Transcriptomic landscapes of effective and failed liver regeneration in humans

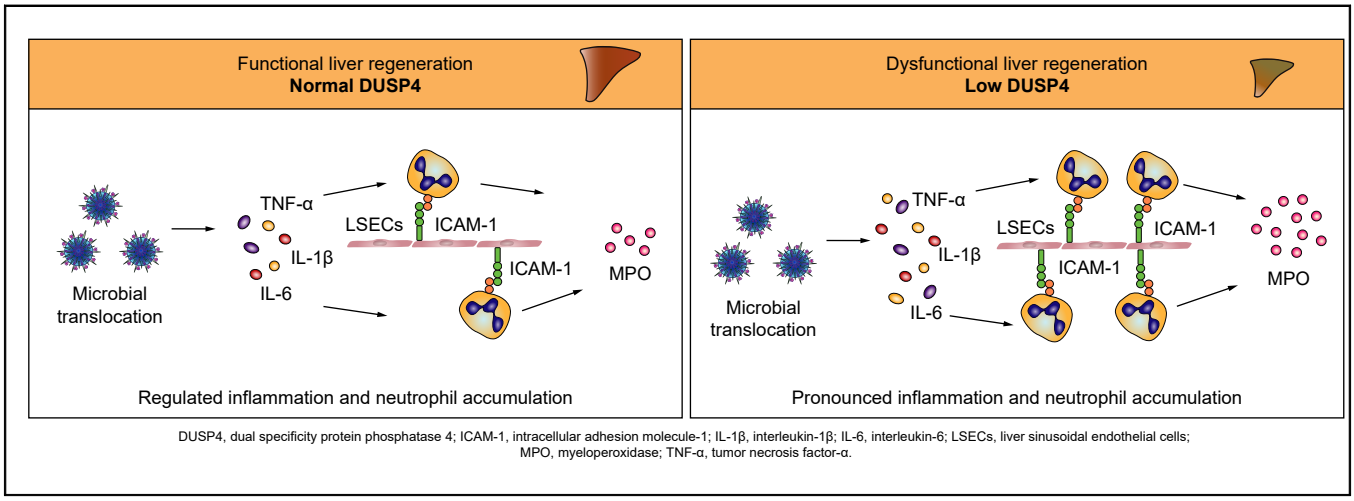
Authors

Patrick Starlinger, Laura Brunnthaler, Chantal McCabe, David Pereyra, Jonas Santol, Jessica Steadman, Matthias Hackl, Susanna Skalicky, Hubert Hackl, Raphael Gronauer, Daniel O'Brien, Renate Kain, Petra Hirsova, Gregory J. Gores, Chen Wang, Thomas Gruenberger, Rory L. Smoot, Alice Assinger

Correspondence

starlinger.patrick@mayo.edu, patrick.starlinger@meduniwien.ac.at (P. Starlinger), smoot.rory@mayo.edu (R.L. - Smoot), alice.assinger@meduniwien.ac.at (A. Assinger).

Graphical abstract



DUSP4, dual specificity protein phosphatase 4; ICAM-1, intracellular adhesion molecule-1; IL-1β, interleukin-1β; IL-6, interleukin-6; LSECs, liver sinusoidal endothelial cells; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α.

Highlights

- We explored circulating and tissue-level alterations associated with functional and dysfunctional liver regeneration (DLR).
- In contrast to experimental animal models, individuals with DLR had an aggravated transcriptional inflammatory response, challenging existing concepts.
- This response appears to be related to decreased levels of DUSP4 in LSECs.

Impact and implications

Using a unique human biorepository, focused on liver regeneration (LR), we explored the landscape of circulating and tissue-level alterations associated with both functional and dysfunctional LR. In contrast to experimental animal models, people with dysfunctional LR demonstrated an aggravated transcriptional inflammatory response, higher intracellular adhesion molecule-1 (ICAM-1) induction, intrahepatic neutrophil accumulation and activation upon induction of LR. Although inflammatory responses appear rapidly after liver resection, people with dysfunctional LR have exaggerated inflammatory responses that appear to be related to decreased levels of LSEC DUSP4, challenging existing concepts of post-resectional LR.

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Research article

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Transcriptomic landscapes of effective and failed liver regeneration in humans

Patrick Starlinger,[1,2,*] Laura Brunnthaler,[3] Chantal McCabe,[4] David Pereyra,[5] Jonas Santol,[6] Jessica Steadman,[1] Matthias Hackl,[7] Susanna Skalicky,[7] Hubert Hackl,[8] Raphael Gronauer,[8] Daniel O'Brien,[4] Renate Kain,[9] Petra Hirsova,[10] Gregory J. Gores,[10] Chen Wang,[4] Thomas Gruenberger,[6] Rory L. Smoot,[1,11,*,†] Alice Assinger[3,†,*]

[1]*Department of Surgery, Division of Hepatobiliary and Pancreatic Surgery, Mayo Clinic, Rochester, MN, USA;* [2]*Center of Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria;* [3]*Center of Physiology and Pharmacology, Institute of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria;* [4]*Department of Quantitative Health Sciences, Mayo Clinic, Rochester, MN, USA;* [5]*Department of Surgery, Medical University of Vienna, General Hospital, Vienna, Austria;* [6]*Department of Surgery, HPB Center, Viennese Health Network, Clinic Favoriten and Sigmund Freud Private University, Vienna, Austria;* [7]*TAmiRNA GmbH, Vienna, Austria;* [8]*Institute of Bioinformatics, Biocenter, Medical University of Innsbruck, Innsbruck, Austria;* [9]*Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria;* [10]*Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA;* [11]*Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA*

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Background & Aims: Although extensive experimental evidence on the process of liver regeneration exists, in humans, validation is largely missing. However, liver regeneration is critically affected by underlying liver disease. Within this project, we aimed to systematically assess early transcriptional changes during liver regeneration in humans and further assess how these processes differ in people with dysfunctional liver regeneration.

Methods: Blood samples of 154 patients and intraoperative tissue samples of 46 patients undergoing liver resection were collected and classified with regard to dysfunctional postoperative liver regeneration. Of those, a matched cohort of 21 patients were used for RNA sequencing. Samples were assessed for circulating cytokines, gene expression dynamics, intrahepatic neutrophil accumulation, and spatial transcriptomics.

Results: Individuals with dysfunctional liver regeneration demonstrated an aggravated transcriptional inflammatory response with higher intracellular adhesion molecule-1 induction. Increased induction of this critical leukocyte adhesion molecule was associated with increased intrahepatic neutrophil accumulation and activation upon induction of liver regeneration in individuals with dysfunctional liver regeneration. Comparing baseline gene expression profiles in individuals with and without dysfunctional liver regeneration, we found that dual-specificity phosphatase 4 (DUSP4) expression, a known critical regulator of intracellular adhesion molecule-1 expression in endothelial cells, was markedly reduced in patients with dysfunctional liver regeneration. Mimicking clinical risk factors for dysfunctional liver regeneration, we found liver sinusoidal endothelial cells of two liver disease models to have significantly reduced baseline levels of DUSP4.

Conclusions: Exploring the landscape of early transcriptional changes of human liver regeneration, we observed that people with dysfunctional regeneration experience overwhelming intrahepatic inflammation. Subclinical liver disease might account for DUSP4 reduction in liver sinusoidal endothelial cells, which ultimately primes the liver for an aggravated inflammatory response.

Impact and implications: Using a unique human biorepository, focused on liver regeneration (LR), we explored the landscape of circulating and tissue-level alterations associated with both functional and dysfunctional LR. In contrast to experimental animal models, people with dysfunctional LR demonstrated an aggravated transcriptional inflammatory response, higher intracellular adhesion molecule-1 (ICAM-1) induction, intrahepatic neutrophil accumulation and activation upon induction of LR. Although inflammatory responses appear rapidly after liver resection, people with dysfunctional LR have exaggerated inflammatory responses that appear to be related to decreased levels of LSEC DUSP4, challenging existing concepts of post-resectional LR.

