded, cells were washed with PBS and new medium was added. At day 7 cells were harvested by incubation with trypsin/EDTA. Therefore, the medium was discarded and 3 ml trypsin/EDTA was added for 5 min at 37°C. Trypsin/EDTA was discarded and 3 ml of fresh one was added for 20 min at 37°C. Cells were mechanically detached from the plate by scraping and resuspended in PBS supplemented with 10% FCS. BMDM were centrifuged and resuspended in full medium without L929 supernatant and seeded for further experiments (5.0 \(\times 10^6\) cells/well for cytokine release experiments and qPCR, 1.0 \(\times 10^6\) cells/well for Western blot analysis).

7.2.8 Plasmids

The plasmids were propagated in the respective bacterial clone, which was pre-cultured in 2 ml LB medium (10 g/l bacto tryptone, 5 g/l bacto yeast, 10 g/l NaCl) with 100 µg/ml of ampicillin for 5-7 hours at 37 °C on a shaker at 220 rpm. This pre-culture was used to inoculate
100 ml ampicillin containing LB-medium and grown overnight. Plasmids were isolated using the PureYield Plasmid Midiprep System (Promega, Mannheim, Germany) according to the manufacturer’s protocol. Plasmid concentration and purity were determined by spectrophotometric analysis at 260 nm and 280 nm, respectively, with a Nano-Drop spectrophotometer (Eppendorf, Hamburg, Germany).

The human FasL luciferase reporter construct (HFLP-Luc), containing 1.2 kb of the FasL promoter, and the empty control plasmid HsLuc have already been described (Kasibhatla et al., 1998). HFLP-Luc with single or double mutated LRH-1 binding sites in the FASLG promoter (HFLP mut BS1, HFLP mut BS2, HFLP mut BS1+2) were generated by site-directed mutagenesis with primers from Table 3, as described in Master thesis of Ann-Kathrin Fuchs. The putative LRH-1 binding sites and in the FasL promoter and their mutation are depicted in Figure 10. The LRH-1 reporter containing 5 copies of the LRH-1 binding motive in the pGL3 basic plasmid (Promega, Mannheim, Germany) has been described previously (Atanasov et al., 2008). The Myc-6xHis-tagged LRH-1 expression plasmid was generated by cloning human LRH-1 into a pcDNA3.1 Myc/His expression vector (Invitrogen). The Flag-tagged LRH-1 expression plasmid was generated by exchanging the Myc-His tag with a 3xFlag epitope tag. A β-galactosidase expression plasmid was used to normalize transfection efficiency (Invitrogen). In Table 2 all plasmids used are listed.

7.2.9 Transfection

HEK 293T cells were transfected by Ca\(^{2+}\)-phosphate-precipitation. By incubation of plasmid DNA with calcium chloride in a buffered saline/phosphate solution, the plasmid DNA can be co-precipitated with calcium phosphate crystals. These fine, plasmid-containing crystals are able to bind to the cell surface of HEK 293T cells and are subsequently taken up by endocytosis. One day prior to transfection, 3.0 x 10^5 HEK 293T cells were seeded in a well of a 6-well plate in 2 ml medium. A total of 1 μg of total plasmid were added to 25 μl 2.5 M solution (final volume: 75 μl). The DNA/CaCl\(_2\) solution was added dropwise to 100 μl 2 x HBS buffer (HEPES-buffered saline: 280 mM NaCl, 10 mM KCl, 1.5 mM Na\(_2\)HPO\(_4\), 12 mM glucose, 50 mM HEPES). The transfection mixtures were incubated for 30 min at RT. Meanwhile, the medium of overnight attached HEK 293T cells was replaced with half as much of fresh DMEM. Afterwards, the mixtures were added dropwise to the cells and 6 h later the transfection medium was removed and replaced by 2 ml fresh medium.
Nucleofection is a transfection method where an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing DNA to be introduced into the cell. This method was applied to transfect Jurkat IT cells and RAW 296.4 cell.

Jurkat IT cells were seeded one day prior transfection at a density of $3.0 \times 10^5$ cells/ml. Cells ($1.0 \times 10^6$ cells) were transfected with a total of 2 µg of plasmid in 100 µl SA-buffer B (150 mM Na$_2$HPO$_4$ pH 7.2, 20 mM HEPES pH 7.2, 15 mM MgCl$_2$, 5 mM KCl, 50 mM Mannitol) in a Gene Pulser Cuvette (BioRad, München, Germany) by electroporation using an Amaxa Nucleofector Device (Lonza, Basel, Switzerland) with program X-005.

RAW296.4 cells were passaged one-day prior transfection by diluting them 1:3. $2.0 \times 10^6$ cells were then transfected with a total of 6 µg of plasmid in 100 µl SA-buffer B by electroporation with Amaxa program D-032.

Electroporated cells were pooled in complete medium and seeded into a tissue culture dishes for further experiments.

### 7.2.10 Luciferase reporter assay

In order to quantify promoter strength, promoters are fused to reporter genes that encode enzymes that can be quantified, such as the firefly luciferase. This enzyme oxidizes D-luciferin in the presence of ATP, oxygen, and Mg$^{2+}$, yielding a fluorescent product that can be quantified by measuring the released light (Smale, 2010). Including coenzyme A in the reaction enhances the sensitivity of the assay and provides a sustained light reaction.

Cells were transiently transfected with expression and luciferase reporter plasmids, while cotransfection of β-galactosidase (β-gal) expression plasmid served for normalization (Kasibhatla et al., 1998). One day after transfection, cells were either control treated or treated with different concentrations of the LRH-1 inhibitors and controls for 2 h, prior to stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 16 h.

Subsequently, 100 µl cold Lysis Buffer (100 mM K$_2$HPO$_4$, 0.2% TritonX-100, pH 7.8) was added per sample and the cells were incubated for 15 min on ice. The lysates were subsequently transferred to a V-bottom 96-well plate and cleared by centrifugation (500 x g, 10 min, RT). Lysates were stored on ice until further usage. Further, 30 µl of the lysates were transferred to a white flat-bottom 96-well plate. Luciferase activities were measured using an Infinite 200 PRO plate reader (Tecan, Crailsheim, Germany) as relative light units (R.L.U.). The measurements were performed well-wise after automatic injection of 50 µl ATP (10 mM ATP in Assay Buffer (20 mM MgCl$_2$, 35 mM Glycyl-glycine)) solution and 50 µl luciferin (270 µM Coenzyme-A (Li-salt), 470 µM Luciferin (K-salt) in Assay Buffer) per well.
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Co-transfection of cells with β-gal and a subsequent β-gal assay was used as an internal transfection control to normalize the luciferase activities of each transfection condition. This colorimetric assay is based on the β-gal mediated hydrolysis of its synthetic substrate o-nitrophenyl β-D-galactopyranoside (ONPG) into galactose and o-nitrophenol. The latter compound has a yellow color and the intensity of the color produced can be used as a measure of the enzymatic rate of β-gal. Therefore, 30 µl of the lysates were transferred to a transparent flat bottom 96-well plate and 105 µl of ONPG (0.2 mg/ml) in Z-Buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM 2-mercaptoethanol) was added to each well of the plate. Plates were incubated for approximately 30-120 min depending on cell type at 37 °C in the dark. Subsequently, the optical density of o-nitrophenol was measured at 405 nm using an Infinite 200 PRO plate reader.

7.2.11 Chromatin immunoprecipitation (ChIP)

With the chromatin immunoprecipitation (ChIP) assay the direct interaction of a protein, such as a transcription factor, with the DNA was investigated. In this multistep process, intact cells were treated with formaldehyde to covalently link protein to DNA, the nucleoprotein complexes are then sheared and the resultant soluble cross-linked DNA-protein complexes were enriched by immunoprecipitation. The retrieved complexes are then analyzed by PCR amplification with gene-specific primers to detect specific DNA binding sites (Gade and Kalvakolanu, 2012).

Jurkat IT cells (1.0 x 10⁷ cells) were transfected with the FLAG-tagged LRH-1 expression vector or pcDNA 3.1 as empty vector control. After 48 h cells were centrifuged at 12’000 rpm for 7 min and resuspended in 1 m full medium. To cross-link the nuclear proteins with the DNA, 9 ml medium supplemented with 1% formaldehyde were added and incubated for 10 min at 37 °C. To quench the cross-linking 1 ml 10x Glycine were added and incubated for 5 min at RT. Cells were centrifuged and washed with ice-cold PBS. Afterwards, cells were resuspended in 1 ml ice-cold PBS supplemented with 10 µl 100x PMSF and 10 µl 100x PIC protease inhibitor cocktail and transferred to Eppendorf tubes. The cells were pelleted at 12’000 rpm for 7 min at 4 °C and the supernatant was discarded. The cell pellet can be stored at -80 °C.

The cells were lysed with 300 µl Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethyl sulfonide fluoride) on ice for 15 min to obtain the cytosolic fractions. Next, 20 µl 10% NP-40 solution were added and vortexed for 10 sec followed by centrifugation at 5’000 rpm for 5 min at 4 °C. The nucleic pellets were washed twice with 300 µl of Buffer A and centrifuged as before and were lysed with 660 µl of Nuclei Lysis Buffer (50 mM TrisHCl pH 8.0, 10 mM EDTA, 1% SDS, phenylmethyl sulfonide fluoride,
complete Protease Inhibitor Cocktail Tablets (Roche)) on ice for 10 min. The samples, 330 µl/1.5 ml Eppendorf tube, were sonicated for 26 cycles (for 30 sec each, high intensity) using a Bioruptor Plus (Diagenode, Seraing, Belgium) followed by centrifugation at high speed in a microcentrifuge for 15 min at 4 °C to pellet the precipitated SDS. 100 µl of the supernatant were diluted in 200 µl Immunoprecipitation Dilution Buffer (16.7 mM TrisHCl, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% TritonX-100) and incubated overnight at 4 °C with 5 µg mouse anti-FLAG antibody or control mouse IgG antibody (Sigma-Aldrich, Steinheim, Germany). Antibodies were immunoprecipitated by incubation with 20 µl of Magna CHIP Protein G magnetic beads (Merck Millipore, Darmstadt, Germany) on a rotation wheel for 2 h at 4 °C (in the cold room). With the help of a magnetic rack, the supernatant was discarded and the beads were washed in 1 ml of High-salt Buffer (50 mM HEPES pH 7.9, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% TritonX-100, 0.1% deoxycholate) four times rotating for 10 min at 4 °C. The last washing step was performed with 1 ml of TE buffer as before. DNA was purified with the IPure kit (Diagenode) using magnetic beads according to the manufacturer’s protocol. The following amplification of the putative LRH-1 binding sites in the FASLG promoter or the SHP promoter (positive control) was performed by PCR. 3 µl DNA of the immunoprecipitated samples and the input were amplified by conventional PCR for 35 cycles with primers stated in Table 3. PCR products were then separated and analyzed on a 1.5% agarose gel by electrophoresis.

7.2.12 Quantitative real-time PCR

In order to analyze gene expression, real-time polymerase chain reaction (RT-PCR), also known as quantitative polymerase chain reaction (qPCR), was performed. For this transcript analyses, 2 x 10⁶ cells were pelleted by centrifugation (400 x g, 5 min, RT) and were lysed in 1 ml peqGOLD TriFast reagent (PeqLab, Erlangen, Germany). Tissue samples were homogenized in 1 ml TriFast using the TissueLyser II (Qiagen, Hilden, Germany) for 3 min and 300 Hz. The lysates were transferred to 1.5 ml centrifuge tubes and kept at -80 °C until further usage. For RNA isolation, 200 µl chloroform was added per 1 ml lysing reagent and samples were vortexed for 1 min. Afterwards, samples were incubated for 3 min at RT and centrifuged (12,000 x g, 4 °C, 15 min) to separate the different phases. To precipitate the RNA, the aqueous layer was transferred to a new 1.5 ml tube and an equal volume of isopropanol was added to each sample. Samples were incubated for 15 min on ice, before repeated centrifugation (12’000 x g, 15 min, 4 °C). To wash the RNA, the supernatant was removed and the pellets were washed twice with 1 ml ice-cold 75% ethanol (in DEPC H₂O; centrifugation: 7’500 x g,
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8 min, 4 °C). The RNA pellets were air-dried for 10 min at 56 °C and subsequently dissolved in 20 µl pre-warmed DEPC H2O (56 °C, 15 min). RNA concentrations were determined using a Nano-Drop spectrophotometer (Eppendorf, Hamburg, Germany). Purified RNA was stored on ice for immediate use or was kept at -80 °C for long-term storage to prevent degradation.

