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Lymphotoxin- β -receptor (LT β R) signaling on hepatocytes is required for liver regeneration after partial hepatectomy

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Abstract: Lymphotoxin- β -receptor deficient (LT β R^{-/-}) and Tumor Necrosis Factor Receptor p55 deficient (TNFRp55^{-/-}) mice show defects in liver regeneration (LR) after partial hepatectomy (PHx) with significantly increased mortality. LT β R and TNFRp55 belong to the core members of the TNF/TNFR superfamily. Interestingly, combined failure of LT β R and TNFRp55 signaling after PHx leads to a complete defect in LR. Here, we first addressed the question which liver cell population crucially requires LT β R signaling for efficient LR. To this end, mice with a conditionally targeted LT β R allele (LT β R^{fl/fl}) were crossed to AlbuminCre and LysozymeMeCre mouse lines to unravel the function of the LT β R on

hepatocytes and monocytes/macrophages/Kupffer cells, respectively. Analysis of these mouse lines clearly reveals that LT β R is required on hepatocytes for efficient LR while no deficit in LR was found in LT β R^{fl/fl} $\times$ LysMCre mice. Second, the molecular basis for the cooperating role of LT β R and TNFRp55 signaling pathways in LR was investigated by transcriptome analysis of etanercept treated LT β R^{-/-} (LT β R^{-/-}/ET) mice. Bioinformatic analysis and subsequent verification by qRT-PCR identified novel target genes (Cyclin-L2, Fas-Binding factor 1, interferon-related developmental regulator 1, Leucyl-tRNA Synthetase 2, and galectin-4) that are upregulated by LT β R/TNFRp55 signaling after PHx and fail to be upregulated after PHx in LT β R^{-/-}/ET mice.

Keywords: AlbuminCre line; etanercept; LT β R; partial hepatectomy; TNFRp55; transcriptome analysis.

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Introduction

LT β R, TNFRp55 and TNFRp75 as well as their corresponding ligands lymphotoxin β (LT $\alpha_1\beta_2$), LIGHT (homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes), tumor necrosis factor (TNF) and lymphotoxin α (LT α_3) are core members of the TNF/TNFR superfamily (Hehlhans and Pfeffer 2005). They are essential for the organogenesis of secondary lymphoid organs, for the maintenance of the architectural integrity of lymphoid tissues (De Togni et al. 1994; Futterer et al. 1998; Neumann et al. 1996; Pfeffer et al. 1993) and, importantly, for liver regeneration (LR) (Anders et al. 2005; Sorg et al. 2016). These cytokines and their cognate receptors play an important role in the generation of immune responses, especially with regard to protective immune functions in infections and are responsible for host damaging effects in sepsis, cachexia, and autoimmune diseases (Croft 2009; Hehlhans and Pfeffer 2005).

To date, defects in LR in LT β R^{-/-} mice have only been described in conventional LT β R deficient mice (Anders et al. 2005; Sorg et al. 2016). Since LT β R^{-/-} mice lack lymph

nodes and Peyer's patches and present with a disorganized spleen structure, the question arises whether the described defects in LR are, at least partially, due to these developmental deficiencies (Futterer et al. 1998; Hehlhans and Pfeffer 2005). Therefore, we used a floxed LTβR^{fl/fl} mouse line (Wimmer et al. 2012a; Wimmer et al. 2012b) and performed partial hepatectomy (PHx) on mouse lines that were conditionally deficient in LTβR expression on hepatocytes (LTβR^{fl/fl} × Alb-Cre [Kellendonk et al. 2000]) and monocytes/macrophages/Kupffer cells (LTβR^{fl/fl} × LysM-Cre [Clausen et al. 1999]), respectively. Our data clearly reveal that LTβR signaling in hepatocytes is required for LR after PHx.