Keywords: Liver regeneration; Human; Inflammation; Neutrophils; DUSP-4.
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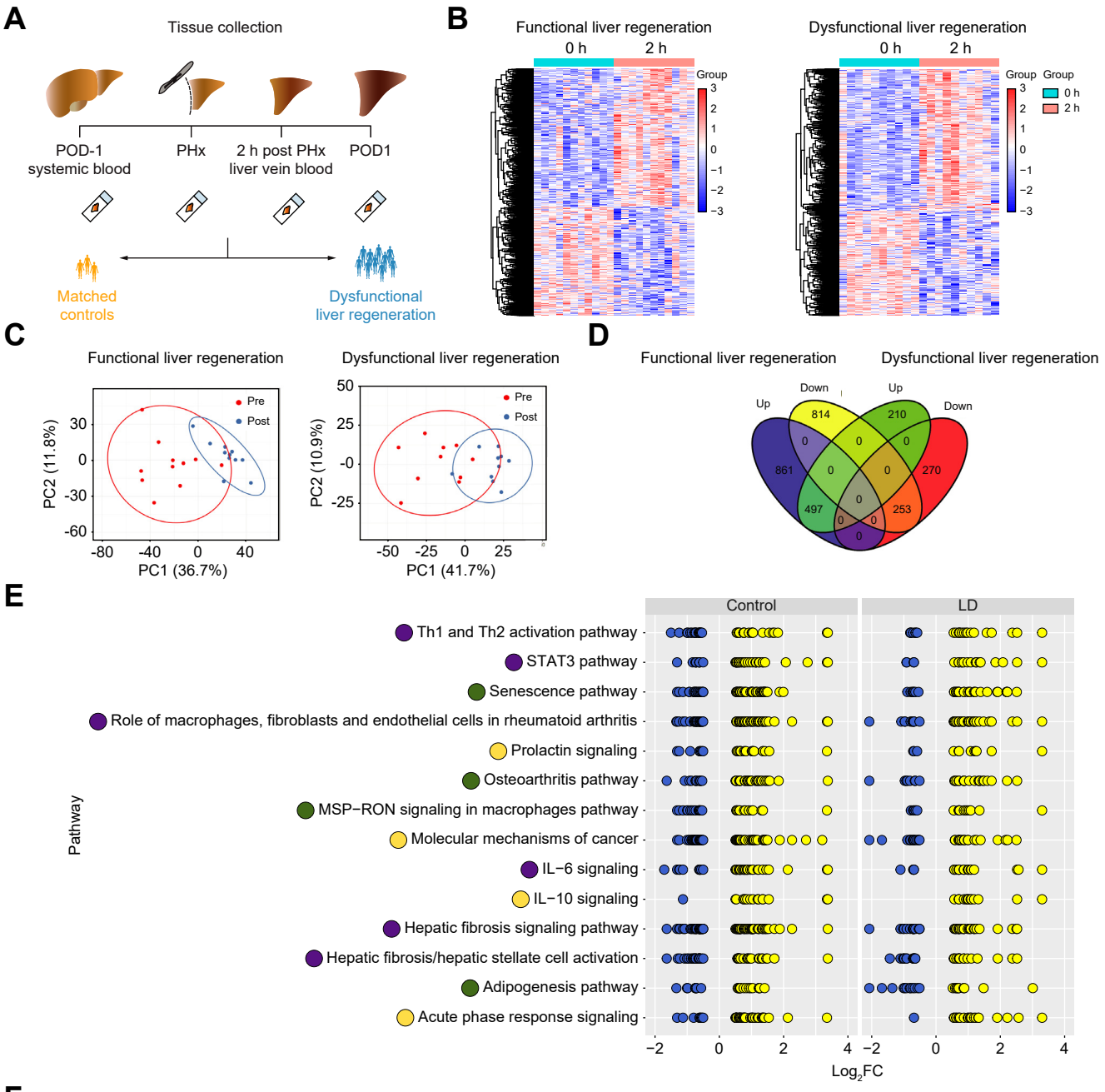
† These authors shared last authorship.

* Corresponding authors. Addresses: Department of HPB Surgery, Mayo Clinic, 200 First Street SW, Rochester 55905, MN, USA; Department of Surgery, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria. Tel.: +43-1-40400-5621 (P. Starlinger); Department of Surgery, Mayo Clinic, 200 First Street SW, 55905 Rochester, MN, USA. Tel.: +1-507-284-1529; fax: +1-507-284-5196 (R.L. Smoot); Institute of Physiology, Medical University of Vienna, Schwarzspanierstraße 17, 1090 Vienna, Austria. Tel.: +43-1-40160-31405 (A. Assinger).
E-mail addresses: starlinger.patrick@mayo.edu, patrick.starlinger@meduniwien.ac.at (P. Starlinger), smoot.rory@mayo.edu (R.L. Smoot), alice.assinger@meduniwien.ac.at (A. Assinger).

Introduction

The liver has remarkable regenerative potential, allowing for extensive surgical treatments of both benign and malignant diseases. Inefficient and/or absent regeneration can lead to post-hepatectomy liver failure, a condition in which the liver cannot functionally meet the physiologic requirements.[1] Overall, this



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Unique FLR

Pathways associted with induced genes	Pathways associted with induced genes
Steroid hormone biosynthesis	Intestinal immune network for IgA production
Ascorbate and aldarate metabolism	**Rheumatoid arthritis**
Pentose and glucuronate interconversions	**Asthma**
Porphyrin and chlorophyll metabolism	**Allograft rejection**
Bile secretion	**Herpes simplex virus 1 infection**
Metabolism of xenobiotics by cytochrome P450	**Antigen processing and presentation**
Retinol metabolism	**Inflammatory bowel disease**
Pathogenic *Escherichia coli* infection	***Staphylococcus aureus* infection**
p53 signaling pathway	Other glycan degradation
NOD-like receptor signaling pathway	Toxoplasmosis

Unique DLR

Pathways associted with induced genes	Pathways associted with induced genes
NF-kappa B signaling pathway	Primary bile acid biosynthesis
IL-17 signaling pathway	**Asthma**
Viral carcinogenesis	Phenylalanine, tyrosine, tryptophan biosynthesis
PPAR signaling pathway	**Antigen processing and presentation**
Cytokine-cytokine receptor interaction	**Allograft rejection**
Epstein-Barr virus infection	Relaxin signaling pathway
Legionellosis	Vascular smooth muscle contraction
Terpenoid backbone biosynthesis	Alcoholism
Human T-cell leukemia virus 1 infection	**Graft-versus-host disease**
TNF signaling pathway	Type I diabetes mellitus

Fig. 1. Intrahepatic gene expression rapidly changes after induction of LR in humans. (A) Sample collection study design for human LR tissue. (B) Heatmap illustration of individuals with and without postoperative DLR. (C) Results of principal component analyses for individuals with and without postoperative DLR, whereby PC1 and PC2, explaining around 50% of the variance and projection into two dimensions, indicate that postoperative samples can be separated from preoperative samples based on their gene expression profiles. (D) Venn diagram for individuals with DLR and controls and further stratified according to gene expression induction (up) and repression (down). (E) The 10 most affected pathways for individuals with and without postoperative DLR (six overlapping, resulting in a total of 14 unique pathways), and only significantly affected genes and their fold change. Yellow indicates an increase, blue indicates a decrease, and dots indicate the top 10 composition of common

condition has a high mortality rate, and there are no approved therapies for either prophylaxis or treatment.[2] Underlying liver diseases associated with inflammation, fibrosis, and cirrhosis (viral hepatitis, chemotherapy associated steatohepatitis (CASH), non-alcoholic steatohepatitis (NASH), and alcoholic steatohepatitis, amongst others) are known to increase the risk of postoperative dysfunctional liver regeneration (DLR) and are frequently present in people requiring hepatic resection.[3] Existing molecular studies aimed at further understanding the regenerative process in the liver and have routinely used murine partial hepatectomy models as a surrogate for human liver regeneration (LR).[3] As these models do not routinely assess regeneration in models of chronic liver disease, it remains unclear how thoroughly these models recapitulate the process of human LR in the complex setting of underlying liver pathology. Several key pathways and molecular modulators have been identified, with inflammation-related pathways/molecules as predominating mechanisms during the immediate stages of LR – the so-called priming phase.[4]

Rodent models have identified inflammation as a critical mechanism of the priming phase of LR.[4] Bacterial translocation and gut-derived endotoxins such as lipopolysaccharide (LPS) have been suggested as one of the major initiating events upon LR, which is accompanied by an acute cytokine release.[4] Liver sinusoidal endothelial cells (LSECs) are the first cells that get in contact with these circulating cytokines, inducing a rapid inflammatory response, including increased intracellular adhesion molecule-1 (ICAM-1) expression.[5] ICAM-1 has been documented to be critically important to intrahepatic neutrophil adhesion.[6] In line with this, ICAM-1 knockout or neutrophil depletion is associated with a dramatic reduction in LR in experimental models.[7] Although these data support a significant role for inflammatory signalling in regulating LR, it remains unclear how these signalling molecules and mechanisms participate in acute human LR. More importantly, it remains unclear how these processes are dysregulated in people with DLR after hepatic resection.