For the conversion of messenger RNA (mRNA) into complementary DNA (cDNA) a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer’s protocol. Therefore, a total amount of 1 µg mRNA in 10 µl H2O was combined with 10 µl 2x Reverse Transcription Master Mix (4.2 µl H2O, 2 µl 10x RT-buffer, 0.8 µl 25 x dNTP mix, 10x RT random primer mix, 1 µl transcriptase). The reaction was run in a peqStar PCR machine using the following settings: lid 110 °C; 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min. Obtained cDNA samples were stored at -20 °C.

Quantitative real-time PCR was used to quantify transcription levels in comparison to the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin. With this method, amplified cDNA is detected in real time using SYBR Green, a fluorescent dye that binds to double-stranded DNA. The resulting DNA-dye-complex has excitation and emission maxima of 494 nm and 521 nm, respectively (Zipper et al., 2004). A thermal cycler with the capacity to detect the fluorescence emitted by the excited SYBR Green. To each well of PCR plate 2 µl diluted cDNA as well as 6 µl H2O, 2 µl primer mix (10 µM forward and reverse primers of each target gene in RNase free water) primers stated in Table 3 and 10 µl Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was added. Plates were sealed with polylefin film and briefly centrifuged at 500 x g before the qPCRs were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and analyzed using the StepOne software. The threshold cycles (Ct) were determined for each gene and first gene expression levels were calculated as relative expression compared to the housekeeping genes. Subsequently, fold changes relative to the control sample were calculated.

7.2.13 Activation-induced cell death assay

Activation-induced cell death was induced as described by Brunner et al. 1995 (Brunner et al., 1995). Viable primary T cells or A1.1 cells were pre-treated with 3d2, SR1848, CsA or anti-FasL antibody for 2 h, and then cultured for 6 h in a density of 3.0 x 10^5 cells/well in anti-CD3 antibody-coated 96-well plates. Subsequently, cells were harvested and stained with Annexin V-FITC as described in 3.2.14. (Apoptosis detection by flow cytometry).
7.2.14 Apoptosis detection by flow cytometry

Flow cytometry allows the simultaneous analysis of multiple characteristics of single cells, such as size, granularity and the relative fluorescence intensity when cells are stained with fluorochromes. During apoptosis phosphatidylserine typically becomes exposed on the cell surface (Schlegel and Williamson, 2001), which can be stained with fluorescently labelled Annexin V, a phosphatidylserine-binding protein, to determine the extent of apoptotic cell death. Therefore, after removal of the medium cells were resuspended in 200 µl FITC-conjugated Annexin V-FITC diluted 1:1000 in Annexin V binding Buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂) and incubated for 15 min in the dark at RT. Afterwards, samples were immediately analyzed by flow cytometry using a BD LSRFortessa (BD Biosciences, Heidelberg, Germany). A population of 10'000 cells was analyzed for each sample. The number of Annexin V positive, apoptotic cells as a percentage of total cells was determined using the FlowJo software.

7.2.15 Life-death staining with acridine orange and propidium iodide

Dual-fluorescence viability can be measured using acridine orange and propidium iodide. Acridine orange and propidium iodide are nucleic acid binding dyes. Acridine orange is permeable to both live and dead cells, and propidium iodide enters dead cells with compromised membranes. Cells stained with acridine orange fluoresce green and all dead nucleated cells fluoresce red.

One day before the experiment, RAW 296.4 cell were seeded at 3.0 x 10⁴ cells/well in a 96-well plate in full medium supplemented with 2% FCS. The cells were treated with LRH-1 inhibitors and after overnight incubation cells were stained with 4 µg/ml acridine orange and 4 µg/ml propidium iodide for 15 min and observed with the ZEISS Axio Observer (ZEISS, München, Germany).

7.2.16 Cytotoxicity assay

Cells undergoing apoptosis are fragmenting their DNA. If the DNA is labelled with ³H-thymidine DNA fragmentation can be assessed by the loss of radioactivity that can be harvested on glass fibre filters. Cytotoxic effector functions of T cells (e.g. FasL) induce DNA fragmentation in target cells. By co-culturing labelled targets with activated T cells cytotoxicity can be measured using this assay.
Jurkat IT target cells were labelled with 10 µCi/ml [methyl-\(^3\)H]-thymidine (Hartmann Analytic, Braunschweig, Germany) for 3 h. Primary T cell blasts or A1.1 cells (effector cells) were serially diluted in a 96-well plate coated with anti-CD3 antibody (1 µg/ml), and treated with 3d2 or CsA for 2 h. Labelled target cells were washed twice, resuspended in complete medium, and 100 µl cell suspension (20'000 cells) were added to the plate and incubated with the effector cells for 18 h. Afterwards, \(^3\)H-thymidine-labelled target cell DNA was harvested onto glass fiber filters using Omnifilter-96 cell harvester (Perkin, Rodgau, Germany Elmer). Consequently, 30 µl Microscint (Perkin Elmer, Rodgau, Germany) was added and beta counts were measured using TopCount Microplate Scintillation Counter (Perkin Elmer, Rodgau, Germany). Subsequently, DNA fragmentation was calculated as % of DNA fragmentation = 100 x (1 - cpm experimental sample/cpm targets only).

7.2.17 \(^3\)H-thymidine incorporation assay (Proliferation)

Cellular proliferation was determined by incorporation of radioactively labelled \(^3\)H-thymidine. One day prior treatment cells (2 x 10\(^4\) cells/well) were seeded into a cell culture plate. After 6 h of treatment with 3d2, cells were labelled with 0.5 µCi [methyl-3H]-thymidine (Hartmann Analytic, Braunschweig, Germany) per well for 18 h. Afterwards, \(^3\)H-thymidine-labelled target cell DNA was harvested onto glass fiber filters using Omnifilter-96 cell harvester (Perkin, Rodgau, Germany Elmer). Consequently, 30 µl Microscint (Perkin Elmer, Rodgau, Germany) was added and beta counts were measured using TopCount Microplate Scintillation Counter (Perkin Elmer, Rodgau, Germany). Subsequently, DNA fragmentation was calculated as % of DNA fragmentation = 100 x (1 - cpm experimental sample/cpm targets only).

7.2.18 MTT assay

MTT assay was used to determine the respiratory activity of the cells. Therefore, cell culture medium was discarded and replaced with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) solution (Sigma-Aldrich), dissolved in complete medium. Plates were incubated under cell culture condition for 1 h. After MTT reduction to purple formazan, the MTT solution was discarded and replaced with 100 µl DMSO (Roth, Karlsruhe, Germany) to dissolve the formazan products. Plates were incubated for 15 min in the dark at RT. After gently mixing of the plates, the intensity of the colored solution was quantified by measuring the absorbance at \(\lambda=562\) nm on at the Infinite 200 PRO plate reader (Tecan, Crailsheim, Germany). Cell death induction (%) was calculated as 100 x (1− (OD experimental mean value (−substrate blank)/OD control mean value (− substrate blank))).
7.2.19 Enzyme-linked immunosorbent assay (ELISA)

With an enzyme-linked immunosorbent assay cytokines in the production of murine pro-inflammatory cytokines in the cell supernatants and serum were determined. For the detection of murine TNFα matched antibodies pairs were used with anti-mouse/rat TNFα, (Biolegend # 506102, 3 μg/ml) as the capturing antibody and biotinylated anti-mouse/rat TNFα (Clone Poly5160, Biolegend # 516003, 1 μg/ml) as the detecting antibody. Murine IL-6 was detected with anti-mouse IL-6 (Biolegend #504501, 1 μg/ml) and biotinylated anti-mouse IL-6 (Biolegend # 504601, 1 μg/ml).

First, the capture antibody was diluted in coating buffer (100 mM NaHCO₃, 33 mM Na₂CO₃, pH 9.5) and a 96-well Nunc Maxisorp plate was coated with 50 μl o/n at 4°C. After washing the ELISA plate 4x with 400 μl of ELISA-Wash (0,05% Tween 20 in PBS) buffer 200 μl of Assay diluent (1% BSA in PBS) were incubated for 1 h at RT to block the unspecific bindings. After the washing procedure, 100 μl of the cytokine standard and the samples 1:2 diluted in Reagent diluent were incubated for 2h at RT. After the washing, 100 μl of the biotinylated detection antibody was incubated for 1 h at RT. After the further washing procedure, 100 μl of the streptavidin conjugated to horseradish-peroxidase (Calbiochem #189733) was incubated for 30 min at RT. After washing the plate 100 μl of TMB Working solution (equal volumes of TMB Substrate A and TMB Substrate B, TMB Substrate Set Biolegend #421101) was incubated for 15-20 min at RT in the dark and the reaction was stopped by addition of 100 μl of 1 M H₂SO₄. The absorption was measured within 30 min at the Infinite 200 PRO plate reader (Tecan, Crailsheim, Germany) at a wavelength of 450 nm (reference wavelength 570 nm).

IL-1β was analyzed with a commercially available kit (R&D Kit DuoSet ELISA Development system Mouse IL-1β # DY401-05) according to the manufacturer’s instructions.

7.2.20 Western blot analysis

Western blot analysis allows detection and quantification of proteins from cell and tissue lysates. Proteins are separated by their size, transferred onto a membrane and detected with specific antibodies which are labelled for visualization over a chemiluminescence reaction. Frozen liver tissue was homogenized in NP-40 lysis Buffer (150 mM NaCl, 50 mM Tris, pH 7.6, 1 mM EDTA and 1% NP-40) using the TissueLyser II (Qiagen, Hilden, Germany) for 3 min and 300 Hz. The protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Darmstadt, Germany). BMDM were lysed in NP-40 lysis Buffer 15 min on ice. Samples diluted within 5x loading Buffer (250 mM Tris-Cl (pH 6.8), 500 mM DTT, 10% SDS, 50% glycerol, 0.5% bromophenol blue) were denatured at 95 °C for 5 min before loading equal amounts on the polyacrylamide gels, consisting of a 12% resolving gel (4 ml Rotiphorese Gel
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30, 2.5 ml 1.5 M Tris (pH 8.8), 100 µl 10% SDS, 100 µl 10% ammonium persulfate, 12 µl TEMED, 3.3 ml H2O) and a 5% stacking gel (830 µl Rotiphoresse Gel 30; 630 µl 1.0 M Tris (pH 6.8), 50 µl 10% SDS, 50 µl 10% ammonium persulfate, 12 µl TEMED, 3.4 ml H2O) on top. PageRuler Prestained Protein Ladder (Thermo Scientific, Darmstadt, Germany) was loaded as a molecular weight standard. Samples were run through the stacking gel with 70 V in SDS Running Buffer (25 mM Tris, 250 mM glycine, 0.1% SDS (w/v), pH 8.3). When samples reached the separation gel, the voltage was increased to 120 V. The electrophoretically separated proteins were then transferred onto polyvinylidene difluoride (PVDF) (Roche, Mannheim, Germany) membranes. Therefore, a stack out of a sponge, two Whatman papers, the PVDF membrane, the SDS-PAGE gel, two Whatman papers and a sponge was prepared and fixed in a Western Blot chamber on ice and covered with Transfer Buffer (192 mM glycine, 25 mM Tris base, 0.1% SDS, 21.5% methanol). For the transfer, a current of 350 mA was applied for 80 min. Subsequently, membranes were blocked with TBS-T (8 g/l NaCl, 0.2 g/l KCl, 3 g/l Tris base, 0.1% Tween) /5% bovine serum albumin for 1 h.

Primary antibodies (see Table 4) were diluted in TBS-T/5% bovine serum albumin and added to the membranes, which incubated overnight at 4 °C. Membranes were washed three times for 5 min with TBS-T and then incubated for 1 h with a horse radish-coupled secondary antibody diluted (1:5’000) in TBS-T. Again, the membranes were washed three times for 5 min with TBS-T. Afterwards, 1 ml ECL solution (2.5 mM Luminol, 0.4 mM p-Coumaric acid, 10 mM Tris, pH 8.5 (add fresh: 0.015% H2O2)) was added directly to the membranes for imaging. Chemiluminescent signals were detected with an Image Quant LAS 4000 (GE Healthcare, Dornstadt, Germany) and images were taken.

7.2.21 ConA-induced hepatitis

Injection (i.v.) of the lectin Concanavalin A (ConA) in mice induces the activation of liver-homing T cells and NK cells, resulting in expression of FasL and consequently in apoptosis of sinusoidal endothelial cells and hepatocytes. This ultimately leads to acute hepatitis, as evidenced by areas with extensive tissue damage in the liver, and increased serum transaminases (Tagawa et al., 1998).