We have established that LTβR^{-/-} as well as TNFRp55^{-/-} mice are deficient in LR with survival rates of about 65 and 30%, respectively (Anders et al. 2005; Shimizu et al. 2009; Sorg et al. 2016). Etanercept, a soluble TNFR2-Fc fusion protein can be used to interfere with TNFR mediated signaling (Tracey et al. 2008) and we could show that etanercept treated LTβR^{-/-} mice (LTβR^{-/-}/ET), which are functionally doubly deficient in LTβR as well as TNFRp55 signaling, reveal a complete LR failure indicated by 100% mortality within 48 h (Sorg et al. 2016). Transcriptome analysis is a powerful tool to unravel the molecular mechanisms of biological processes (Wang et al. 2009). We therefore performed transcriptome analysis in LTβR^{-/-}/ET animals 24 h after PHx to identify novel target genes that could play a crucial role in LR. And, indeed, we could identify a set of differentially transcribed genes such as Cyclin-L2 (Ccnl2), Fas-Binding factor 1 (Fbfl), Galectin-4 (Lgals4), interferon-related developmental regulator 1 (Ifrd1), and Leucyl-tRNA Synthetase 2 (Lars2) which are induced to a significantly lower degree after PHx in mice without functional TNFRp55/LTβR signal transduction pathways.

Results

Expression of the LTβR on hepatocytes is crucial for efficient liver regeneration

To elucidate whether expression of the LTβR on hepatocytes is essential for LR a mouse line with a conditionally targeted LTβR allele (LTβR^{fl/fl}) (collaboration with T. Hehlhans, Regensburg) (Wimmer et al. 2012a) was crossed to AlbuminCre (Kellendonk et al. 2000), and LysozymeMCre (Clausen et al. 1999) mouse lines to unravel the functions of the LTβR on hepatocytes and monocytes/macrophages/Kupffer cells, respectively. LTβR^{fl/fl} × AlbCre mice and controls (LTβR^{fl/+} × AlbCre, LTβR^{fl/fl}, and WT control mice) were

subjected to PHx as described previously (Sorg et al. 2016) and survival was monitored. Interestingly the survival of PHx in LTβR^{fl/fl} × AlbCre mice was markedly decreased compared to control mouse lines (Figure 1). These data clearly show that LTβR signaling is required on hepatocytes and indicate that the regenerative capacity of hepatocytes depends on LTβR initiated transcriptional responses which are mediated by both classical and alternative NFκB signaling pathways (Hehlhans and Pfeffer 2005; Oeckinghaus et al. 2011).

Interestingly, determination of aspartate amino transferase (AST), alanine amino transferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) alanine in surviving LTβR^{fl/fl} × AlbCre mice indicate that liver regeneration is still incomplete as late as 24 days after PHx when LTβR signaling is deficient in hepatocytes since these enzymes are still elevated in LTβR^{fl/fl} × AlbCre mice compared to WT controls (Figure 2). Even though the differences are not significant, these results fit the observation that a percentage of surviving LTβR^{fl/fl} × AlbCre mice takes noticeably longer to recover after PHx (data not shown) and warrants further analysis of the kinetics of these serum enzymes in LTβR^{fl/fl} × AlbCre mice after PHx.

Transcriptome analysis of LTβR/TNFRp55 regulated genes after PHx

In order to identify novel target genes that are involved in TNFRp55 and LTβR mediated signaling during LR,

Figure 1: Survival of mice that lack LTβR expression on hepatocytes (LTβR^{fl/fl} × AlbCre) and WT control mice after PHx. PHx was performed as described previously (Sorg et al. 2016) with minor modifications. After anesthesia, laparotomy was performed and first the right and left upper liver lobes together with the gall bladder were ligated and resected followed by ligation and resection of the left lower liver lobe. Mice received analgesia subcutaneously before PHx and 24 and 48 h post PHx. Survival was monitored daily up to day 20 post PHx. WT controls: *n* = 4, LTβR^{fl/fl}: *n* = 7, LTβR^{fl/+} × AlbCre: *n* = 7, LTβR^{fl/fl} × AlbCre: *n* = 12.