Herein, we examine both the baseline transcriptomic signature and the acute transcriptomic response to a regenerative stimulus in the human liver stratified by clinical patient outcome. The results indicate a rapid increase, within 2 h, of genes associated with several important signalling pathways predominantly involved in inflammation. When stratifying the differential responses based on the final liver regenerative outcomes (functional regeneration *vs*. liver dysfunction), we noted a pronounced increase in inflammatory pathway components such as ICAM-1 in individuals with DLR. This increased induction of inflammatory signalling and ICAM-1 was accompanied by augmented intrahepatic neutrophil accumulation. Circulating cytokine profiling supported the transcriptomic changes observed. Further, individuals with DLR had a pronounced and prolonged elevation of circulating myeloperoxidase (MPO) levels, a marker for neutrophil activation. Most importantly, evaluating baseline transcriptomic signatures, we found that people who developed DLR post-surgery had lower levels of dual-specificity phosphatase 4 (DUSP4), a critical modulator of inflammatory response in endothelial cells,[8] which thereby appeared to already be primed for an overwhelming inflammatory response.

Materials and methods

Study cohorts

A total of 154 participants were recruited. Participants undergoing liver resection were followed prospectively over a postoperative time period of 90 days. Blood samples were assessed perioperatively, namely, 1 day before surgery, before liver resection in the portal vein, 2 h after induction of LR in the liver vein (draining the regenerating liver lobe), and 1 (POD1) and 5 days after liver resection (POD5). Essential patient-related data, including baseline characteristics, surgical procedure, perioperative routine laboratory parameters, and baseline liver pathology, were assessed and recorded as illustrated in Table S3 (sequencing cohort, N = 21), Table S4 (detailed intraoperative dynamics cohort, N = 46), and Table S5 (perioperative validation cohort, N = 108).

The study was conducted in adherence to the Declaration of Helsinki and was approved by the responsible institutional ethics committee (Medical University of Vienna). Ahead of participation, informed consent was obtained from all participants (EK 16-253-0117 and EK 14-122-0714).

Optimised blood sample preparation and assessment

Optimised plasma preparation was applied as previously described.[9–14]

Definition and classification of postoperative liver dysfunction

Postoperative DLR was diagnosed following the criteria issued by the International Study Group of Liver Surgery.[15] Of note, participants reaching normal serum bilirubin or prothrombin time values before POD5, or were discharged early because of good clinical performance and hence had no further blood collection, were considered as 'functional LR' (FLR). This definition has been well established to be associated with multiple outcome measures after liver resection. Most importantly, it is associated not only with fulminant post-hepatectomy liver failure, but also with delayed decompensation after hepatic resection. This classification for dynamic postoperative liver function recovery was therefore used to define FLR and DLR.

RNA extraction and RNA sequencing analysis

The miRNeasy Mini Kit (Qiagen, Germany) was applied to isolate total RNA, including small RNAs, from frozen tissue samples. RNA sequencing libraries were generated using the QuantSeq 3′ protocol (Lexogen, Austria) according to the manufacturer's recommendation. Sequencing was performed on a NovaSeq6000 SP100 Flowcell, achieving 750 million reads, or >15 Mio reads per sample. The raw RNA sequencing paired-end reads for the samples were processed through the Mayo RNA sequencing bioinformatics pipeline, MAP-RSeq version 3.1.4.[16] Briefly, MAP-RSeq uses the very fast, accurate, and splice-aware aligner STAR (illustrated in Fig. S1)[17] to align reads to the reference

(purple), FLR (yellow), and DLR (green) pathways. (F) The top 10 pathways affected by genes that were uniquely upregulated or downregulated in individuals with either FLR or DLR. Pathways predominantly associated with inflammation are bolded. DLR, dysfunctional LR; FLR, functional LR; LD, liver dysfunction; Log_2FC, log_2 fold change; LR, liver regeneration; NOD, Nucleotide-binding oligomerization domain; PC1, principal component 1; PC2, principal component 2; PHx, partial hepatectomy; POD1, 1 day after liver resection; PPAR, peroxisome proliferator-activated receptor; TNF, tumour necrosis factor.

human genome build hg38. Gene and exon expression quantification was performed using the Subread package to obtain both raw and normalised (reads per kilobase per million mapped reads) reads.[18] Comprehensive quality control analyses were run on the aligned reads to assess the quality of the sequenced libraries. All samples were processed in randomised order. Sequencing was performed on a single equimolar pool in one sequencing run to avoid batch effect.

Using the raw gene counts report from MAP-RSeq, genes that are differentially expressed between the postoperative (2 h) and preoperative (0 h) groups, separately for FLR and DLR, were assessed using the Bioconductor package edgeR 2.6.2 in a paired fashion based on a negative-binomial model by taking the patient into account and performing likelihood ratio tests.[19] Genes that have sufficiently large counts to be retained in a statistical analysis were selected using filterByExpr. Values of p were adjusted based on the false discovery rate (FDR) according to the Benjamini–Hochberg method. Normalisation factors representing library sizes were calculated with the trimmed mean of M values (TMM), and gene counts were scale normalised and log2 transformed (log counts per million [logCPM]). The number of genes expressed in a condition was determined by an average expression value of logCPM greater than 1. Genes that were more than 2-fold changed at an FDR of <5% were considered significantly differentially expressed. Unsupervised hierarchical clustering analysis was performed on z-score-transformed normalised data for the most significantly upregulated or downregulated genes. Principal component analyses (PCAs) were performed on normalised data including all expressed genes.

Mouse model and LSEC and hepatocyte isolation

Details to the NASH mouse model and cell isolation can be found in the Supplementary information and have also been described elsewhere.[20]

Immunofluorescence staining

A detailed description of immunofluorescence staining can be found in the Supplementary information.

Spatial transcriptomics

A detailed description of spatial transcriptomics can be found in the Supplementary information.

Electron microscopy

Details on electron microscopy can be found in the Supplementary information.

Statistical and pathway analyses

Details on statistical and pathway analyses can be found in the Supplementary information.

Results

Marked changes in the global transcriptome occur rapidly following a regenerative stimulus in the human liver

Transcriptome changes occurring in the setting of murine LR have previously been defined.[21] We sought to understand the changes occurring at an immediate time point (2 h) after induction of LR in humans (Fig. 1A). Ten individuals with postoperative DLR were matched according to baseline characteristics to 11 individuals with FLR (Table S1). RNA sequencing analyses resulted in about 10.5 million high-quality sequencing reads per sample, from which could be on average 76% uniquely mapped to the reference genome. Normalised expression values demonstrated uniformity in both patient groups with and without liver dysfunction, and more than 12,000 genes were found to be expressed in each of the condition (postoperative 2 h and preoperative 0 h) of both groups. Unsupervised hierarchical clustering (Fig. 1B) showed significant gene expression changes 2 h after induction of LR. PCAs demonstrated a relevant amount of heterogeneity at baseline, particularly in the DLR group. However, the components appeared to shift comparably during early LR in both groups (Fig. 1C). The DLR and FLR groups had distinctly significantly regulated genes (>2-fold change, FDR <5%). However, all genes induced in both groups showed the same direction of induction (up) or repression (down) (Fig. 1D). Thus, transcriptomic changes occur rapidly after LR induction, and directions of transcriptomic changes appear similar between individuals with FLR and those with DLR.