ConA was diluted in sterile endotoxin-free PBS. Male mice were pretreated with 3d2 (50 mg/kg body weight, dissolved in 10% DMSO in PBS) by i.p. injection 1 h before the ConA injection (i.v. 10 mg/g body weight). Six hours after the challenge, mice were sacrificed and serum samples were collected. Liver samples were snap-frozen in liquid nitrogen and then stored at -80 °C for RNA isolation and protein assays, or immersed in 4% formaldehyde in PBS for 24 h at 4 °C for histology and cleaved caspase 3 immunohistochemistry. Furthermore,
serum was centrifuged twice at 1’000 x g for 10 min. TNF-ELISA and alanine aminotransferase (ALT) in the sera was measured using a colorimetric kit (Teco Diagnostics, Anaheim, CA, USA) according to the manufacturer’s instructions.

**7.2.22 Histology and immunohistochemistry**

Tissue sections were generated from formalin-fixed and paraffin-embedded liver tissue. For histological analysis, they were stained with hematoxylin and eosin. Cleaved caspase 3 as a marker of apoptotic cells was detected by immunohistochemistry as described previously (Jakob et al., 2008).

Mouse liver tissue was immersed in 4% formaldehyde in PBS for 24 h at 4°C and for long-term storage transferred to 0.4% formaldehyde solution. Tissues were embedded in paraffin using the Microm STP 120 spin tissue processor (ZEISS, München, Germany). Sections at 4-5 μm thickness were cut on a rotary microtome Hyrax C 50 (ZEISS, München, Germany) and transferred to Superfrost plus slides (Thermo Scientific).

For deparaffinization, the slides were heated up to 60 °C. The tissue sections were hydrated by immersing in xylene for 5 min and afterwards in 100% ethanol for 5 min, followed by 5 min in 90%, 80% and 70% ethanol, three times for each step. Sections were rinsed under running tap water for 5 min.

For the hematoxylin and eosin staining, sections were first stained in hematoxylin for 5 min followed by rinsing under running tap water for 5 min. Next, sections were stained with eosin for 4 min followed by rinsing under running tap water for 5 min.

For the cleaved caspase 3 immunohistochemistry, after the deparaffinization and hydration, sections were first immersed in TBS Buffer (Tris-buffered saline: 25 mM, pH 7.5). For antigen retrieval, sections were transferred into a glass jar with Na-citrate buffer (10mM, pH 6.0) with 6 zeolites and were microwaved 3x for 5 min. After cooling down, sections were immersed in TBS Buffer. The endogenous peroxidase activity is quenched by incubation with a peroxidase blocking solution (1% H2O2 in TBS) for 10 min and immersed in TBS Buffer for 5 min. The sections were blocked with 100µl antibody dilution buffer (5% goat serum in TBS) for 30 min. Afterwards, the sections were incubated with 50 µl rabbit cleaved caspase 3 antibody (1:100 dilution in antibody dilution buffer) in a humidified chamber overnight at 4 °C. Sections were immersed in TBS Buffer and were washed in fresh TBS for 10 min 3x while shaking. Next, the sections were incubated with 50 µl biotinylated secondary goat anti-rabbit antibody (1:100 dilution in antibody dilution buffer) in a humidified chamber for 2 h at RT. Again, sections were immersed in TBS Buffer and were washed in fresh TBS for 10 min 3x while shaking. The immunoperoxidase detection system Vectastin ABC Kit (Vector Laboratories, CA, US) was
prepared according to the manufacturer’s protocol and sections were incubated with 100 µl ABC reagent for 30 min at RT. Sections were immersed in TBS Buffer and were washed in fresh TBS for 10 min 3x while shaking. Next, the Peroxidase Substrat Kit (ImmpACT DAB, Vector Laboratories, CA, US) was prepared according to the manufacturer’s protocol and sections were incubated with 50 µl peroxidase substrate for 10 min until the tissue gets a brown colour. Sections were rinsed under running tap water for 5 min and counterstained with hematoxylin (Sigma-Aldrich, Steinheim, Germany) for 10 sec followed by another 5 min of rinsing by running tap water.

For the dehydration of the hematoxylin and eosin, as well as cleaved caspase 3 stainings, the sections were dehydrated with ascending graded alcohols 70%, 90%, 100% ethanol for 30 sec each and immersed in xylene for 5 min 3x. Last, sections were mounted in mounting medium Fluoroshield (Sigma-Aldrich, Steinheim, Germany).

7.2.23 Statistical analysis

Student’s t-test, multiple t-test and ordinary one-way ANOVA were performed on Prism7 software (GraphPad Software, La Jolla, CA, USA) to define significant differences between experimental groups. A P-value of <0.05 was regarded as significant.
8 RESULTS

8.1 LRH-1 expression in the T cell lineage

The orphan nuclear receptor LRH-1 has been reported to be predominantly expressed in endoderm-derived organs, such as liver, pancreas and the intestine. In these tissues, LRH-1 expression is extensively studied and multiple effector functions are described (Bookout et al., 2006). Importantly, LRH-1 negatively regulates the rapid inflammatory response of acute phase proteins in the liver (Venteclef and Delerive, 2007) and is crucial in the regulation of immune responses in the intestinal mucosa, where it contributes to the intestinal epithelial barrier integrity by intestinal stem cell proliferation and it determines the synthesis of immunoregulatory glucocorticoids (Mueller et al., 2006). However, little is currently known whether LRH-1 is also expressed in tissues of mesodermal or hematopoietic origin. Of interest, Benod et al. observed in an immunohistochemical analysis of LRH-1 expression in human pancreatic tumors that the immune cells infiltrating the tumor tissue were positively stained for LRH-1 (Benod et al., 2011).

LRH-1 expression was therefore analyzed in different organs of wild type mice. High LRH-1 expression levels in liver tissue and the colon were confirmed. Interestingly, low but detectable LRH-1 mRNA levels were observed in primary and secondary lymphatic organs, such as the mesenteric lymph nodes, spleen, and thymus (Figure 8A). LRH-1 was also detected in highly purified splenic CD4+ and CD8+ T cells, as well as in the murine T cell hybridoma cell line A1.1 (Figure 8B). Thus, the LRH-1 expression is not restricted to endodermal tissue, but is also found to be in the T cell lineage.

![Figure 8: LRH-1 gene expression in lymphatic tissues and T cells](image-url)
RESULTS

A) Relative LRH-1 mRNA expression in liver, colon, mesenteric lymph nodes (MLN), spleen and thymus. Mean values from 3 C57BL/6 mice are shown. Each data point represents an individual mouse. B) Relative LRH-1 mRNA expression in total spleen, sorted splenic CD4+ and CD8+ T cells, and A1.1 T cell hybridoma cells. Mean values are shown for spleen and sorted cells (n = 3-5), where dots represent an individual mouse.

8.2 FASLG is a direct transcriptional target of LRH-1

To understand the potential role of LRH-1 in the adaptive immune system, especially in the regulation of T cell effector functions, first putative LRH-1 target genes were screened using a bioinformatics approach performed by Ann-Kathrin Gaiser. The screening revealed two putative LRH-1 binding sites in the human FASLG promoter with the consensus sequence NN AGGTCA NN, one sense-orientated at position -734, and one anti-sense orientated at position -387 (Figure 9). Since FasL is an extrinsic cell death receptor ligand and a key molecule in T cell apoptosis, further investigations were taken in order to validate FASLG as a potential LRH-1 target gene.

![Figure 9: Schematic presentation of the FASLG promoter](image)

LRH-1 binding sites (BS1 and BS2) in the FASLG promoter and nucleotides mutated by site-directed mutagenesis.

Thus, it was analyzed whether LRH-1 directly binds to these putative binding sites using chromatin immunoprecipitation (ChIP). Human leukemic Jurkat IT T cells were transiently transfected with a control plasmid or a FLAG-tagged LRH-1 expression construct, and subsequently nuclear proteins binding to genomic DNA were cross-linked. Afterwards, LRH-1 bound to specific DNA sequences was immunoprecipitated using anti-FLAG antibodies. LRH-1 binding to the putative consensus sequences in the FASLG promoter or the SHP promoter, a known LRH-1 target gene (Lee et al., 1999), was analyzed using sequence-specific PCR. LRH-1 was specifically precipitated from its binding site in the SHP promoter, in contrast to the negative IgG isotype control, as it could be seen by the bands of the SHP PCR product in immunoprecipitated ChIP eluate, while the IgG controls reveal no bands (Figure 10A). Similarly, the sequence of the proposed binding site 2 (BS2) could be specifically detected in
the ChIP eluate, whereas no amplification of the binding site 1 (BS1) was observed. These findings indicate that LRH-1 is specifically binding to the FASLG promoter.

In T cells FasL transcription is regulated by several well-described transcription factors interacting with the FasL promoter and leading to its activation (Kavurma and Khachigian, 2003). Therefore, the role of LRH-1 in the transcriptional regulation of FasL expression was analyzed using luciferase promoter reporter constructs. Co-transfection of Jurkat IT cells with an LRH-1 expression vector resulted in a significant activation of a 1.2 kb sequence of the human FASLG promoter compared to the control plasmid (Figure 10B). Subsequently, the two putative binding sites of LRH-1 in the FasL promoter were mutated (as indicated in Figure 9). Three base pairs of the predicted core binding sites were exchanged by site-directed mutagenesis. The introduced mutations have already been shown to abrogate LRH-1 binding in the CYP11B1 promoter (Mueller et al., 2006). The mutation of both LRH-1 consensus sequences in the FASLG promoter strongly reduced LRH-1-induced FASLG promoter activity (Figure 10B). To study the importance of the individual binding sites single mutations in the FASLG reporter constructs were introduced. Notably, mutation of BS1, but not BS2 resulted in significantly reduced FASLG promoter activity (Figure 10C). These findings demonstrate FasL as an LRH-1 target gene in T lymphocytes, since LRH-1 directly regulates FASLG promoter activity via specific response elements.
A) Chromatin immunoprecipitation (ChIP) of LRH-1 was done using anti-FLAG antibodies or control IgG, and binding to the SHP promoter (SHP), or to BS1 and BS2 in the FASLG promoter was detected using sequence-specific PCR. Jurkat IT cells were transfected with control plasmid (pcDNA) or FLAG-tagged LRH-1 (LRH-1). IP, immunoprecipitation; TF, transfection. One representative experiment out of 3 is shown. For reporter assays in B) and C) For reporter assay Jurkat IT cells were transfected with a control luciferase plasmid (HsLuc), the wild type human FASLG luciferase promoter reporter (HFLP) or the reporter with mutated BS1, BS2 or both. The cells were co-transfected with a control expression plasmid (pcDNA) or an LRH-1 expression plasmid (LRH-1). Luciferase reporter activity was measured and normalized to luciferase control plasmid. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (unpaired t-test; * P-value < 0.05; ** P-value < 0.01; *** P-value < 0.001).

8.3 Pharmacological inhibition of LRH-1 activity

LRH-1 transcriptional activity fulfills important regulatory functions in early differentiation and embryogenesis, therefore the genomic deletion of LRH-1 results in embryonic lethality (Rausa et al., 1999, Pare et al., 2004, Gu et al., 2005b, Wagner et al., 2010). Thus, mice with a T cell-specific deletion of LRH-1 were generated by breeding a transgenic mouse, which carries the gene for the Cre recombinase under the CD4 promoter, with a mouse that has its LRH-1 gene floxed on both alleles (Sauer, 1998). Since thymocytes undergo a double-positive stage during thymic development, both CD4+ and CD8+ T cells are depleted of LRH-1. The analysis of splenocytes revealed much less single positive CD4+ and a reduction in CD8+ T cells in LRH-1 deficient cKO cells when compared with the floxed control cells (Figure 11). Due to the different distribution of CD4+ and CD8+ T cells, a genetic model cannot be used to further address the role of LRH-1 in the regulation of FasL expression in T cells and associated functions.

![Figure 11: Phenotype of splenocytes in mice with LRH-1 deleted in T cells](image)

Dot blot of flow cytometry analysis of isolated T cells from floxed mice LRH-1 L2/L2 and T cell specific LRH-1 knockout mice LRH-1 cKO after stained with anti-CD4 and anti-CD8 antibodies (data from one experiment).
In this regard, Benod et al. described a series of pharmacological inhibitors with LRH-1-specific inhibitory functions (Benod et al., 2013). Most notably, compound 3d2 showed strong LRH-1 inhibitory effect, whereas its close homologue SF-1 was not affected. Thus, this pharmacological inhibitor was further evaluated to study the role of LRH-1 in the regulation of FasL in T cells. In order to test the capacity of 3d2 in inhibiting LRH-1 activity, the human embryonic kidney cell line HEK 293T was co-transfected with an LRH-1 expression vector and an LRH-1 responsive reporter construct containing 5 repeats of an LRH-1 consensus sequence. As predicted, 3d2 showed a dose-dependent inhibition of LRH-1 reporter activity as observed by the decrease in relative light units (Figure 12A). In contrast, the compound 7 (cpd7), the structural homologue of 3d2, did not show any inhibitory effects on LRH-1 activity, confirming the specificity of 3d2 for LRH-1 (Figure 12B).