Figure 2: Elevated liver enzyme amounts in sera of LTβR^{fl/fl} × AlbCre mice on day 24 after PHx. Enzyme amounts were determined for aspartate amino transferase (AST), alanine amino transferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) in sera using the automated biochemical analyzer Spotchem EZ SP-4430. Shown are mean values + SD of $n = 3$ for LTβR^{fl/fl} × AlbCre mice and $n = 4$ for WT controls.

regenerating livers were removed 24 h after PHx from LTβR^{-/-}/ET (10 mg/kg etanercept 24 h before PHx) and control (WT) mice. Principle component analysis (PCA) of LTβR^{-/-}/ET animals compared to WT controls could separate all animals analyzed into two groups according to their genotype/treatment (Figure 3). Bioinformatic analysis identified 209 genes that were significantly up- or down-regulated in livers of LTβR^{-/-}/ET mice compared to WT controls (p (FDR) ≤ 0.05 ; $|FC| \geq 2$; Supplementary Table 1). A total of six genes, significantly down-regulated in livers of LTβR^{-/-}/ET mice after PHx and deemed to be of interest in the context of LTβR/TNFR regulated LR were selected for further analysis: Ccn12, Fbf1, growth arrest and DNA damage inducible gamma (Gadd45y), Ifrd1, Lars2, and

Lgals4 (Table 1). Their expression profiles 24 h post PHx were verified by qRT-PCR (Figure 4). Importantly, LTβR/TNFRp55 regulated transcripts which were significantly down-regulated in livers of LTβR^{-/-}/ET mice 24 h after PHx and could be independently confirmed encompassed Ccn12, Fbf1, Ifrd1, Lars2, and Lgals4. Expression of Gadd45y was lower in LTβR^{-/-}/ET mice but not significantly so (Figure 4).

Discussion

While it has been known for quite some time that LTβR ligands and the LTβR contribute significantly to efficient LR after PHx (Anders et al. 2005; Sorg et al. 2016; Tumanov et al. 2009), the exact role of the LTβR in LR has not been clearly defined yet. One important step in this direction was to identify the cell lineage on which the expression of the LTβR is required for LR. To this end, conditional gene

Figure 3: Principal component analysis (PCA) of LTβR^{-/-}/ET mice compared to WT controls 24 h post PHx. PCA based on transcriptome analysis of livers from LTβR^{-/-}/ET ($n = 5$) and WT controls ($n = 2$) 24 h post PHx. The plot shows the projection of the samples onto the two-dimensional space spanned by the first and second principal components of the covariance matrix. The expression levels used as input are normalized log Counts Per Million (CPM) values.

Table 1: Genes identified via RNAseq as potential LTβR/TNFRp55-regulated genes in liver regeneration.

Gene	FC	p-Value (FDR corrected)	RPKM mean WT	RPKM mean LTβR ^{-/-} /ET
Ccn12	-2.99	1.57×10^{-6}	10.51	3.70
Fbf1	-3.26	6.64×10^{-5}	2.39	0.58
Gadd45y	-2.34	7.05×10^{-3}	53.06	17.58
Ifrd1	-3.61	6.35×10^{-3}	11.00	2.66
Lars2	-32.70	0	329.11	9.63
Lgals4	-5.03	1.57×10^{-6}	2.94	0.63

PHx was performed on WT controls and LTβR^{-/-} mice treated with etanercept (10 mg/kg, s.c.) 24 h before PHx (LTβR^{-/-}/ET). 24 h after PHx mice were sacrificed and liver tissue was harvested. RNA was isolated, followed by RNAseq as described in the materials and methods section. Subsequently, bioinformatic analysis was performed and six genes were selected that seemed likely candidates for LTβR/TNFRp55 regulation after PHx (Ccn12, Fbf1, Gadd45, Ifrd1, Lars2, and Lgals4). Fold change (FC), p -Values (FDR corrected) and mean RPKM (Reads Per Kilobase Million) for WT and LTβR^{-/-}/ET mice are listed for these six genes. WT $n = 2$; LTβR^{-/-} $n = 5$.