Inflammation-associated pathways are predominantly induced after induction of LR

Ingenuity Pathway Analysis (IPA) revealed significant differences in affected pathways. The 10 most significantly affected pathways (in both groups) are illustrated in Fig. 1E. We could observe that inflammation-associated pathways were over-represented. To obtain a more detailed picture, we assessed the log fold induction of genes significantly affected in the top 10 pathways within our two groups. As Fig. 1E illustrates, most of the identified pathways were significantly affected in both patient groups. However, the intensity of activation (fold induction) as well as the balance of upregulated and downregulated genes within respective pathways significantly differed between groups. This led us to hypothesise that, within individuals with and those without postoperative DLR, known pathways are affected in both patient groups; however, the intensity of activation might determine FLR. As we further assessed the genes that were uniquely upregulated or downregulated in FLR or DLR and compared the top 10 pathways associated with these genes, we found that, again, in individuals with DLR, uniquely upregulated genes clustered around critical inflammatory pathways (Fig. 1F). Of particular interest appeared the involvement of uniquely induced genes in the NF-κB signalling pathway as well as the tumour necrosis factor (TNF) signalling pathway, both critically involved in inflammation during the priming phase of LR.

Most affected genes are associated with inflammation, cell adhesion, and cell growth control

Unbiased pathway analyses using IPA or gene interactomes rely on predefined databases that lack direct relation to LR. To address this, we assessed the 15 genes with the highest induction or reduction during this early phase of LR and aimed to address their specific role during LR as described in rodent models (Fig. 2, N = 21; a complete list of significantly affected genes can be found in Table S1). Fig. 2A illustrates the documented roles of these 15 genes during liver pathophysiology (references are listed in the Supplementary information – 'References associated with Fig. 2'), highlighting the role of the most regulated genes in inflammation, cell adhesion, and cell growth control. To further evaluate the top three regulated genes in each functional group, spatial transcriptomics was used. In two

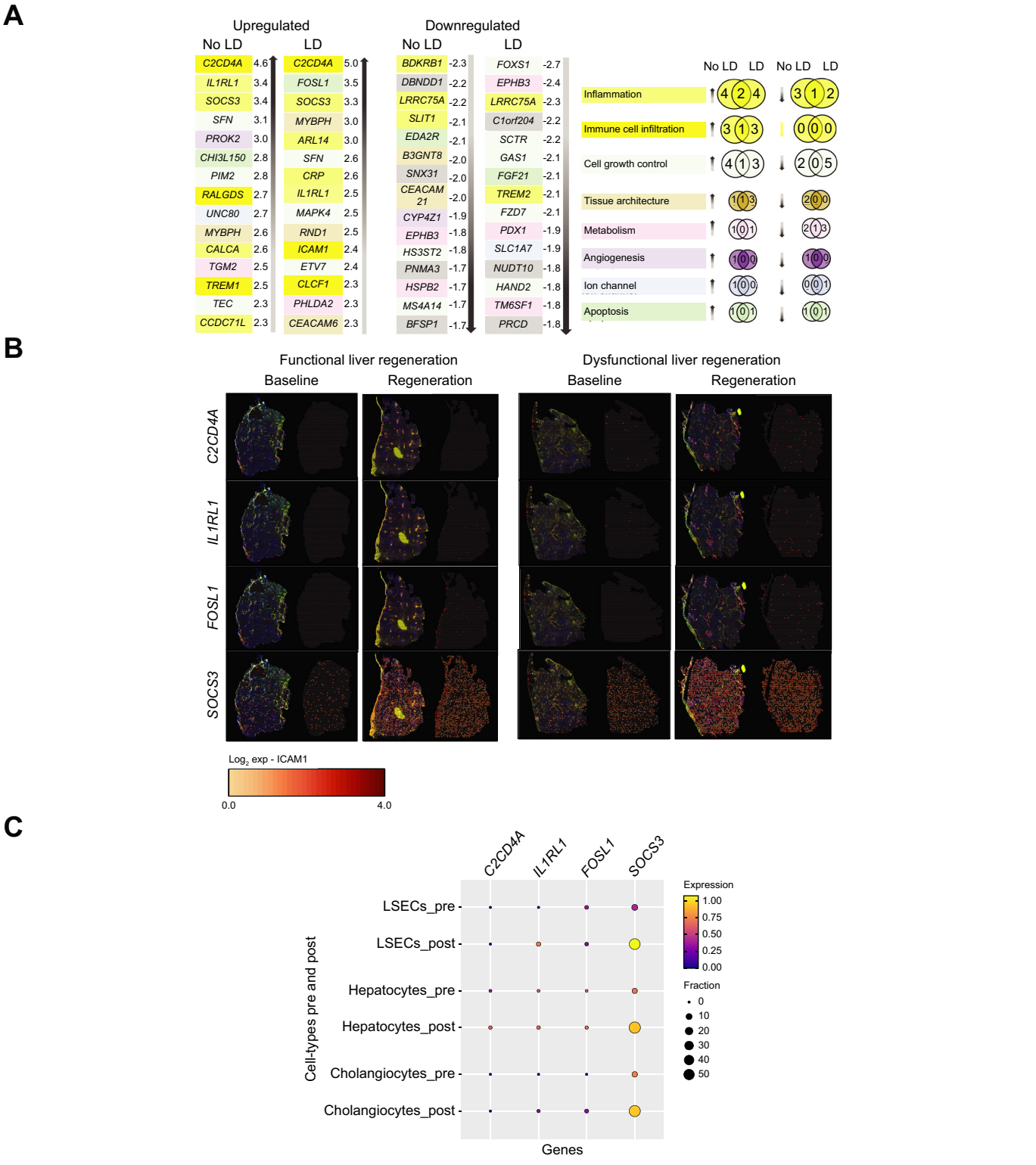

Fig. 2. Most regulated genes are involved in multiple processes that are known to be relevant for LR but predominantly involve inflammatory processes. (A) The 15 most affected (upregulated *vs.* downregulated) genes are illustrated for individuals with and without postoperative DLR and associated with their known functions in liver (patho)physiology (n = 21). The Supplementary information contains the respective references indicating individual gene functions. Grey fields represent genes with unknown functions in the liver. (B) Special transcription validation and assessment of gene expression location of the three most upregulated genes in two representative individuals (with and without DLR), which is also quantified with respect to expressing cell type in (C). DLR, dysfunctional LR; ICAM-1, intracellular adhesion molecule-1; LR, liver regeneration.