Next, the effect of pharmacological inhibition of LRH-1 activity was investigated in Jurkat IT cells. PMA-induced MAP kinase activation has been reported to promote LRH-1 phosphorylation and activation of its transcriptional activity (Lee et al., 2006). Furthermore, PMA in combination with ionomycin activates T lymphocytes (Villalba et al., 1999). Interestingly, confirming LRH-1 expression also in human Jurkat IT cells, TCR stimulation with PMA plus ionomycin increased LRH-1 mRNA levels (Figure 13A). In LRH-1 luciferase reporter studies, PMA plus ionomycin as well as LRH-1 overexpression induced LRH-1 activity in Jurkat IT cells, which both was inhibited by treatment of cells with 3d2 (Figure 13B and C).

Figure 12: Validation of LRH-1 inhibitor 3d2
A) For reporter assay HEK293T cells were co-transfected with an LRH-1 luciferase reporter construct in combination with control plasmids (pcDNA) or LRH-1 expression plasmid. Cells were treated with increasing concentrations of the LRH-1 inhibitor 3d2. Luciferase reporter activity was measured and normalized to luciferase control plasmid. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA ***P-value < 0.001). B) HEK293T cells were co-transfected with an LRH-1 luciferase reporter construct and an LRH-1 expression plasmid. Cells were treated with buffer control (UT), 3d2 or cpd7 equally concentrated (60 μM). Inhibition of the LRH-1 transcriptional activity by 3d2 and cpd7 is depicted as percentage compared to control cells. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (unpaired t-test **P-value < 0.01; ns, not significant).
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Figure 13: Pharmacological modulation of LRH-1 expression and activity in Jurkat IT T cells

A) LRH-1 expression was analyzed by quantitative PCR in Jurkat IT cells stimulated with control buffer, or PMA (50 ng/ml) and ionomycin (500 ng/ml) for 18 h. B) For reporter assay Jurkat IT cells were transfected with an LRH-1 luciferase reporter construct, treated with control buffer (UT) or 3d2 (15 μM), and stimulated with control medium or PMA/ionomycin (50/500 ng/ml) for 18 h. Luciferase reporter activity was measured and normalized to luciferase control plasmid. Mean values of triplicates ± SD of a representative experiment (n=2) are shown (unpaired t-test *P-value < 0.05). C) For reporter assay, Jurkat IT cells were co-transfected with an LRH-1 luciferase reporter construct, and control plasmids (pcDNA) or an LRH-1 expression plasmid. Cells were treated with 3d2 (15 μM) or buffer control (UT) for 18 h. Luciferase reporter activity was measured and normalized to the luciferase control plasmid. Mean values of triplicates ± SD of a typical experiment (n=2) are shown (unpaired t-test ***P-value < 0.001).

These data illustrate that also the human leukemic Jurkat IT cells express LRH-1 and that LRH-1 expression, as well as activity, is induced upon activation with PMA and ionomycin. Interestingly, co-treatment with the compound 3d2 efficiently reduced LRH-1 activity in T cells and even inhibited the endogenous LRH-1 levels in activated T cells without a transient LRH-1 overexpression.

8.4 Pharmacological inhibition of LRH-1 activity restricts FASLG promoter activity and expression

In T cells, TCR stimulation after binding to an antigen-derived peptide bound to MHC leads to a signalling cascade that activates transcription factors such as NFAT, EGR-2 and -3, AP-1 or NFκB, leading to FasL upregulation (Kavurma and Khachigian, 2003). This effect can be mimicked pharmacologically by PMA plus ionomycin (Villalba et al., 1999). After the successful validation of the pharmacological LRH-1 inhibition in human T cells, next the effect of LRH-1 inhibition on activation- and LRH-1-induced FasL expression was tested.

Gene expression analysis demonstrates that FasL mRNA expression was strongly induced after activation by PMA plus ionomycin (Figure 14A). Interestingly, overexpression of LRH-1 as well
as PMA and ionomycin stimulation induced human FASLG promoter activity (Figure 14B). 3d2 treatment inhibited the basal activation-induced as well as LRH-1 overexpression-induced FASLG promoter activity in a dose-dependent manner. Similarly, pharmacological inhibition of LRH-1 by 3d2 decreased PMA and ionomycin-induced FasL expression as seen in the control transfected cells (Figure 14C). Overexpression of LRH-1 in turn further increased FasL mRNA expression and was significantly inverted by 3d2.

In summary, these findings demonstrate that the pharmacological inhibition of LRH-1 by 3d2 results in reduced activation- and LRH-1-induced FASLG promoter activation in Jurkat IT cells. This transcriptional regulation translates further into FasL mRNA expression.

**Figure 14:** LRH-1 inhibition by 3d2 restricts FASLG promoter activity and expression

A) Human FasL mRNA expression of Jurkat IT cells after treatment with PMA/ionomycin (50/500 ng/ml) for 18 h was determined using quantitative PCR. Mean values of triplicates ± SD are shown. B) For reporter assay, Jurkat IT cells were co-transfected with a FASL luciferase promoter reporter, and control plasmids (pcDNA) or an LRH-1 expression plasmid. Cells were then stimulated with PMA/ionomycin (50/500 ng/ml) and the indicated concentrations of 3d2 for 18 h. Luciferase reporter activity was measured and normalized to luciferase control plasmid. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (unpaired t-test; **P-value < 0.01). C) Human FasL mRNA expression of Jurkat IT cells transfected with control plasmid (pcDNA) or LRH-1 expression plasmid was determined using quantitative PCR. Cells were pretreated with buffer control (UT) or 3d2 (20 μM) and stimulated with PMA/ionomycin (50/500 ng/ml). Mean values of triplicates ± SD of a representative experiment (n=2) are shown (unpaired t-test; **P-value < 0.01).

### 8.5 LRH-1 inhibition blocks activation-induced FASL expression and associated T cell suicide

FasL plays an important role in the regulation of T cell homeostasis. Restimulation of previously activated T cells results in a rapid induction of FasL expression and consequently the cell-autonomous induction of FasL/Fas-mediated suicide (Brunner et al., 1995), which is referred to as AICD (Figure 15). Hence, FasL/Fas-mediated apoptosis contributes to peripheral T cell homeostasis (Fischer et al., 2000). Therefore, it was investigated how inhibition of LRH-
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1 and associated regulation of FasL expression affects AICD in T cell hybridoma cells and primary T lymphocytes.

Figure 15: AICD

Scheme presenting the FasL-mediated activation-induced cell death (AICD) upon T cell receptor activation (TCR) by anti-CD3 antibody (aCD3).

TCR stimulation with immobilized anti-CD3 results in high mRNA expression levels of FasL in the murine T cell hybridoma cell line A1.1 (Figure 16A). Similar as seen in Jurkat IT cells, 3d2 treatment resulted in a strong decrease of activation-induced FasL expression in A1.1 cells. However, this was not observed in cells treated with the structural homologue cpd7 used as negative control (Figure 16A). Furthermore, the effect of 3d2 on the function of FasL in mediating AICD in A1.1 cells was investigated. When A1.1 cells were activated with immobilized anti-CD3 for 18 h, a strong apoptosis induction was observed by Annexin V staining (Figure 16B) (Brunner et al., 1995). Along with the observation that LRH-1 inhibition by 3d2 blocks activation-induced FasL expression, a dose-dependent inhibition of AICD was observed, as can be seen by a reduced percentage of Annexin V positive cells in a dose-dependent manner. In order to confirm that AICD is dependent on FasL a neutralizing anti-FasL antibody was used (Wasem et al., 2003). Indeed, the anti-FasL antibody treated samples showed no apoptosis induction after stimulation with anti-CD3. To further validate the role of LRH-1 in activation-induced FasL expression we employed another recently described small molecule repressor of LRH-1, SR1848 (Corzo et al., 2015). The data demonstrate that activation-induced FasL expression in A1.1 cells was also strongly inhibited when treated with 5 μM SR1848 and that the inhibition was even more pronounced than treatment with 20 μM 3d2 (Figure 16C). Although toxic effects at higher concentrations were observed, low-dose treatment of SR1848 significantly decreased AICD (Figure 16D). Supporting this, similar results were also obtained for the 3d2 treatment of primary murine T cells. Isolated spleen cells were therefore activated with the lectin ConA for 24 h and expanded using the cytokine IL-2 for 4 days in order to generate T cell blasts. In the same way, 3d2 treatment of anti-CD3 restimulated T cells substantially inhibited the induction of FasL mRNA expression (Figure
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AICD was as well dose-dependently inhibited by 3d2 in primary murine T cells. It is well known that activation-induced FasL expression is transcriptionally controlled by NFAT (Anel et al., 1994, Brunner et al., 1996). Accordingly, treatment of primary murine T cells with CsA which inhibits the NFAT activator calcineurin (Brunner et al., 1996), completely abrogated the FasL-mediated AICD and was giving proof of FasL as mediator for AICD (Figure 16F). These experiments with pharmacological LRH-1 inhibition show that LRH-1 regulates murine FasL expression and consequently FasL-mediated AICD.

Figure 16: LRH-1 inhibition blocks activation-induced FasL expression and associated T cell suicide

A) FasL mRNA was assessed by quantitative PCR. A1.1 cells were pre-incubated for 2 h with control buffer (UT), 3d2 or cpd7 (20 μM) and then stimulated with plate-bound anti-CD3 (1 μg/ml) for 6 h. Mean values of triplicates ± SD of three independent experiments are shown (unpaired t-test; **P-value < 0.01; ns, not significant). B) Annexin V-FITC was analyzed by flow cytometry. A1.1 cells were pre-incubated for 2 h with 3d2 at indicated concentrations or 5 μg/ml FasL antibody, followed by stimulation with plate-bound anti-CD3 (1 μg/ml) for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA ***P-value < 0.001). C) FasL mRNA expression was assessed by quantitative PCR. A1.1 cells pre-incubated for 2 h with control buffer (UT), 20 μM 3d2 or 5 μM SR1848, and stimulated with plate-bound anti-CD3 (1 μg/ml) for 6 h. Mean values...
of triplicates ± SD of a representative experiment (n=2) are shown (unpaired t-test ***P-value < 0.001). D) Annexin V-FITC was analyzed by flow cytometry. A1.1 cells were pre-incubated for 2 h with indicated concentrations of SR1848, followed by stimulation with plate-bound anti-CD3 (1 μg/ml) for 18 h (one-way ANOVA ***P-value < 0.001). E) FasL mRNA expression was assessed by quantitative PCR. Primary murine T cell blasts were pre-treated with control buffer (UT) or 3d2 (20 μM), and stimulated with plate-bound anti-CD3 (1 μg/ml) for 4 h. Mean values of triplicates ± SD are shown. F) Annexin V-FITC was analyzed by flow cytometry. Primary murine T cell blasts were pre-incubated for 2 h with indicated concentrations of 3d2 or 100 nM CsA, and then stimulated with plate-bound anti-CD3 (1 μg/ml) for 6 h. Mean values of triplicates ± SD of a representative experiment (n=2) are shown (one-way ANOVA **P-value < 0.01; ***P-value < 0.001).

### 8.6 LRH-1 inhibitor 3d2 reduces FasL-mediated cytotoxicity

FasL is known to play an important role in cell-mediated cytotoxicity. The interaction of membrane-bound FasL with the Fas receptor on target cells activates a caspase cascade leading to apoptosis (Figure 17A) (Suda et al., 1993). Consequently, the cytotoxic effect of activated effector cells on Fas receptor-expressing and FasL-sensitive target cells, such as Jurkat IT cells was investigated. Jurkat IT cells were therefore labelled with ³H-thymidine to assess the apoptosis-induced DNA fragmentation by harvesting the cells onto glass fiber filters. Jurkat IT target cells were co-cultured with A1.1 effector cells for 18 h at different effector-target ratios.

Activation of A1.1 cells by immobilized anti-CD3 induced a strong apoptosis induction in Jurkat IT cells (Figure 17B). In contrast, no apoptosis induction was observed in target cells without stimulation. In line with our previous results, treatment of activated A1.1 cells with 3d2 significantly inhibited FasL-mediated cytotoxicity as seen in all effector-target ratios. Furthermore, the FasL-dependency of this cytotoxic effect was confirmed by the specific inhibitor CsA. In addition, similar results were obtained when FasL-mediated cytotoxicity was analyzed in primary murine T cells. Only anti-CD3-activated primary T cells were able to induce apoptosis in Jurkat IT target cells whereas 3d2 treatment inhibited the effect of FasL cytotoxicity in a dose-dependent manner (Figure 17C).