Figure 4: Confirmation of novel LTβR/TNFRp55 regulated genes identified by RNAseq. qRT-PCR analysis of the novel LTβR/TNFRp55 regulated genes (*Ccnl2*, *Fbf1*, *Gadd45y*, *Ifrd1*, *Lars2*, and *Lgals4*) in liver tissue from LTβR^{-/-}/ET (*n* = 2) compared to WT controls (*n* = 5) 24 h post PHx. RNA was extracted from livers of LTβR^{-/-}/ET and control mice 24 h after PHx. cDNA transcription was performed and qRT-PCR with primer pairs for the indicated transcripts was performed. Expression of each tested gene of LTβR^{-/-}/ET mice was normalized to the respective gene expression in WT controls. ns: not significant, **p* ≤ 0.05, ***p* ≤ 0.05, ****p* ≤ 0.01.

targeting was employed (Rajewsky et al. 1996). Our data clearly demonstrates that mice that lack LTβR expression on hepatocytes show clear deficits in LR, indicating that LTβR signals in hepatocytes are crucial. Furthermore, in a global transcriptome analysis by RNAseq novel LTβR/TNFRp55 regulated genes important for LR were successfully identified.

Membrane bound LTβR ligand LTα₁β₂ is delivered by T cells into the regenerating liver and is essential for successful LR after PHx (Tumanov et al. 2009). Our data suggest that hepatocytes are the direct interaction partner of these T cells in early events after PHx and that the LTα₁β₂ mediated effects are not conveyed indirectly by intermediate signaling effects as discussed by Tumanov et al. (2009). LTβR^{-/-} mice show decreased survival rates after PHx (Anders et al. 2005; Sorg et al. 2016), however, regeneration kinetics in surviving LTβR^{-/-} mice are similar to WT controls: After 7–10 days post PHx liver weight and AST (aspartate aminotransferase) and ALT (alanine aminotransferase) amounts are comparably low in both genotypes (data not shown). In contrast, in LTβR^{fl/fl} × Alb-Cre mice AST and ALT amounts are still increased on day 24 post PHx compared to WT controls. This is in line with initial observations that LR is delayed in these animals in so far as that they seem to take longer to regain their pre-surgery general health status. Some individual animals present with jaundice and slower weight gain (data not shown). This set of experiments establishes that LTβR signaling on hepatocytes is necessary for efficient LR.

Expression of the LTβR on cells of the myeloid lineage, on the other hand, does not seem to play a role for LR, since LTβR^{fl/fl} × LysMCre mice conditionally targeted in the myeloid compartment, including liver resident macrophages/Kupffer cells show survival rates similar to WT control mice (data not shown). This implies, that while Kupffer cells play an important role in efficient LR after PHx (Elchaninov et al. 2019; Nishiyama et al. 2015), these Kupffer cell mediated functions appear not to be directly dependent on LTβR signaling. It might be of interest to note that there is data that suggests that LysMCre mice do not (completely) delete floxed genes in Kupffer cells/liver macrophages. Maeda et al. (Maeda et al. 2005) show incomplete deletion of IKKβ in Kupffer cells of Δmyc (IKKβ^{fl/fl} × LysMCre) mice, on the other hand, there is a set of more recent publications that use the LysMCre system to demonstrate efficient recombination mediated by LysMCre in Kupffer cells: Lanaya et al. for EGFR (Lanaya et al. 2014), Morán-Salvador for PPARγ (Moran-Salvador et al. 2013), Lodder et al. for ATG5 (2015), Lee et al. for caNrf2 (2016), Manakkat Vijay et al. for TLR9 (2019), Van Campenhout et al. for IRE1α (2020), and Li et al. for GSDMD (2020).