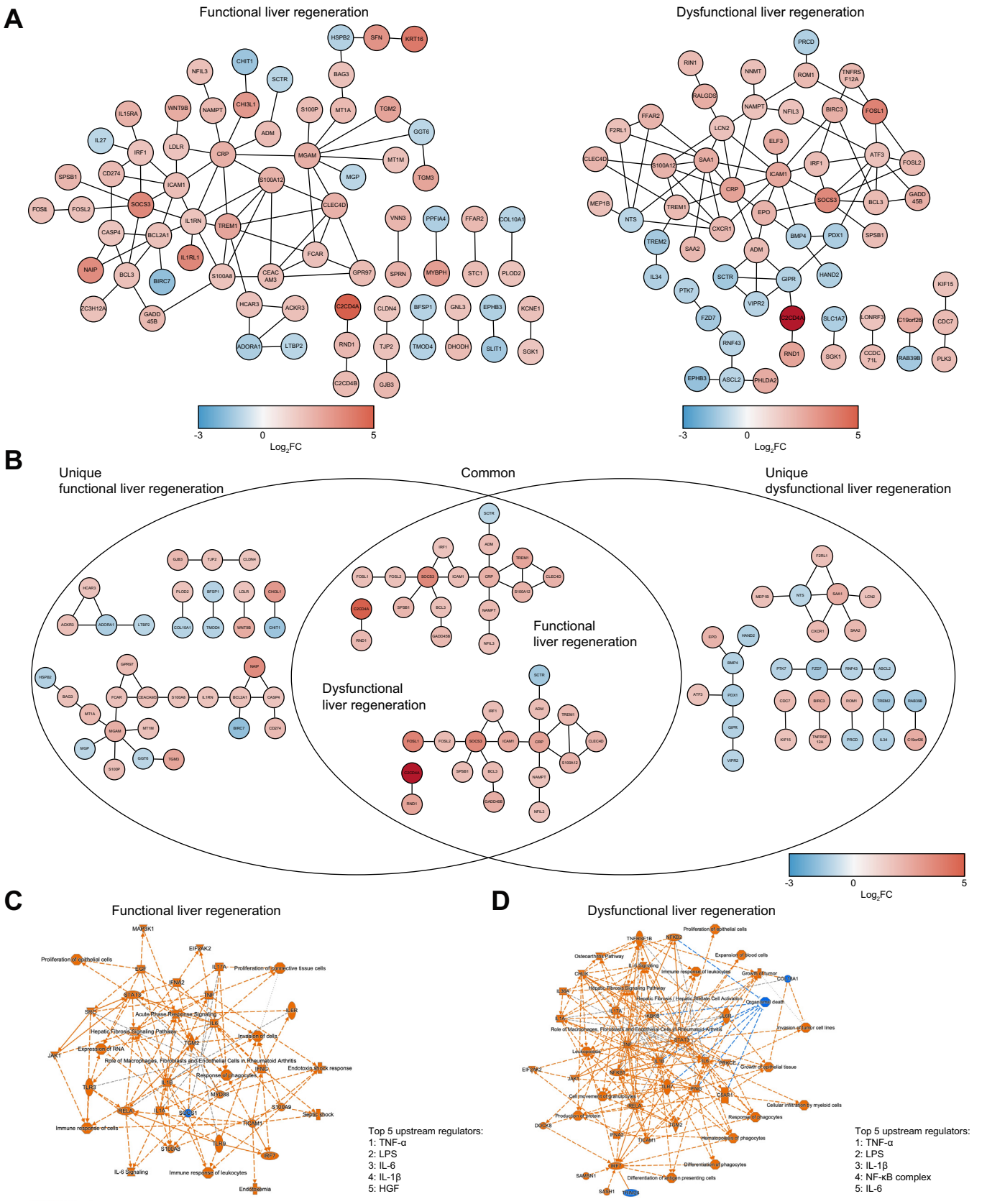

Fig. 3. STRING as well as upstream regulator analyses cluster around inflammatory processes during the priming phase of human LR. (A) STRING analyses (including only significantly regulated genes with a fold change of >1.5) are illustrated for individuals with and without postoperative DLR and colour coded according to the degree of change (red = induction, blue = reduction; see Fig. S2 for induction levels). (B) STRING assessment unique to individuals with or without postoperative DLR as well as STRING associations affected in both groups (middle part). IPA upstream regulator assessment is illustrated (C) for individuals with postoperative FLR and (D) for individuals with postoperative DLR. The top five regulators are also illustrated for each group. DLR, dysfunctional LR; FLR, functional LR; IPA, Ingenuity Pathway Analysis; LR, liver regeneration; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TNF, tumour necrosis factor.

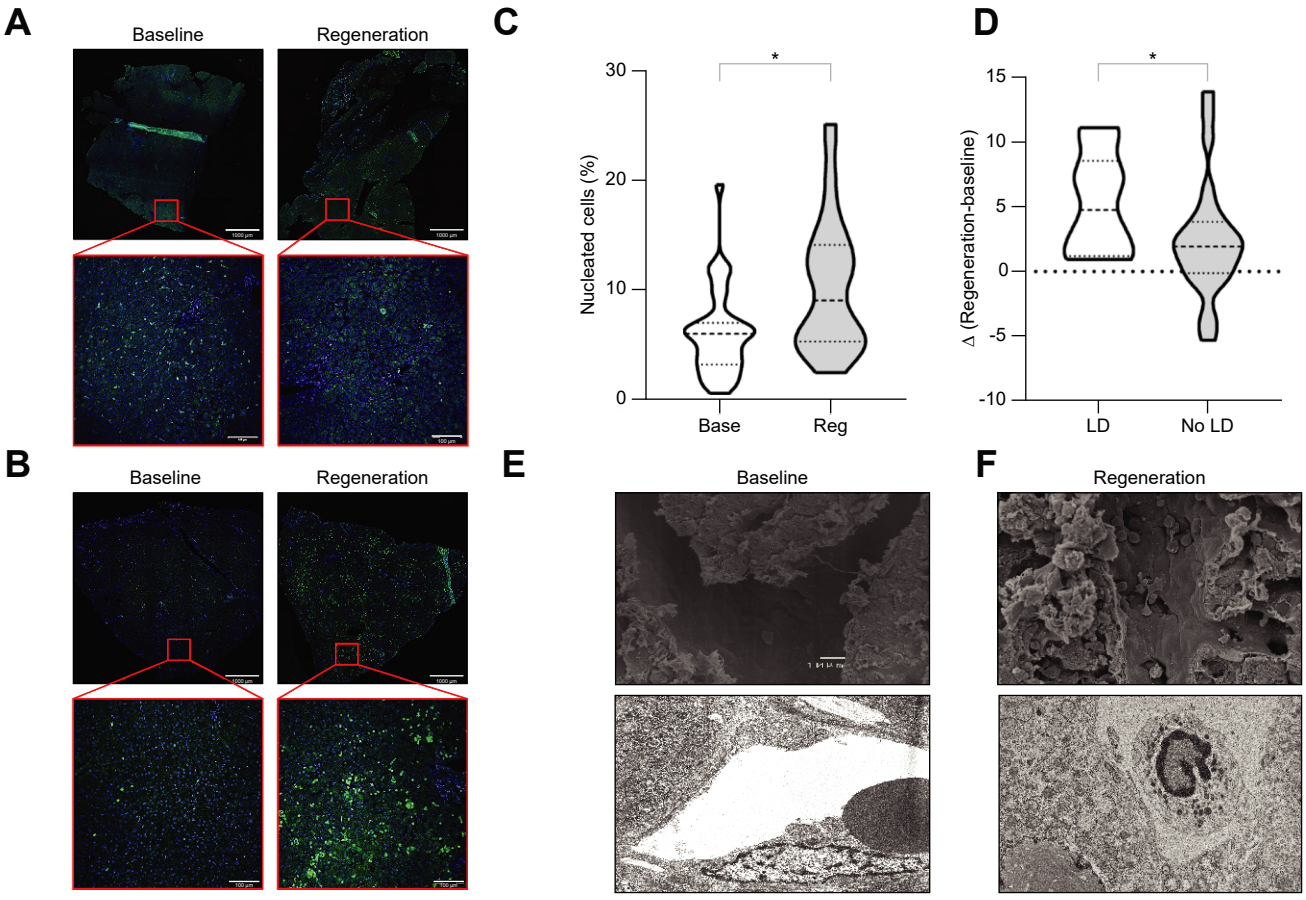

Fig. 4. Neutrophils rapidly accumulate in the liver during the priming phase of human LR. Intrahepatic neutrophil accumulation was assessed using immunofluorescence analysis at baseline and after resection (during the priming phase of LR, n = 46). Representative samples are given (A) for individuals with postoperative FLR and (B) for patients with postoperative DLR. (C) Neutrophil accumulation during LR was compared with baseline (Wilcoxon signed-rank test, p <0.001), and (D) the delta between these time points was evaluated between individuals with and without DLR (Mann–Whitney U test, p = 0.019). Transmission electron microscopy was used to confirm intrahepatic neutrophil accumulation during the priming phase of LR: (E) baseline and (F) regeneration. *p <0.05, **p <0.005. Base, baseline; DLR, dysfunctional LR; FLR, functional LR; LR, liver regeneration; Reg, regeneration.

representative individuals, one with DLR and the other without DLR, spatial transcriptomic approaches also demonstrated that these genes were induced in liver tissue upon the priming phase of human LR (Fig. 2B).

Functional protein association networks and IPA upstream regulator assessment further highlight the relevance of inflammation during the priming phase of human LR

To further explore possible functional associations, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) network analyses were used. As illustrated in Fig. 3A, networks of FLR and DLR showed significant overlap, again centring around inflammatory processes (Fig. 3B illustrates common and uniquely regulated genes between groups). Once more, the induction of inflammation-associated genes appeared increased in patients with DLR (Fig. 3A and B; a list of significantly induced genes and their absolute induction can be found in Table S2).

Similarly, assessing predicted upstream regulators using IPAs, multiple cytokines were observed to be critically involved in the process of early gene expression in our cohort (Fig. 3C [FLR] and D [DLR]). Highest-activation z-scores were achieved for TNF-α, LPS, IL-1β, NF-κB complex, and IL-6 in patients with DLR and TNF-α, LPS, IL-6, IL-1β, and hepatocyte growth factor in patients with FLR, again putting inflammation in the centre of the priming phase of human LR.