These data confirm that inhibition of LRH-1 activity by 3d2 blocks activation-induced FasL expression and strongly reduces associated FasL-mediated cytotoxicity.
RESULTS

Figure 17: LRH-1 inhibitor 3d2 prevents FasL-mediated cytotoxicity

A) Scheme showing the FasL-mediated cytotoxicity assay. B) The percentage of DNA fragmentation in target cells was assessed by 3H-thymidine labelled DNA. A1.1 cells were pre-incubated for 2 h with control buffer (UT), 20 μM 3d2 or 100 nM CsA, then stimulated with plate-bound anti-CD3 (3 μg/ml), and co-cultured with Fas-sensitive target cells at indicated effector-target ratios for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (multiple t-test ***P-value < 0.001). C) The percentage of DNA fragmentation in target cells was assessed by 3H-thymidine labelled DNA. Primary murine T cell blasts were pre-incubated for 2 h with indicated concentrations of 3d2 and stimulated with plate-bound anti-CD3 (3 μg/ml). T cells were co-cultured with Fas-sensitive target cells at an effector-target ratio of 1:1 for 18 h. Mean values of triplicates ± SD of a representative experiment (n=2) are shown (one-way ANOVA *P-value < 0.05; **P-value < 0.01).

8.7 LRH-1 inhibitor 3d2 protects from FasL-mediated liver immunopathology

FasL-mediated cytotoxicity and associated tissue destruction play a critical role in immunopathologies, such as T cell-mediated hepatitis. Hepatocytes are exquisitely sensitive to Fas-mediated apoptosis and rapidly die upon treatment with FasL (Suda and Nagata, 1997). Thus, the in vivo activity of 3d2 in a mouse model of FasL-mediated liver toxicity was investigated.

Intravenous (i.v.) injection of lectin ConA in mice induces the activation of liver-homing T cells and NK cells, resulting in expression of FasL and consequently, in apoptosis of sinusoidal endothelial cells and hepatocytes (Takeda et al., 2000, Bajt et al., 2000). This effect ultimately leads to acute hepatitis, as evidenced by areas with extensive tissue damage in the liver and
increased serum transaminases (Figure 18A, C, G) (Tagawa et al., 1998). FasL-dependency of ConA-induced hepatotoxicity was confirmed by the reduced liver damage of FasL mutant (gld) mice (Figure 18A) (Takahashi et al., 1994). Although the use of 3d2 has been reported previously in zebrafish (Nissim et al., 2016), so far it had never been applied to mammals in order to inhibit LRH-1-regulated processes. Even though the liver expresses high levels of LRH-1, mice injected intraperitoneal (i.p.) with 50 mg/kg body weight 3d2 alone, tolerated 3d2 well with a minor increase in serum transaminases (Figure 18C). More importantly, ConA treatment alone induced an extensive liver damage as evidenced by a profound increase in serum transaminases, caspase 3 activation, apoptosis and histological liver damage (Figure 18A, C, F, G). In contrast, 1 h pretreatment with 3d2 almost completely abrogated all signs of liver damage and hepatitis (Figure 18C-G). Furthermore, in line with the in vitro experiments described above, this protective effect of 3d2 appears to be mediated by inhibition of LRH-1-regulated FasL expression and associated liver damage, as ConA-induced FasL expression in liver tissue was completely reversed by 3d2 (Figure 18B). The presented data indicate that LRH-1 regulates activation-induced FasL expression in T cells, and that pharmacological inhibition of LRH-1 has a potential in therapeutic applications on FasL-mediated liver immunopathology.
RESULTS

Figure 18: LRH-1 inhibition protects from FasL-mediated liver damage

A) Serum ALT levels of wild type (wt) and gld (FasL mutant) mice injected with ConA (10 mg/g body weight) for 6 h. Data of individual mice (n=1-6) and mean values ± SD are shown (unpaired t-test; *P-value < 0.05; ns, not significant). B) FasL mRNA expression in liver tissue was measured by quantitative PCR. Wild type mice were pre-treated with 3d2 (50 mg/kg body weight) and challenged with ConA (10 mg/g body weight) or PBS. (n=3, unpaired t-test; *P-value < 0.05). C) Serum ALT levels of wild type mice pre-treated with 3d2 (50 mg/kg body weight), challenged with ConA (10 mg/g body weight) determined after 6 h (n=3-8, unpaired t-test; *P-value < 0.05; **P-value < 0.01.
value < 0.01). D) Mice were treated as in C) and caspase 3 activation (19 and 17 kD fragments) in liver tissue was analyzed by Western blotting. E) Densitometry analysis of the experiment shown in D. Mean values of triplicates ± SD are shown. F) Immunohistochemical detection of apoptotic, cleaved caspase 3-positive cells in liver sections from mice treated with PBS, ConA- or ConA plus 3d2 (scale bar, 150 μm). G) Hematoxylin/Eosin staining of liver sections from mice treated with PBS, ConA- or ConA plus 3d2 (scale bar, 300 μm, magnification 150 μm).

8.8 LRH-1 inhibitor 3d2 decreases TCR- and LPS-induced TNFα production in splenocytes

Pro-inflammatory processes are known to be regulated by the cytokine TNFα, which is produced by activated macrophages, CD4⁺ and CD8⁺ T cells, natural killer cells, dendritic cells and other immune and non-immune cells (Wajant et al., 2003). Since a correlation between LRH-1 and the pro-inflammatory cytokine TNFα was already described in the context of intestinal inflammation (Schoonjans et al., 2005, Coste et al., 2007), and TNFα is a close homolog of FasL, the role of LRH-1 on TNFα expression was investigated. Therefore, TNFα protein levels were measured after pharmacological LRH-1 inhibition by 3d2 in combination with different immune cell activating stimuli in isolated splenocytes, which are a very heterogeneous cell population consisting of T and B lymphocytes, dendritic cells and macrophages (Mebius and Kraal, 2005). The TCR was activated by immobilized anti-CD3 or PMA and ionomycin, while LPS was used to trigger TLR4-mediated immune cell activation.

The treatment of splenocytes using the indicated stimuli increased TNFα secretion (Figure 19A). Interestingly, 3d2-mediated LRH-1 inhibition prevented immune and T cell activation by PMA/ ionomycin, anti-CD3, as well as LPS as observed by decreased TNFα protein levels (Figure 19A). The LPS-induced and therefore also macrophage-mediated TNFα production was further confirmed by a dose-dependent 3d2-mediated inhibition of LPS-induced TNFα production, while the structural homolog cpd7, which served as negative control, did not show any reduction (Figure 19B). The results showed that LPS-induced TNFα was inhibited by 3d2 highlighting the relevance of LRH-1 in activated splenocytes.
RESULTS

Figure 19: LRH-1 mediates TCR- and LPS- induced TNF production in splenocytes

A) Murine TNFα was analyzed by ELISA. Splenocytes were pre-incubated for 2 h with 3d2 (30 μM), followed by stimulation with PMA/ionomycin (10/100 ng/ml), plate-bound anti-CD3 (1 μg/ml) and LPS (30 ng/ml) for 18 h. Mean values of triplicates ± SD of a representative experiment are shown (unpaired t-test *P < 0.05; ***P < 0.001). B) Murine TNFα was analyzed by ELISA. Splenocytes were pre-incubated for 2 h with 3d2 at indicated concentrations, followed by stimulation LPS (30 ng/ml) for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA ***P < 0.001).

8.9 3d2-mediated inhibition of LRH-1 activity in macrophages

LRH-1 expression in macrophages was recently described (Lefevre et al., 2015). After the 3d2 mediated inhibition of TNFα in splenocytes stimulated with LPS the question arises whether 3d2 directly targets LRH-1 in macrophages or affects T cells, which have been activated by macrophages. Therefore, it was tested whether LRH-1 activity in macrophages can be manipulated by 3d2.

First, it was confirmed that LRH-1 mRNA expression was detectable in ex vivo differentiated bone-marrow derived macrophages (BMDM) and the murine RAW 264.7 macrophage cell line in comparison to liver and spleen (Figure 20A). In order to analyze the transcriptional activity of endogenous LRH-1, RAW 264.7 cells were transfected with an LRH-1 luciferase reporter containing 5 copies of the LRH-1-binding motive. Indeed, active LRH-1 was observed compared to the empty backbone reporter control (Figure 20B). In contrast, transcriptional LRH-1 activity was significantly inhibited in transfected RAW 264.7 cells after treatment with the LRH-1 inhibitor 3d2, whereas the control treatment using cpd7 did not show any inhibitory effects (Figure 20C). The presented results hereby confirm the expression of LRH-1 in macrophages, which can be targeted by 3d2.
RESULTS

Figure 20: LRH-1 activity in macrophages is inhibited by 3d2

A) Relative LRH-1 mRNA expression in liver, spleen, bone-marrow-derived macrophages (BMDM) from C57BL/6 mice and RAW 264.7 cells. B) Endogenous LRH-1-dependent transcriptional activity of RAW 264.7 cells transfected with the LRH-1 luciferase reporter construct containing the LRH-1-binding-motive (5xRE LRH-1) or an empty luciferase reporter (pGL3). Luciferase reporter activity was measured and normalized to luciferase control plasmid. Mean values of triplicates ± SD of a typical experiment (n=3) are shown. C) Percent inhibition of the LRH-1 transcriptional activity by 3d2 and cpd7 was analyzed. RAW 264.7 cells were transfected with an LRH-1 luciferase reporter construct and treated with control buffer (UT), 3d2 or cpd7 (40 μM). Mean values of triplicates ± SD of a typical experiment (n=3) are shown (unpaired t-test *P < 0.05; ns, not significant).

8.10 LRH-1 inhibition reduces induction of pro-inflammatory cytokines in macrophages

In order to investigate whether the reduced TNFα levels in splenocytes are due to a direct effect of 3d2 on LRH-1 in macrophages, cells were treated with 3d2 and stimulated with LPS. Subsequently the secretion of TNFα as well as the pro-inflammatory cytokines IL-6 and IL-1β were analyzed.

As expected, the mRNA expression of TNFα, IL-6, and IL-1β in RAW 264.7 cells was induced in a dose-dependent manner using LPS stimulation (Figure 21A, B and C). Of interest, specific LRH-1 inhibition by 3d2 decreased the expression TNFα as well as IL-6 and IL-1β mRNA levels in LPS-stimulated macrophages (Figure 21A, B, and C). Consequently, LPS-induced TNFα and IL-6 protein levels in the supernatant of RAW 264.7 cells were also decreased dose-dependently by the 3d2-mediated LRH-1 inhibition (Figure 21D, E). Of note, IL-1β protein levels were not detected in RAW 264.7 cells (Figure 21F). In order to assess IL-1β protein in the supernatant of BMDM, cells were treated with ATP for 1 h prior to the ELISA analysis. TLR activation induces the synthesis of the inactive pro-IL-1β. However, the release of mature IL-1β requires the inflammasome to stimulate caspase 1 activation, which cleaves pro-IL-1β.
Therefore, ATP is needed to trigger the activation of the inflammasome in response to LPS (Franchi et al., 2009). In BMDM the synthesis of the pro-inflammatory cytokines was dose-dependently inhibited by 3d2 as shown similarly with RAW 264.7 cells (Figure 21G-I). Furthermore, the small molecule repressor of LRH-1, SR184 (Corzo et al., 2015), was used as an alternative mode of LRH-1 inhibition in order to confirm the specificity for LRH-1. Thus, RAW 264.7 cells pre-treated with SR1848 showed a significant decrease in their TNFα production (Figure 21J). Moreover, significant inhibition of TNFα was already detectable at low concentrations of 0.1 μM SR1848 confirming thereby a higher efficiency compared to 3d2 (Figure 21J).