While LTβR activates the canonical RelA(p50/p65) and the alternative NFκB pathway via the NIK-IKKα-mediated processing of p100 and activation of p52/RelB target genes (Ware 2008), the TNFRp55 signals only via the canonical NFκB pathway via TRAF2 in order to activate RelA(p50/p65) via IκB degradation and also couples into the caspase cascade (Locksley et al. 2001; Norris and Ware 2007).

Since the absence of TNFRp55 mediated signaling in TNFRp55^{-/-} mice can be compensated by recombinant IL-6 (Yamada et al. 1997) it would be interesting to investigate whether the absence of LTβR mediated signaling could also be compensated by recombinant IL-6. The NFκB pathways control many cellular responses and p100 synthesis and the two forms of NFκB regulate distinct sets of genes (Ware 2008). Interestingly, as mentioned above, LTβR^{-/-} as well as TNFRp55^{-/-} mice show decreased survival after PHx (Anders et al. 2005; Shimizu et al. 2009; Sorg et al. 2016). This makes it difficult to study effector mechanisms that are induced early after PHx as one cannot predict which mouse will survive. To circumvent this ambiguity, LTβR^{-/-}/ET mice, which are functional LTβR/TNFRp55 double knock-outs and show a complete lack of LR and die within 48 h after PHx (Sorg et al. 2016) appear to be a suitable approach. Therefore, the LTβR^{-/-}/ET PHx model was used to unravel the transcriptional repertoire dependent upon LTβR/TNFRp55 signaling for LR. Employing this approach, we could successfully identify five gene transcripts in this study that are under control of LTβR/TNFRp55 signaling.

Notably, none of these identified transcripts have been reported as NF κ B responsive genes in <http://www.bu.edu/nf-kb/gene-resources/target-genes> and additional sources (Bunting et al. 2007; Liu et al. 2003; Naamane et al. 2007; Shelest et al. 2003) up to now. This might be due to the specific functions and cell populations required for LR, which have not been studied before. Thus, our distinctively identified and selected genes comprise a novel set of LT β R/TNFR responsive genes that are dependent on the combined LT β R/TNFRp55 signal transduction pathways and are putatively highly relevant for LR. Here, in the following sections, we individually report on these transcripts and attempt to provide information on putative functions in liver biology and LR:

Ccnl2

Cyclins are described to play an important role in the regulation of cell proliferation (Yang et al. 2004). Certain cyclins have been identified as critical modulators of transcription (Bregman et al. 2000; Miller and Cross 2001). Ccnl2 has also been reported to be involved in the regulation of cell cycle progression and in the signal transduction of the apoptosis pathway (de Graaf et al. 2004). Since hepatocyte proliferation after PHx is a highly synchronized event, Ccnl2 might be required for tight control of cell cycle progression.

Fbf1

Fbf1 is a protein that can interact with the cytosolic domain of the murine CD95 receptor, a member of the TNF/TNFR receptor superfamily and bears no sequence similarity to the known CD95 adaptor proteins (Schmidt et al. 2000). Its structural similarities to plectin and trichohyalin point to a possible role as a scaffold protein that links CD95 and the cytoskeleton which could in turn indicate a role in the extensive reorganization of the microarchitecture of liver tissue, especially mediated by hepatocytes, necessary for successful LR.

Gadd45y

Gadd45y is a family member of the growth arrest and DNA damage-inducible gene family 45 (Hoffmeyer et al. 2001). While no data for a role of Gadd45y in LR after PHx is available, previous studies with ectopically overexpressed

Gadd45y in various cell lines implicated a function in negative growth control. While our qRT-PCR results do not show significant down-regulation of Gadd45y, growth control is an important issue in LR, as the extensive cell division machinery has to be tightly regulated to prevent avoid hepatomegaly (Anders et al. 2005).