Exceeding neutrophil infiltration and activation is associated with DLR

The majority of the identified pathways and interactome analyses as well as several of the most altered genes were associated with intrahepatic inflammation and immune cell accumulation. Immunofluorescence analyses showed a significant increase of intrahepatic neutrophils already during this early period of LR (Fig. 4A and B show representative samples of individuals with FLR and those with DLR, respectively; Fig. 4C illustrates quantification; p <0.001). When further quantifying intrahepatic neutrophil accumulation, we observed that individuals with DLR presented with a significantly higher intrahepatic neutrophil accumulation compared with individuals with FLR (Fig. 4D; p = 0.019). Intrahepatic neutrophil accumulation was further confirmed using scanning and transmission electron microscopy (Fig. 4E and F).

Given the fact that multiple cytokines serve as upstream regulators of LR and are critically involved in this process in mice, we profiled selected cytokines, described to be involved during rodent LR, in our participants. Although we observed rather

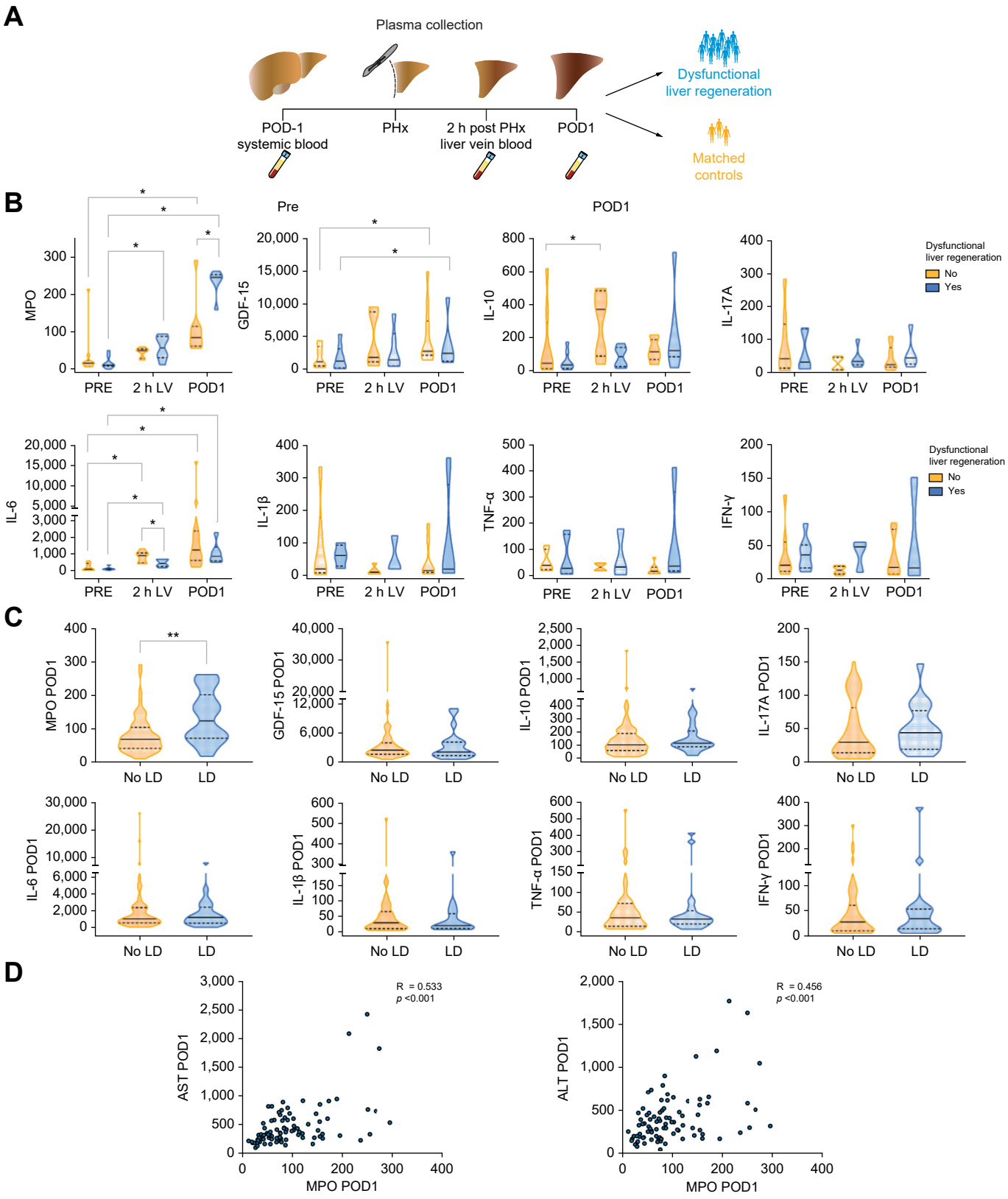

Fig. 5. Perioperative cytokine profiling reveals only moderate differences in circulating cytokines but significant heterogeneity of MPO (as a neutrophil activation marker) in patients with and those without DLR. The dynamics of multiple cytokines were assessed before the operation (PRE), 2 h after ligation of the portal vein (2 h LV) (with blood taken from the liver vein, draining the regenerating liver lobe), and on POD1: (A) study design and (B) respective values (n = 46, MPO POD1: Mann–Whitney *U* test, $p = 0.002$). (C) To validate this observation in a larger cohort (n = 108), circulating cytokine levels were compared in individuals with and without DLR (DLR - MPO PRE to 2 h LV, $p = 0.012$; FLR - MPO PRE to POD1, $p < 0.001$; DLR - MPO PRE to POD1, $p = 0.003$; DLR - GDF-15 PRE to

dramatic acute increases of assessed cytokines, the majority of assessed cytokines did not significantly differ in patients with DLR or FLR (Fig. 5A). However, MPO, as a marker of neutrophil activation, significantly increased exclusively in individuals with DLR 2 h after induction of LR and showed significantly higher levels on POD1. To validate these results, we assessed circulating cytokine levels preoperatively as well as on POD1 in a larger cohort of 108 participants. Again, MPO was significantly higher in individuals with postoperative DLR, whereas none of the other assessed cytokines differed significantly between patient groups (Fig. 5B). When we further assessed aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as markers for hepatocyte demise, we found that increased MPO levels were indeed associated with increased intrahepatic cell destruction in the course of ongoing LR, presumably as a consequence of an overwhelming intrahepatic inflammatory response (Fig. 5C).

Baseline differences in gene expression identifies DUSP4 as significantly dysregulated in DLR

When we compared genes with a greater than 1.5-fold difference at baseline, we found that only four protein-coding genes were differentially expressed (heatmap illustration is given in Fig. 6A, N = 21). Of those, DUSP4, which is known to be involved in endothelial cell activation, showed the highest expression value. DUSP4 levels inversely correlated with MPO levels on POD1 (Fig. 6B), and individuals with reduced preoperative intrahepatic DUSP4 expression showed significantly higher MPO levels on POD1 (Fig. 6B), but no correlation could be observed at baseline, suggesting an aggravated intrahepatic neutrophil activation, given the reduced expression of DUSP4. In the liver, DUSP4 is predominantly expressed in LSECs (https://www.proteinatlas.org/ENSG00000120875-DUSP4/tissue). Using spatial transcriptomics and concomitant LSEC staining, we could confirm in a representative sample (one individual with DLR and one individual with FLR) that individuals with DLR did indeed respond in an aggravated LSEC ICAM-1 expression upon induction of LR (Fig. 6C; baseline DUSP4 expression of 11.1 fragments per kilobase of transcript per million mapped reads [FPKM] in the individual with FLR and 2.1 FPKM in the individual with DLR), which is in line with our bulk sequencing data. To confirm LSEC localisation and its reduction on the protein level, we further performed immunofluorescence stainings for DUSP4, documenting predominant LSEC specificity (two representative samples are given for individuals with FLR [left] and two for individuals with DLR [right] in Fig. 6D) and its reduction in individuals with postoperative DLR (Fig. 6D, right; FLR: 293.4 mean fluorescence intensity [MFI] *vs*. DLR: 137.2 MFI). Ultimately, we quantified DUSP4 expression using spatial transcriptomics data and were able to document predominant colocalisation with LSECs, as shown in Fig. 6E. Subsequently, we aimed to assess the potential underlying mechanism of DUSP4 reduction. Accordingly, we performed evaluations in a validated mouse model of NASH and a mouse model of inferior vena cava ligation (IVCL). Compared with regular chow-fed age-matched controls, LSECs from NASH or IVCL mice demonstrated a significant reduction of DUSP4 (chow *vs*. NASH LSECs, $p = 0.007$; control *vs*. IVCL LSECs, $p = 0.049$; Fig. 6F). In line with previous reports, no DUSP4 expression was observed in hepatocytes (Fig. 6F).