In summary, these data show that LRH-1 is involved in the regulation of LPS-mediated expression of pro-inflammatory cytokines in macrophages since the specific inhibition of LRH-1 attenuates LPS-mediated effects.
Results

Figure 21: LRH-1 inhibition in macrophages attenuates the induction of pro-inflammatory cytokines

TNFα (A), IL-6 (B) and IL-1β (C) mRNA expression was determined by quantitative PCR in RAW 264.7 cells after pre-incubation for 2 h with control buffer (UT) or 40 μM 3d2 and subsequent stimulation with LPS at indicated concentrations for 18 h. Mean values of triplicates ± SD of three independent experiments are shown (unpaired t-test; **P < 0.01; ***P < 0.001; ns = not significant). Murine TNFα (D), IL-6 (E) and IL-1β (F) secreted protein levels were analyzed by ELISA. RAW 264.7 cells were pre-incubated for 2 h with 3d2 at indicated concentrations and stimulated with LPS at indicated concentrations for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA *P < 0.05; **P < 0.01; ***P < 0.001). Murine TNFα (G), IL-6 (H) and IL-1β (I) secreted protein levels were analyzed by ELISA. BMDM cells were pre-incubated for 2 h with 3d2 at indicated concentrations and stimulated with LPS at indicated concentrations for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA *P < 0.05; **P < 0.01; ***P < 0.001). J) Murine TNFα secreted protein levels were analyzed by ELISA. RAW 264.7 cells were pre-incubated for 2 h with SR1848 at indicated concentrations and then stimulated with LPS (100 ng/ml) for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA ***P < 0.001).
8.11 The LPS-signalling pathway is not affected by the 3d2 treatment

Since pharmacological inhibition of LRH-1 in macrophages revealed an overall decrease in the LPS-mediated induction of pro-inflammatory cytokines, the LPS signalling pathway was analyzed in detail in order to exclude an unspecific effect of 3d2. In general, LPS stimulation leads to activation of the MAPK family, which is composed of the ERK 1/2, p38 and JNK pathways and activates the transcription factor AP-1 (Lu et al., 2008). Additionally, LPS stimulation activates the IKK complex, which phosphorylates IκBα. This triggers IκBα ubiquitination and subsequent degradation resulting in the release of NFκB subunits p50 and p65, which are able to translocate to the nucleus (Gilmore, 2006). Upon activation, AP-1 and NFκB can thereby induce the transcription of pro-inflammatory cytokines. The described LPS signalling pathway is depicted in Figure 22.

![Figure 22: Schematic presentation of LPS signalling](image)

LPS signalling includes the activation of the MAPK family members, ERK 1/2, p38 and JNK pathways activating the transcription factor AP-1. LPS activates in parallel the IKK complex, which phosphorylates IκBα and activates the transcription factor NFκB. As a result, the pro-inflammatory cytokines TNF, IL-6, and IL-1β are produced.

BMDM were pre-treated for 2 h with 3d2, the negative control cpd7 or with the mitogen-activated protein kinase (MEK) inhibitor U0126, which blocks the ERK pathway (Scherle et al., 1998), and subsequently were stimulated with LPS for 30 min and 60 min. Specific phosphorylation of ERK 1/2, p38 and JNK proteins was detected as an indicator of activation upon LPS stimulation. ERK 1/2 phosphorylation was detectable after LPS stimulation and U0126 as positive control specifically blocked this activation pathway (Figure 23, top line). The same activation pattern was observed for p38 and JNK phosphorylation, while U0126, as expected, did not affect their pathways (Figure 23, second and third line). IκBα was degraded...
30 min after LPS stimulation, which confirms the activation of the NFκB pathway (Figure 23, bottom line).

In all, the activation patterns of the LPS signalling pathway were not affected by 3d2-mediated LRH-1 inhibition indicating that LRH-1 mediates the induction of pro-inflammatory cytokines in a different way.

![Figure 23: LPS-signalling pathway is not impaired by 3d2 treatment](image)

Phospho-specific ERK1/2, phospho p38, phospho JNK and IκB alpha were analyzed by Western blotting using tubulin as a loading control. BMDM were pre-incubated for 2 h with control buffer (UT), 3d2 or cpd 7 (20 μM), or U0126 (10 μM) and then stimulated with 100 ng/ml LPS for 30 and 60 min (representative experiment shown).

### 8.12 3d2-mediated effect on apoptosis, proliferation, respiration and cell morphology

Interesting observations were made during experimentation with RAW 264.7 cells treated for 48 h with 3d2 since they unexpectedly revealed a major pH difference in their supernatant. The assay medium, which contains phenol red as pH indicator, of untreated cells turned yellow indicating a decreased pH due to cell waste products, such as lactate (Seyfried, 2012). However, cells treated with 3d2 showed in a dose-dependent manner a loss of this change towards low pH indication. It is well known that sensitivity to pharmacological intervention can vary between different cell types (Kenakin, 2017). In T cells, the LRH1 inhibitor 3d2, but
especially SR1848, induced apoptosis at high concentrations (see Figure 16B, D, F). One could speculate that 3d2 leads to cell death induction in macrophages which correlates with a reduction of metabolic waste products.

Repeating these observations experimentally, the broad kinase inhibitor staurosporine was included as a positive control for cell death (Gescher, 2000). Indeed, the 3d2 treated wells at high concentrations did not change to an acidic pH after 48 h in culture, which was comparable to the staurosporine treated apoptotic cells (Figure 24A). In order to assess whether LRH-1 inhibition leads to cell death induction, RAW 264.7 cells were analyzed after 3d2 and SR1848 treatment for 18 h. However, no apoptosis induction measured by Annexin V staining was observed compared to the untreated control (Figure 24B). In addition, a dual cell life-death staining using the nucleic acid-binding dyes acridine orange (viable cells, green fluorescence) and propidium iodide (dead cells, red fluorescence) was performed and confirmed previous observations. The microscopic analysis of cells treated with the highest concentrations of the LRH-1 inhibitors did not reveal changes in the viability compared to the untreated control (Figure 24C). An alternative explanation for the missing pH shift in 3d2 treated samples, could be a defect in proliferation. LRH-1 was described to be involved in the proliferation of pancreatic, hepatic and in intestinal cells and SR1848 revealed an inhibition of proliferation in multiple cancer cell lines (Botrugno et al., 2004, Corzo et al., 2015). The proliferation index was analyzed by [3H]-thymidine incorporation in RAW 264.7 cells after 18 h of 3d2 treatment, but did not show differences between the control and the treated samples (Figure 24D). Interestingly, macrophage proliferation was blocked by LPS since they exit from the cell cycle during the M1 differentiation in order to save energy, a process which is induced by c-Myc suppression (Cocks et al., 1992, Liu et al., 2016).

In all, the missing pH shift in the 3d2 treated cells or even the reduction of the pro-inflammatory cytokine levels cannot be explained by apoptosis or proliferation.
RESULTS

Figure 24: Pharmacological LRH-1 inhibition prevents the pH shift of the culture medium, without affecting cell death or proliferation

A) Representative image of pH changes in cultures of RAW 264.7 cells after incubation with 3d2 at indicated concentrations (μM) and 2 μM staurosporine (S) for 48 h (n=3). B) Cell death was analyzed by Annexin V staining and flow cytometry. RAW 264.7 cells were incubated with 3d2 or SR1848 at indicated concentrations for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown. C) Life-death staining of RAW 264.7 cells treated as in B) and stained with acridine orange (green) and propidium iodide (red) (scale bar, 150 μm). D) Proliferation index was assayed in RAW 264.7 cells incubated with 3d2 at indicated concentrations and LPS 30 ng/ml. After 6 h cells were labelled with [3H]-thymidine and after 18 h cells were harvested. Mean values of triplicate'es ± SD of a representative experiment (n=3) are shown.

In order to further characterize RAW 264.7 cells after pharmacological LRH-1 inhibition, the mitochondrial respiration of RAW 264.7 cells was analyzed after 3d2 treatment for 18 h by using MTT assay. This colorimetric assay monitors the reduction of the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan precipitations in viable cells as indication of cellular respiration (Riss et al., 2004). The results show how cells stimulated with LPS presented higher respiratory activity compared to the control, while after
RESULTS

3d2 treatment LPS stimulated and unstimulated cells showed a decrease of respiratory activity in a dose-dependent manner (Figure 25A). Notably, cells treated with the control substance cpd7 were unaffected (Figure 25B). Xu et al. described LRH-1 as a critical regulator of the mitochondrial glutaminase 2 enzyme and its influence on the glutamine-induced metabolism and signalling pathway in hepatic cancer cells (Xu et al., 2016). Glutamine is metabolized through glutaminolysis to produce α-ketoglutarate (α-KG), which enters the tricarboxylic acid (TCA) cycle to generate ATP (DeBerardinis et al., 2008). In order to test whether the reduced respiratory capacity observed after LRH-1 inhibition is due to a blockage of this pathway, the measurement was repeated with α-KG or L-glutamine (L-Glut) supplementation. However, these supplementations were not able to restore the reduced respiratory activity after 3d2 treatment since the 3d2 mediated decrease of respiratory activity remained (Figure 25C). Pharmacological LRH-1 inhibition reduces cellular respiration, but this effect is independently of α-KG or L-glutamine generation.

Figure 25: 3d2-mediated LRH-1 inhibition reduces respiratory capacity

Respiratory activity of RAW 264.7 cells was analyzed by MTT. Cell were pre-incubation for 2h with 3d2 A) or cpd7 B) at indicated concentrations and then stimulated with LPS (30 ng/ml) for 18 h. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA *P < 0.05; ***P < 0.001). C) RAW 264.7 cells in complete medium (control), α-KG and L-Glut supplementation (2 mM) were pre-incubated for 2 h with 3d2 at indicated concentrations and then stimulated with LPS (30 ng/ml) for 18 h. The percentage of respiratory activity was normalized to the untreated control. Mean values of triplicates ± SD of a representative experiment (n=3) are shown (one-way ANOVA ***P < 0.001).

Distinct morphological changes after LPS stimulation can serve as a marker for macrophage activation (Williams and Ridley, 2000). Activated cells form lamellipodia, which are cytoskeletal protein actin projections on the leading edge of the cell, and show extensive cell spreading (Pi et al., 2014). Of interest, morphological analysis of the experimental RAW 264.7 cells revealed a similar phenotype in LPS-stimulated cells (Figure 26). While unstimulated cells showed an oval shape with few lamellipodia, the LPS stimulated cells became larger, more flattened and had more lamellipodia. On the contrary, 3d2-treated cells did not show any signs of LPS-mediated morphological changes. They were roundly shaped, grew in a three-dimensional manner, suggesting to present some macrophage activation defects.
RESULTS

Figure 26: Morphological changes after LPS stimulation
Representative microscopy images of RAW 264.7 cells were pre-incubated for 2 h with 3d2 (40 μM) and then stimulated with LPS (30 ng/ml) for 18 h (scale bar, 150 μm).

In summary, these results show for the first time, that LRH-1 is expressed in primary and secondary lymphatic tissues, as well as in CD4+ and CD8+ T cells. Moreover, LRH-1 directly binds to its binding sites in the FASLG promoter and thereby drives FASLG promoter activity. Pharmacological inhibition of LRH-1 decreases activation-induced FasL mRNA expression, as well as FasL-mediated activation-induced T cell apoptosis and T cell cytotoxicity. Furthermore, in a mouse model of ConA-induced and FasL-mediated hepatitis pharmacological inhibition of LRH-1 resulted in decreased hepatic FasL expression and a significant reduction of liver damage.

LRH-1 has an immunoregulatory role, not only just in the adaptive immune system by specifically targeting FasL in T lymphocytes, but also in the innate immune system promoting a pro-inflammatory phenotype in macrophages. Here we showed that pharmacological LRH-1 inhibition is also an effective tool in modulating LRH-1 activity, since it is able to reduce the transcription activity and the targeted protein levels of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β. However, the exact mechanism of LRH-1 regulating pro-inflammatory cytokine production after LPS stimulation needs to be further investigated. The obvious lack of cell activation observed by microscopy in 3d2 treated cell is in line with the reduced acid waste production and the decreased respiratory activity, although neither apoptosis nor proliferation was impaired in RAW 264.7 cells.

The newly discovered role in immune regulation gives many ideas for further investigations in order to establish LRH-1 as a potential pharmacological target in immunopathologies.
9 DISCUSSION

9.1 LRH-1 expression and immunoregulatory effector functions

At present, it is widely accepted that the NR LRH-1 is a transcriptional master regulator involved in a large variety of different processes (Stein and Schoonjans, 2015). Next to its critical role in development, as underlined by the early embryonic lethality of LRH-1-deficient mice (Pare et al., 2004), LRH-1 has important regulatory roles in glucose and lipid metabolism in the liver, female reproduction in the ovaries, cell proliferation, and tumor development in the pancreas (Benod et al., 2011) and the intestine (Botrugno et al., 2004, Schoonjans et al., 2005). Importantly, LRH-1 plays a crucial role in the regulation of inflammation and immune responses in the intestinal mucosa. It regulates not only the intestinal stem cell proliferation contributing to the intestinal epithelial barrier integrity, but also induces the expression of steroidogenic enzymes and associated synthesis of immunoregulatory glucocorticoids (Mueller et al., 2006). As a consequence, deletion of LRH-1 in the intestinal epithelium results in increased susceptibility to experimentally induced colitis (Coste et al., 2007). Also in the liver LRH-1 has anti-inflammatory properties by targeting IL-1 receptor antagonist and therefore negatively regulating proteins involved in the acute phase response (Ventecllef and Delerive, 2007). Previously, a study of LRH-1 in pancreatic cancer presented by Benod et al. coincidentally revealed the expression of LRH-1 in infiltrating immune cells (Benod et al., 2011). Nevertheless, it was Lefèvre and colleagues who described a direct evidence of LRH-1 expression in hematopoietic cells, providing a role for LRH-1 in IL-13-induced macrophage polarization (Lefevre et al., 2015).