Ifrd1

It has been reported that Ifrd1 (Tis7/PC4) is a critical mediator of both the cell-autonomous regulation of osteoblastogenesis and osteoblast-dependent regulation of osteoclastogenesis (Iezaki et al. 2016) influencing the acetylation status of the p65 component of NF κ B. Thus, it appears to be an interesting gene for the analysis of its contribution for LR, where NF κ B signaling is essential.

Lars2

Intriguingly, a report describes the discovery of three clinical cases of infantile hepatopathy that carry mutations in Lars1, which was therefore designated as the infantile liver failure syndrome type 1 (ILfs1) gene (Casey et al. 2012). A Lars1 mutation in zebrafish caused an ILFS1-like phenotype with acute liver failure and was developed as a potential model for ILFS1 (Wang et al. 2018). A mitochondrial Lars2 point mutation leads to hearing loss and premature ovarian failure (Pierce et al. 2013). Therefore, it will be interesting to see whether the mitochondrial Lars2 is involved in infantile liver syndromes and which functions are mediated by Lars2 in LR.

Lgals4

Hepatocyte progenitor cell (HPC) proliferation is significantly reduced in Galectin 3^{-/-} mice and these mice fail to form a HPC niche, with reduced laminin formation (Hsieh et al. 2015). While no information on the role of Lgals4 in LR is available to date, it was reported that higher serologic levels of Lgals4 are associated with more aggressive characteristics of hepatocellular carcinomas in patients (Cai et al. 2014).

Thus, the comprehensive RNAseq approach to analyze liver tissue after PHx in LT β R^{-/-}/ET mice in this study has identified interesting LT β R/TNFR regulated transcripts that are potentially highly relevant with regard to the modulation of LR or its failure. Conventional and conditional gene-

deficient mouse lines of these newly identified genes undergoing PHx will clarify the role of these transcripts in LR in the future and will shed new light on the molecular mechanisms of TNF superfamily functions in the liver.

Materials and methods

Animals

This study was carried out in strict accordance with the German Animal Welfare Act. The protocol was approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Permits number 8.87-50.10.34.08.295 and 84-02.04.2013.A462). All efforts were made to minimize suffering of laboratory animals. TNFRp55^{-/-} and LTβR^{-/-} mice were generated as described previously (Futterer et al. 1998; Pfeffer et al. 1993) and had been backcrossed for at least 10 generations onto a C57BL/6N background. LTβR^{+/-} mice were used as WT controls. Mice were housed under specified pathogen-free conditions in Central Unit for Animal Research and Animal Welfare Affairs of the Heinrich-Heine-University of Düsseldorf and were between 10 and 14 weeks old at the time of surgery.

Partial hepatectomy

70 % PHx was performed according to Greene et al. (Greene and Puder 2003) with slight modifications. Briefly, under isoflurane anesthesia laparotomy was performed. First, the right and left upper liver lobes including the gall bladder were ligated and resected, followed by ligation and resection of the left lower liver lobe. Mice received 5 mg/kg Carprofen (Rimadyl®, Pfizer, Würselen, Germany) subcutaneously 30 min pre PHx (s.c.), and 24 and 48 h post PHx. Mice were sacrificed at the time points indicated in the individual experiments.

Etanercept treatment

Lyophilized etanercept (Enbrel®, Pfizer) was reconstituted with PBS to a final concentration of 10 mg/ml for injection. Mice were subcutaneously (s.c.) injected with 10 mg/kg etanercept 24 h before PHx.

Blood and tissue collection

Mice were anesthetized with 100 mg/kg Ketamin and 10 mg/kg Xylazine (both Vétoquinol GmbH, Ravensburg, Germany), weighed, and bled via the *vena cava inferior* on the days post PHx as indicated. Serum was obtained by coagulating the blood (30 min at room temperature). Serum was collected after two centrifugation steps (10 min, 8000×g). Liver samples were stored at -80 °C for RNA extraction.