Discussion

Our data provide insights into a previously undescribed landscape of the dramatic transcriptomic changes occurring rapidly after a liver regenerative stimulus in the human liver. Although rodent models suggest an indispensable role of inflammatory responses during the priming phase of LR, we observed that participants who developed DLR rather responded with an exaggerated induction of inflammation-related genes as well as an increased intrahepatic neutrophil accumulation and activation. Importantly, our data suggest that people with DLR may be primed for an overwhelming inflammatory response as these individuals exhibited decreased levels of DUSP4. Supporting this possibility, we could document a significant reduction of DUSP4 in LSECs in a mouse model of chronic liver disease. Accordingly, although processes such as inflammation might indeed be critical during the priming phase of LR in rodent models, overactivation of these processes might ultimately be harmful in humans and more relevant as a pathophysiologic phenomenon in people also with underlying liver disease. Ultimately, our results identify DUSP4 in LSECs as a potential therapeutic target to preoperatively optimise individuals to avoid an overwhelming immune response.

Comparing individuals with FLR and DLR, we identified that regulation of inflammatory responses appears to be a key regulatory hub in all individuals. In line with existing experimental findings that inflammatory processes are critically involved during the priming phase of LR, we found that inflammatory processes indeed predominated during this initial regenerative period. Gut-derived endotoxins such as LPS have been implicated as an important factor in the initiation of LR after partial hepatectomy.[22] Our pathway analyses did further support the hypothesis that translocation of LPS mediated a TNF, IL-1β, and IL-6 response via the activation of the NF-κB pathway, ultimately leading to inflammatory responses within the liver predominated by STAT3 activation and further SOCS3 expression. In parallel, endotoxaemia and cytokines have been shown to upregulate ICAM-1 expression in endothelial cells, which has been suggested to significantly contribute to cytokine expression, particularly via the accumulation of leucocytes in the liver sinusoids.[7,23] Comparing participants with FLR and DLR, we observed only moderate differences in circulating cytokines. However, we observed that genes uniquely increasing in patients with DLR were associated with NF-κB and TNF signalling, suggesting a critical intrahepatic dysregulating of inflammatory responses in these patients. Further, we found that patients developing DLR showed an increased ICAM-1 induction, experienced an aggravated intrahepatic neutrophil accumulation, and had increased circulating MPO levels (as a marker of neutrophil

POD1, $p = 0.032$; FLR - GDF-15 PRE to POD1, $p = 0.022$; FLR - IL-10 PRE to 2 h LV, $p = 0.018$; DLR - IL-6, PRE to 2 h LV, $p = 0.012$; FLR - IL-6, PRE to 2 h LV, $p = 0.012$; DLR - IL-6, PRE to POD1, $p = 0.006$; and FLR - IL-6, PRE to POD1, $p < 0.001$ [all Wilcoxon signed-rank test], and FLR *vs*. DLR - MPO: POD1, $p = 0.014$; and FLR *vs*. DLR - IL-6: 2 h LV, $p = 0.043$ [both Mann–Whitney *U* test]). (D) The correlation of POD1 MPO with transaminases (ALT/AST) (Pearson correlation coefficient, MPO POD1 and AST POD1, $R = 0.533$, $p < 0.001$; MPO POD1 and ALT, $R = 456$, $p < 0.001$). $^{*}p < 0.05$, $^{**}p < 0.005$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DLR, dysfunctional LR; FLR, functional LR; GDF-15, Growth differentiation factor 15; IFN-γ, interferon-γ; LR, liver regeneration; MPO, myeloperoxidase; POD1, 1 day after liver resection; TNF, tumour necrosis factor.