In this study, we further extended these investigations on the role of LRH-1 in immune cells and we were able to show for the first time LRH-1 expression in primary and secondary lymphoid tissues, and in mature T cells. Moreover, the described LRH-1 expression in macrophages was confirmed in BMDM and in the murine macrophage cell line RAW 264.7. This work further addressed the question regarding the LRH-1 targeted genes in immune cells and their effector function in the immune system.

9.2 LRH-1 regulates FASLG promoter activity

Here we show the first evidence regarding the LRH-1-dependent regulation of the expression of a critical T cell effector molecule, i.e. FasL. As a member of the TNF and death ligand family,
DISCUSSION

FasL interacts with its cognate receptor Fas to initiate apoptosis, which is involved in a variety of different processes, but most notably the regulation of T cell homeostasis and cytotoxicity (Brunner et al., 2003). When previously primed T lymphocytes become restimulated via the TCR they rapidly undergo AICD, which involves activation-induced transcriptional upregulation of FasL, and cell autonomous FasL/Fas-mediated suicide (Brunner et al., 1995, Brunner et al., 1996, Dhein et al., 1995). Thus, FasL-induced T cell death has been implicated in the regulation of T cell homeostasis and the deletion of autoreactive T and B cells (Rathmell et al., 1995). Accordingly, gld FasL mutant mice show defects in T cell homeostasis, severe lymphoproliferative disorders and autoimmune diseases (Takahashi et al., 1994). Furthermore, FasL is recognized as an important cytotoxic effector molecule, particularly involved in the induction of tissue damage during immunopathological disorders, such as acute GvHD (Lin et al., 1998) and hepatitis, as shown here (Tagawa et al., 1998).

LRH-1 was proven to regulate FASLG transcription via direct interaction with the FASLG promoter. Overexpression of LRH-1 in Jurkat IT cells increased FASLG promoter activity, whereas deletion of in silico-predicted binding sites resulted in a significant reduction of LRH-1-driven or activation-induced FASLG promoter activity. Furthermore, specific binding of LRH-1 to the promoter was confirmed by ChIP analysis. Although two putative binding sites were identified, only mutation of BS1 resulted in a significant inhibition of LRH-1-induced FASLG promoter activity. This result indicates that BS2 is either not important or compensated by BS1. However, LRH-1 binding to the FASLG promoter was detected using BS2-specific primers, but not for BS1. This controversial results may be explained by the fact that these putative binding sites are only 300 base pairs apart, but the fragmentation of the chromosomal DNA results in fragments of 250 to 1000 base pairs. Thus, specific co-immunoprecipitation of BS1 may also result in DNA fragments, which contain both binding sites and as PCR detection of BS2 appears to be more sensitive than that of BS1, BS2 instead of BS1 is preferentially detected by PCR. Nonetheless, these data indicate that LRH-1 specifically binds to the FASLG promoter and that mutation of putative LRH-1 binding sites results in reduced FASLG promoter activity.

The mutation of both putative LRH-1 binding sites did not completely abrogate LRH-1-induced FASLG promoter activity when overexpressed in Jurkat IT cells. This observation either suggests additional so far unrecognized LRH-1 binding sites within the FASLG promoter, or that LRH-1 might also indirectly regulate the expression of FasL. So far, LRH-1 is known to regulate cell proliferation via the transcriptional regulation of cyclin D1 and E1 (Botrugno et al., 2004), and c-Myc (Benod et al., 2011). In previous studies, an interesting correlation
between proliferation and FasL expression was demonstrated (Brunner et al., 2000, Kasibhatla et al., 2000, Torgler et al., 2004). Most notable is the fact that activation of resting T cells results in only low levels of FasL, whereas restimulation of primed proliferating T cells causes rapid and massive induction of FASLG transcription. This phenomenon likely ensures that FasL is not expressed in the lymph nodes and the spleen upon activation of resting or naive T cells, but only when they become reactivated at the effector sites. This observation is related to the role of the proliferation activating factors c-Myc and cyclin B1/Cdk1 in the regulation of FASLG transcription (Brunner et al., 2000, Torgler et al., 2004), and could likely extend to LRH-1. FASLG transcription is regulated by activation- and stress-signal induced factors like NFAT, NFκB, AP-1 and EGR-2 and-3, while transcription factors like c-Myc and likely also LRH-1 may rather have a modulating and enhancing effect, linking FASLG transcription to proliferation. Thus, while LRH-1 on one hand directly regulates FASLG transcription via specific binding sites in the promoter, it may also indirectly control FasL expression via the regulation of c-Myc (Botrugno et al., 2004, Benod et al., 2011).

### 9.3 LRH-1 as pharmacological target

Unfortunately, testing the role of LRH-1 in the transcriptional control of FasL in a genetic mouse model is hampered by the fact that embryonic deletion of LRH-1 results in lethality (Pare et al., 2004). Thus, splenocytes of mice with a conditional LRH-1 deletion in T cells were analyzed and revealed much less single positive CD4+ and a reduction in CD8+ T cells in LRH-1-deficient mice when compared with floxed control mice. Therefore, FasL-regulated T cell homeostasis and cytotoxicity can currently not be tested in LRH-1-deficient T cells, since compared to the floxed control mice LRH-1-deficient mice have significantly less viable T cells as well as they show a different distribution of CD4+ and CD8+ T cells. Further investigations by Seitz et al. (unpublished data) will elucidate the role of LRH-1 in T cell development, proliferation and apoptosis to explain this dramatic phenotype.

To overcome this problem, the possibilities of pharmacological inhibition were exploited to study the role of LRH-1 in FasL expression in immune cells. Of interest, in vitro pharmacological inhibition of LRH-1 by the inhibitors 3d2 and even more pronounced by SR1848 resulted at high doses in extensive cell death induction, suggesting a role of LRH-1 in the regulation of T cell survival by a yet unknown mechanism. Nonetheless, at lower doses both inhibitors, as well as the inactive control substance cpd7, proved to be helpful to investigate the effects of a pharmacological intervention on LRH-1-regulated FASLG transcription and associated T cell effector functions. Of interest is the observation, that 3d2 (Benod et al., 2013) has to be
applied to the immune cells in higher concentrations as SR1848 (Corzo et al., 2015). This observation was supported by a recent publication, which compares multiple LRH-1 agonists and antagonists in their capacity to modulate LRH-1 target gene expression and depending on their mode of action are differently efficacious (de Jesus Cortez et al., 2016). Thus, here we reported for the first time the use of the LRH-1 inhibitor 3d2 in a mammalian in vivo model of FasL-induced liver damage. ConA injection resulted in a rapid induction of FasL expression and associated liver damage, which was almost completely blocked in gld mice, confirming a major role of FasL in this immunopathology. Importantly, 1 h 3d2 pretreatment resulted in a significant protection of mice from ConA-induced liver damage, as seen in reduced serum transaminase levels and reduced liver cell death. These findings show that LRH-1 is indeed an accessible target for pharmacological intervention of T cell-dependent and FasL-mediated immunopathologies.

Most notably, LRH-1 inhibition in T cells appears to be without any obvious side effect on the liver, which expresses high levels of LRH-1. Mice injected with 50 and 100 mg/kg body weight 3d2 alone only showed a very mild increase of serum transaminases (Figure 18C, and data not shown) and no induction of pro-inflammatory TNF levels in the serum (Figure 27)

![Figure 27: Serum TNF level of 3d2 treated and ConA challenged mice](image)

Serum TNF levels of wild type mice were challenged with 6 h ConA (black bars) or treated with 3d2 (grey bars) in the indicated concentrations. Individual data points were shown.

The relative, low LRH-1 expression in T cells compared to its abundant expression in hepatocytes gives the opportunity to target a therapeutic window. Low doses of 3d2 would permit efficient inhibition of LRH-1 and associated effector functions in T cells, whereas inhibitory effects in hepatocytes are minimal without disturbing any vital functions of LRH-1 in the liver. Thus, pharmacological inhibition of LRH-1 may represent an interesting therapeutic approach in the treatment of T cell-dependent and FasL-mediated
immunopathologies, such as hepatitis and acute GvHD. Nevertheless, furthermore investigations are necessary to understand the role of LRH-1 in the immune system in order to define it as a pharmacological target.

9.4 Controversial role of LRH-1 in pro-inflammatory cytokine regulation

Cytokines are small secreted proteins, which regulate the interactions and communication between cells, especially in the immune system. TNFα is the prototypic member of a large cytokine family, the TNF ligand family, which comprises also FasL (Wajant et al., 2003). TNFα has pro-inflammatory properties and is produced by macrophages, lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue. A pro-inflammatory activity of LRH-1 was observed after treating splenocytes with 3d2, which inhibited the cytokine biosynthesis of TNFα upon TCR as well as TLR4 stimulation.

The question arose whether macrophages, as the main responders to LPS via TLR4 activation, represent another direct target for pharmacological treatment of 3d2. It was shown that T cells can also be indirectly activated in vivo through LPS-mediated stimulation of macrophages and their release of type I interferon (Tough et al., 1997). Although, another study reported the need for co-stimulatory signals by primed APC to induce T cell activation by LPS (Mattern et al., 1998). The recently described LRH-1 expression in macrophages (Lefevre et al., 2015) was confirmed in this study and indeed, we showed that LRH-1 can also be pharmacological inhibited in cells of the innate immunity. Interestingly, after LPS stimulation 3d2 treated macrophages were not only restricted in their production of TNFα, but also the pro-inflammatory cytokines IL-6 and IL-1β.

The interpretation of these data is challenging considering the original publication of LRH-1 expression in macrophages and the reported influence of LRH-1 on macrophage polarization (Lefevre et al., 2015). Classical M1 macrophages produce pro-inflammatory effector molecules, such as reactive oxygen species and nitrogen intermediates, as well as pro-inflammatory cytokines (Martinez and Gordon, 2014). Alternatively activated macrophages are classified according to their abundant anti-inflammatory IL-10 levels. They resolve inflammation by removal of apoptotic cells by phagocytosis and secretion of tissue repair mediators. Lefèvre et al. described LRH-1 as a critical anti-inflammatory component in the signalling cascade that drives alternative macrophage activation (Lefevre et al., 2015). This is in line with former publications, where the pro-inflammatory factors TNFα and LPS decreased
DISCUSSION

LRH-1 expression in murine colon tumorigenesis models, as well as a report about an inverse correlation of inflammation with LRH-1 in patients suffering from inflammatory bowel disease (Schoonjans et al., 2005, Coste et al., 2007). Furthermore, Venteclef et al. identified LRH-1 as a negative regulator of the hepatic acute-phase response by inhibiting IL-1β and IL-6 stimulated gene expression in hepatocytes (Venteclef and Delerive, 2007). These observations are contrary to the present data, where after LPS induced M1 macrophage polarization the pharmacological LRH-1 inhibition decreased the pro-inflammatory cytokine biosynthesis.

In order to find an explanation for the opposed results, the LPS signalling pathway was analyzed to exclude a possible defect in the activation process, which would decrease the cytokine transcription. But neither the phosphorylation of the MAPK family consisting of p38, JNK and ERK1/2 nor the degradation of IκB revealed influences of 3d2 treatment on their activity. Our data revealed that pharmacological LRH-1 inhibition at high concentration was cytotoxic in T cells and SR1848 treatment showed an inhibition of proliferation in multiple cancer cell lines (Corzo et al., 2015). To observe further the reduction of pro-inflammatory cytokines after pharmacological LRH-1 inhibition, cell death and proliferation were also analyzed in macrophages, but no changes upon pharmacological LRH-1 inhibition were detected.