Serum biochemistry and cytokine quantification

Serum was tested for concentrations of aspartate transaminase (AST), alanine transaminase (ALT), lactose dehydrogenase (LDH) and alkaline phosphatase (ALP) using the automated biochemical analyzer

Spotchem EZ SP-4430 (Arkray, Amstelveen, Netherlands) and the Spotchem EZ Reagent Strips Liver-1, Amylase and ALP.

RNAseq analysis

Total RNA was extracted from liver tissue using a standard TRIzol™ protocol. Total RNA samples used for transcriptome analyses were quantified (Qubit RNA HS Assay, Thermo Fisher Scientific) and quality measured by capillary electrophoresis using the Fragment Analyzer and the 'Total RNA Standard Sensitivity Assay' (Agilent Technologies, Inc. Santa Clara, USA). All samples in this study showed RNA Quality Numbers (RQN) with a mean of 8.1. The library preparation was performed according to the manufacturer's protocol using the Illumina® 'TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold'. Briefly, 1.5 µg total RNA were used as input for rRNA depletion. Next steps were fragmentation, the synthesis of cDNA, adaptor ligation and library amplification. Bead purified libraries were normalized and finally sequenced on the HiSeq 3000/4000 system (Illumina Inc., San Diego, USA) with a read setup of 1 × 150 bp. The bcl2fastq tool was used to convert the bcl files to fastq files as well for adaptor trimming and demultiplexing.

Transcriptomics analysis

Data analyses on fastq files were conducted with CLC Genomics Workbench (version 10.1.1, Qiagen, Venlo, NL, USA). The reads of all probes were adaptor trimmed (Illumina TruSeq) and quality trimmed (using the default parameters: bases below Q13 were trimmed from the end of the reads, ambiguous nucleotides maximum 2). Mapping was performed against the *Mus musculus* (GRCm38) (March 24th, 2017) genome sequence. After grouping of samples (two respective five biological replicates) according to their respective experimental condition, multi-group comparisons were made and statistically determined using the Empirical Analysis of DGE (version 1.1, cut-off = 5). The resulting *p*-values were corrected for multiple testing by FDR (|FC| ≥ 2). A *p*-value of ≤ 0.05 was considered significant.

Quantitative RT-PCR

Total RNA from macerated liver tissue was isolated using TRIzol reagent (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using 2 µg of total RNA with Moloney murine leukemia virus reverse transcriptase and oligo (dT) primer (both Life Technologies). RT-PCR (40 cycles) was performed in triplicate. Primer sequences used were synthesized by Metabion (Martinsried, Germany), based on the Universal ProbeLibrary (Roche, Mannheim, Germany) and detection was carried out with the corresponding probes (*β-actin*: 5'TGACAGGATGCAGAAGGAGA, 3'CGCTCAGGAGGAGCAATG, probe 106; *Ccnl2*: 5'GGTCCGCTTCTCCAAAGAG, 3'GGAGCACCTCGGTGTACCT, probe 25; *Fbfl*: 5'GCAGGCAGTCAGTCAGCA, 3'AGGCTTTCTCCCTTAGGGTCT, probe 7; *GADD45*: 5'GGATAACTTGCTGTTCGTGGA, 3'AAGTTCGTGCAGTGCTTTCC, probe 7; *IFRD1*: 5'TTTGGCACTTCTGTTTG AATTG, 3'GGGTCAAAGAATCCATATCTTCAT, probe 6; *Lars2*: 5'GGCTTTGGAGTGGGTGAAC, 3'GTCCGTCTCTCTTTTGCTGA, probe 51; *Lgals4*: 5'CAGCTGCAGCCCCATATC, 3'TCATCTGTGGAGGGTTGTACC, probe 3. Results are expressed relative to expression in untreated WT mice normalized to *β-actin* (2^{-ΔΔCT}).

Statistical analysis

Quantifiable data are expressed as means + SD. Statistical analysis was performed using the GraphPad Prism 5.01 software applying Student's *t*-test.

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