Of interest, RAW 264.7 cells in culture produced acid metabolites such as lactate, which led to a decrease in the pH of the cell culture medium, what in turn was inhibited by 3d2 treatment. Moreover, a reduced mitochondrial respiration after pharmacological LRH-1 inhibition was observed. A recent review from Kelly and O'Neill summarizes the importance of metabolic changes in macrophages upon LPS stimulation (Kelly and O'Neill, 2015). Interestingly, as known from cancer cells, also resting macrophages show that most of the consumed glucose is converted into lactate which is similar to the Warburg effect (Newsholme et al., 1987). Upon LPS stimulation of TLR4, a range of Warburg metabolism promoting metabolic changes occur in macrophages or dendritic cells, which lead to ATP production and increased biosynthesis. It has been shown that especially citrate, as a substrate of the TCA cycle, is critical for early macrophage activation after LPS stimulation (Infantino et al., 2011) and increases the lipid synthesis (Infantino et al., 2013). A decreased fatty acid biosynthesis, in turn, limits the expansion of ER and Golgi membranes and was shown to consequently inhibit the protein biosynthesis, including the generation of cytokines, in dendritic cells (Everts et al., 2014). Another TCA cycle intermediate, succinate, is also involved in macrophage activation by LPS, as it induces the glycolytic metabolism and promotes inflammation by transcriptional activation of IL-1β (Tannahill et al., 2013). Our observations of a reduction in lactate
production and mitochondrial respiration after pharmacological LRH-1 inhibition is in line with the hypothesis of LRH-1 regulating the macrophage metabolism and could even explain the reduced pro-inflammatory cytokine levels. This hypothesis is supported by findings describing that LRH-1 promotes proliferation and carcinogenesis in the liver by modulating metabolic changes via the expression of the enzyme mitochondrial glutaminase 2 (Xu et al., 2016). However, the supplementation with key molecules of the glutamine metabolism, such as L-glutamine and α-KG, did not abolish the 3d2 effect, and therefore does not support this hypothesis. Nevertheless, the microscopic observations of an impaired macrophage activation after 3d2 treatment, gives an explanation for the reduced respiratory capacity, the lactate production as well as the biosynthesis of pro-inflammatory cytokines. In macrophages, the NR LRH-1 might have therefore rather a general function in the activation process of M1 macrophages or in regulating the metabolism than a direct influence on the cytokine synthesis itself. It would be worthwhile to continue the work on investigating LRH-1 targets, which are early regulators in LPS activation or metabolism.

Artyomov et al. discussed the importance of macrophage origin for modulation of metabolic programming and the influence of inflammatory responses (Artyomov et al., 2016). Macrophages are a very heterogeneous cell population, which depending of their origin are adapted to the needs of the surrounding tissue. By comparing BMDM with peritoneal macrophages they revealed an opposite mitochondrial respiration following LPS stimulation. Moreover, gene expression data demonstrated a significant variability in basal metabolic programming between macrophages from distinct tissues (Artyomov et al., 2016). Since Lefèvre et al. used peritoneal macrophages to study the role of LRH-1 in macrophage polarization showing contradicting results to our data where BMDM were investigated, it is tempting to speculate that the key explanation for these discrepancies might be metabolic differences of macrophages depending on their tissue environment.

The connection between immune and metabolic responses influences diseases such as diabetes, cardiovascular diseases and cancer. A better understanding of immunometabolism may suggest approaches to better treat these diseases (Schertzer and Steinberg, 2014). Collective data suggested LRH-1 as a potential therapeutic target for several diseases, including cancer (Nadolny and Dong, 2015), inflammatory bowel disease (Kostadinova et al., 2014), diabetes (Lee et al., 2011) and fatty liver disease (Stein et al., 2017). Interestingly, all these diseases share features of metabolic and immunologic disturbances. In this work, the influence of the pharmacological LRH-1 inhibitors 3d2 and SR1848 on immune cells and immunopathologies was studied. Of interest, 3d2 inhibited T cell-dependent and FasL-
mediated liver pathology in *in vivo* experiments. Therefore, LRH-1 presents a promising target for FasL-mediated pathologies, such as hepatitis and acute GvHD. Furthermore, these data reveal an anti-inflammatory effect of pharmacological LRH-1 inhibition in macrophages, which is of great interest for inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease or atherosclerosis (Parameswaran and Patial, 2010). Especially the inhibitory effect on TNFα, as a key molecule in orchestrating pro-inflammatory signalling, should be further investigated in order to elucidate whether LRH-1 is a new target to modulate TNFα biosynthesis by pharmacological intervention. The findings of this work together with the development and optimization of new LRH-1 ligands which effectively and selectively target this NR (de Jesus Cortez et al., 2016, Corzo et al., 2015) and a better understanding of the structural characteristics of the ligand binding pocket (Musille et al., 2016), will improve the understanding of LRH-1 as therapeutic target.
10 OUTLOOK

Although this work revealed interesting and promising data, the gain of new findings correlates strongly with an increase of questions. Clearly, further research is needed in order to understand the role of LRH-1 in immune regulation.

For example, even though LRH-1 was discovered as a new direct transcriptional regulator of FasL and subsequent FasL effector functions, the data are based on pharmacological intervention and should be further verified on a genetic level. Neither a systemic nor a conditional knockout of LRH-1 in mice can be used due to lethality or the phenotype of reduced T cell numbers in the periphery lymphoid organs. Therefore, an *ex vivo* knockout of LRH-1 in primary T cells would be a suitable tool to confirm the effects of pharmacological inhibition. A review from Freeley and Long reports about different ways to deliver siRNAs into primary T cells, such as the use of electroporation/nucleofection, viral vectors, peptides/proteins or nanoparticles (Freeley and Long, 2013). Of great interest, these technologies are used for clinical gene transfer to redirect polyclonal T cells towards tumor targets (Tey, 2014). A first attempt of knocking out LRH-1 with an adenovirus Cre system in primary T cells revealed no results due to massive cell death induction (data Anna-Lena Geiselhöringer). Since cell death induction was similarly observed upon application of high LRH-1 inhibitor concentrations, this could indicate a pro-survival role of LRH-1 in T cells. Nevertheless, the establishment of an improved knockout or knockdown protocol of LRH-1 in primary T cells will be required in order to verify and understand the results of the pharmacological intervention.

Besides the direct regulatory role of LRH-1 in *FASLG* transcription via specific binding sites in the promoter, further studies will be required to elucidate whether LRH-1 may also indirectly control FasL expression via the regulation of c-Myc, and subsequently a pro-proliferative mechanism. Investigations in this direction can be expected to provide important insights in the phenotype of mice with T cells lacking LRH-1 and to explain their reduced cell number in the peripheral lymphoid organs.

The first *in vivo* application of the LRH-1 inhibitor 3d2 in mice has been successfully conducted to show a liver-protective effect on FasL-mediated T cell-induced hepatitis. However, further experiments are required to test the *in vitro* more potent LRH-1 antagonist SR1848, in order to determine whether the same effects can be observed, even at lower concentrations. This is of great interest when it comes to investigations of LRH-1 in a chronic hepatitis model with
an experimental timeframe of up to 10 days. The lymphocytic choriomeningitis virus (LCMV) is such a model system for virus-mediated hepatitis in mice, which causes apoptosis in hepatocytes and is cleared from the liver by activated CD8⁺ T cells in a perforin- and FasL-dependent manner within two weeks (Zinkernagel et al., 1986). To monitor the participation of LRH-1 in such a FasL-dependent disease during the initiation, the acute hepatitis, as well as the recovery phase, repetitive injections with the LRH-1 inhibitor are required, and in order to avoid hepatocyte toxicity, low concentrations would be preferred.

Hepatocytes are the targeted cells during hepatitis that undergo apoptosis. In addition to these in vivo experiments, the identification of the role of LRH-1 in hepatocytes should be assessed to exclude that 3d2 protects liver cells instead of targeting FasL expression in T cells. Therefore, mice with a hepatocyte-specific deletion of LRH-1 were generated by breeding a transgenic mouse, which carries the gene for the Cre recombinase under the albumin promoter with a mouse that has the LRH-1 gene floxed. These LRH-1-Alb-Cre mice can then be used in order to test whether LRH-1 deleted hepatocytes are protected from the ConA or LCMV induced hepatitis. Results from these experiments would confirm or reject the hypothesis that the observed 3d2-mediated protection of liver damage is due to a T cell mediated LRH-1 inhibition and therefore inhibited FasL-mediated cytotoxicity in the liver.

In order to shed light into the role of LRH-1 in macrophages, further in vivo experiments are required that help to understand the complex and dynamic interplay of immune cell interactions with the surrounded tissue. LPS activated macrophages revealed a reduced level of pro-inflammatory cytokines, such as TNFα due to pharmacological LRH-1 inhibition. Injection of LPS and N-galactosamine (GalN) induces a TNF/TNF receptor-mediated hepatitis in mice (Kaufmann et al., 2009). While LPS activates intrahepatic macrophages and the release of pro-inflammatory cytokines, GalN is metabolized by hepatocytes and sensitizes them by inhibition of their transcriptional capacity. In an animal experiment where mice are pre-treated with the pharmacological LRH-1 inhibitor 3d2 and then challenged with LPS/GalN, the influence of LRH-1 on the development of TNF-dependent and macrophage-mediated liver pathology can be observed.

Importantly, further studies will be required in order to understand the contradictory results in the pro-inflammatory properties of LRH-1 in this work in comparison with previously published data. A repetition of the experiments with different macrophages subsets, especially with peritoneal macrophages as they were used in the other researchers work, will ensure comparable results to the previous published work. Such an experiment could confirm
the report that BMDM have an opposite mitochondrial respiration compared to peritoneal macrophages following LPS stimulation (Artyomov et al., 2016).

Observations such as the reduced respiratory activity of 3d2-treated macrophages or the shift in the pH of cell culture medium possibly induced by changes in the lactate production, suggests a role of LRH-1 on macrophage metabolism. Further research is needed in order to confirm these findings and to define the possible LRH-1 targets, which can prove such a hypothesis. Interestingly, by using an extracellular flux analyzer, the metabolic characteristics of activated macrophages in combination with LRH-1 inhibition could be assessed in more detail. This instrument uses pH and oxygen sensors to measure the extracellular acidification-and oxygen consumption- rate, which can be related to glycolytic and mitochondrial oxidative metabolism (Van den Bossche et al., 2015). Activation of macrophages with LPS upregulates glycolysis in order to promote the biosynthesis of lipids, nucleic acids, and proteins in addition to the generation of ATP (Kelly and O'Neill, 2015). At the same time, mitochondrial respiration is suppressed in LPS activated BMDMs and this is thought to be a consequence of a broken TCA cycle (Tannahill et al., 2013). This extracellular flux analyzer would convincingly prove the effect of pharmacological compounds, such as 3d2 or SR1848, on the macrophage’s metabolic phenotype. It will further support the link of LRH-1 to metabolism and the biosynthesis of pro-inflammatory cytokines. This will help to clarify how macrophage metabolic programming is regulated and influences inflammatory responses (Artyomov et al., 2016).

Further studies will be required to discover direct targets of LRH-1 and to explain the reduced pro-inflammatory cytokine levels in activated T cells and macrophages. Experiments such as microarray analysis were already successfully applied to elucidate LRH-1 targets in cancer (Kramer et al., 2016, Xu et al., 2016). The transcriptomes of LRH-1 inhibitor-treated and untreated activated T cells and macrophages can be compared and the expression of cell activation, metabolism and inflammation genes can be analyzed. Gene sets, which show a significant reduction or enrichment after pharmacological LRH-1 inhibition, can then be further analyzed in in vitro experiments to identify LRH-1 direct targets.
11 CONCLUSION

This work contributed to elucidate the currently almost unknown role of LRH-1 in the immune system by proving evidence of the existence of the NR LRH-1 in cells of hematopoietic origin. The expression of LRH-1 was confirmed in macrophages and for the first time was also described in CD4$^+$ and CD8$^+$ T cells, where the expression is even further induced upon T cell activation. The transcriptional activity of LRH-1 was demonstrated in the cells of the innate and adaptive immunity and was successfully blocked by pharmacological LRH-1 inhibition.

In T cells, the death receptor ligand FasL was identified as an LRH1 targeted gene with direct binding sites to its promoter. Furthermore, specific pharmacological inhibition of LRH-1 resulted in reduced LRH1 activation- and LRH-1-induced FasL expression in T cells and inhibited FasL-mediated AICD and cytotoxicity. The importance of LRH-1-mediated regulation of FasL in T cells was confirmed in vivo by the profound inhibition of ConA-induced and FasL-dependent liver damage using the LRH-1 inhibitor 3d2. Nevertheless, the contribution of a potential pro-proliferative effect of LRH-1 on FasL expression, the role of LRH-1 on T cell survival or the impact of LRH-1 inhibition in a chronic model of hepatitis are questions arising from this work.

In macrophages, pharmacological LRH-1 inhibition revealed a significant reduction of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β. Therefore, further investigations are needed to understand the LRH-1-dependent metabolic changes upon LPS activation and their impact on cytokine biosynthesis. In addition, it would be interesting to identify LRH-1-targeted genes in macrophages and to find out whether depending to the macrophage origin these targeted genes differ.

This work has advanced the understanding of targeting and modulating LRH-1 by pharmacological compounds. 3d2 and SR1848 were tested in different immune cell subsets and revealed interesting differences in their inhibitory capacity as well as cytotoxicity. Importantly, the first in vivo application of 3d2 in mammals illustrated that pharmacological inhibition of LRH-1 may represent a novel strategy in the treatment of FasL-mediated immunopathologies.
12 PUBLICATION

13 ACKNOWLEDGEMENTS

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