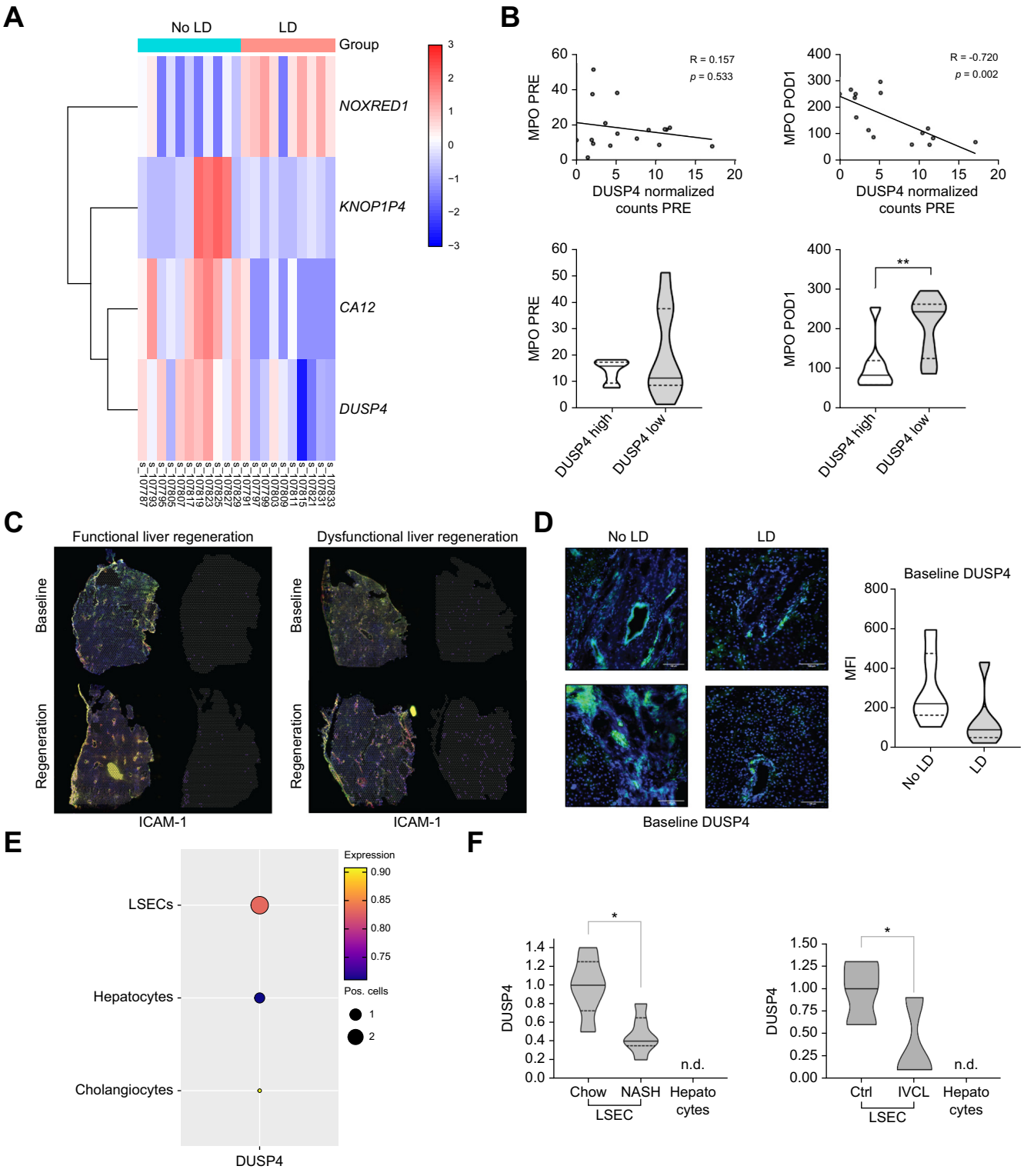

Fig. 6. Intrahepatic baseline DUSP4 reduction affects postoperative LR and is associated with neutrophil activation. (A) Heatmap illustration indicating genes significantly affected (1.5-fold difference) in individuals with and without DLR (derived from our sequencing cohort, n = 21). (B) Correlation of baseline DUSP4 expression with preoperative (PRE) and postoperative (POD1) MPO (Pearson correlation coefficient, DUSP4 PRE normalised counts, MPO POD1, R = -0.720, p = 0.002) (top). Further, MPO levels (before liver resection and POD1) were compared in individuals with high *vs.* low DUSP4 expression (Mann–Whitney U test, DUSP4 high/low MPO POD1, p = 0.002) (bottom). (C) Spatial transcriptomics are illustrated for ICAM-1 expression in an individual with FLR and in an individual with DLR at baseline and after induction of LR. (D) DUSP4 immunofluorescence staining is shown, illustrating predominant expression in LSECs, at baseline in two representative samples of patients with FLR and two with DLR and is further quantified on the right (Mann–Whitney U test; DUSP4, p = 0.064). (E) Spatial transcriptomics confirmed that DUSP4 expression predominantly colocalised with LSECs. (F) DUSP4 expression of isolated LSECs from NASH and IVCL mice compared with age-matched controls (chow-fed mice) (Mann–Whitney U test; chow *vs.* NASH, p = 0.007; Ctrl *vs.* IVCL, p = 0.049). DUSP4 expression was not

activation) after induction of hepatic regeneration. This further underlines the potential deleterious effects of an overwhelming inflammatory response in individuals with DLR. It is important to note that not a single gene that was donwregulated in DLR was induced in FLR during the induction of LR. Although this would have been of particular interest for our analyses, this indicates that it appears that processes are not 'missing' or even 'counter-regulated' in individuals with DLR, but it appears to be the amount of induction that plays a critical role in DLR.

Neutrophils are the most abundant immune cells and are involved in the immediate response to infection and inflammation. Of interest, during LR, their role is regarded as pro-regeneratory. In particular, neutrophil depletion is well documented to result in reduced LR in mice,[7] and similar results could be observed when ICAM-1, one of the major adhesion molecules for neutrophils, was depleted.[7] Depletion of these mechanisms was associated with a significant reduction of IL-6 and TNF, and administration of IL-6 could restore LR in this model.[7] Similarly, neutrophils may indirectly evoke a positive effect on liver repair via shifting macrophages into a pro-regenerative phenotype via reactive oxygen species.[24] However, specifically in acute processes such as ischaemia/reperfusion injury, observed during liver surgery, neutrophils promote liver injury.[25] During acute liver injury, neutrophils rapidly accumulated within the liver and mediate excessive immune responses in liver tissue, resulting in aggravated intrahepatic cellular demise.[26,27] Further, experimental models have documented the necessity of tight regulation of initial inflammatory processes during the priming phase of LR. As an example, the simple duration of IL-6 exposure critically affects liver injury and repair in mice, with adverse effects observed after prolonged exposure.[28] In our analyses, we found that individuals with and without DLR only marginally differed in their circulating cytokine levels, even if they were assessed directly in the liver vein, draining the regenerating liver lobe. The fact that intrahepatic neutrophil accumulation was significantly higher in individuals with DLR and that the neutrophil activation marker MPO was the only elevated marker at POD1 in these individuals (also in the larger validation cohort) suggests a rather deleterious effect of excessive intrahepatic neutrophil accumulation in human LR. This was further supported by the association of MPO with postoperative AST and ALT, indicating increased intrahepatic cell death. This is in line with one of our previous reports that suggests that individuals with underlying liver disease experience an exhausted response of counter-regulative/immunosuppressive mechanisms that leads to an overwhelming immune response, ultimately inhibiting LR.[29]

In this context, it is important to note how human LR significantly differs from conventional rodent LR models. First, classical knockout or treatment models combined with partial hepatectomy use livers without underlying hepatic pathology most typically. In clinical routine, however, only a minority of people do not have any form of underlying liver disease. NASH, CASH, cirrhosis, or simply advanced age are known pathologies that significantly affect intrahepatic pathophysiology. Second, inflow occlusion or simple parenchymal compression during liver surgery causes ischaemia/reperfusion. Both these aspects are closely linked to intrahepatic inflammation and oxidative stress, which might explain our observation that participants with DLR showed an aggravated intrahepatic inflammatory response and in particular neutrophil accumulation/activation.

Indeed, our results suggest that the underlying liver parenchyma may represent a main determinant on how the liver will react to a regeneratory stimulus. Although classical liver histology was unable to detect any differences between individuals with and without DLR, we found that only four protein coding genes (DUSP4, NOXRED1, CA12, and KNOP1P4) were highly (>1.5-fold) differentially expressed within groups. DUSP4, which was significantly lower in individuals with DLR, was of particular interest as in the liver it is predominantly expressed in LSECs, where it is associated with inflammatory and oxidative stress responses in LSECs.[8,30] According to the Human Protein Atlas, LSECs express 43.4 protein-coding transcripts per million (pTPM), whereas this value is more than 80 times lower in hepatocytes (0.5 pTPM), which was supported by our mouse results, where we were unable to detect DUSP4 in hepatocytes compared with a high expression in isolated LSECs. Ischaemia/reperfusion injury in DUSP4$^{-/-}$ hearts leads to a significant upregulation of NOX4 and concomitantly increased oxidative stress.[8] DUSP4 overexpression, conversely, was able to reduce oxidative stress.[8] DUSP4 seems to negatively regulate mitogen-activated protein kinase activation, also mediating anti-inflammatory effects.[31] Targeted DUSP4 knockdown in endothelial cells enhanced TNF-α-mediated ERK1/2 pathway activation and resulted in increased adhesion molecule expression such as ICAM-1.[30] Of interest, ICAM-1 was significantly elevated in individuals with DLR (2.3-fold *vs.* 1.7-fold in individuals with FLR, p <0.001), reaching number 11 of the most regulated genes in these individuals (number 109 in individuals with FLR), suggesting that this mechanism is presumably critical in the process of DLR. This is of particular interest as ICAM-1 increases in the lining of liver sinusoids upon toxic injury of the liver, mediating intrahepatic neutrophil accumulation. Upon its deficiency, neutrophils not only adhered significantly less frequently, but also their trans-endothelial migration was significantly inhibited, resulting in decreased intrahepatic inflammation and toxicity.[6] Ultimately, we were able to document that DUSP4 was significantly reduced in LSECS from NASH and IVCL mouse models. This further suggests that DUSP4 reduction, which is most likely caused by chronic liver disease, might be of critical relevance in the observed increased intrahepatic inflammation and in particular the aggravated neutrophil accumulation, as we observed in individuals with DLR. The underlying mechanism of DUSP4 reduction associated with inflammation is an important area of future investigation.

Within these analyses, we are able to demonstrate that, within only 2 h of induction of LR, the human liver induces a

detectable (ND) in isolated hepatocytes of any of the models. *p <0.05, **p <0.005. Ctrl, control; DLR, dysfunctional LR; DUSP4, dual-specificity phosphatase 4; FLR, functional LR; ICAM-1, intracellular adhesion molecule-1; IVCL, inferior vena cava ligation; LR, liver regeneration; LSEC, liver sinusoidal endothelial cell; MFI, mean fluorescence intensity; MPO, myeloperoxidase; NASH, non-alcoholic steatohepatitis; POD1, 1 day after liver resection.

magnitude of different responses and our results represent a unique dataset of early genes affected during the priming phase of human LR. These early responses centred around inflammation, suggesting this process as a key regulatory hub during the priming phase of LR also in humans. However, although in knockout models, several of these processes are seemingly indispensable for the induction of LR, in the human setting, rather an excessive inflammatory response with an increased intrahepatic neutrophil accumulation seems to be of relevance given its association with DLR. Importantly, particularly baseline LSEC DUSP4 might be critically relevant in this process and might represent an attractive new therapeutic target.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CASH, chemotherapy associated steatohepatitis; DLR, dysfunctional LR; DUSP4, dual-specificity phosphatase 4; FDR, false discovery rate; FLR, functional LR; FPKM, fragments per kilobase of transcript per million mapped reads; ICAM-1, intracellular adhesion molecule-1; IPA, Ingenuity Pathway Analysis; IVCL, inferior vena cava ligation; logCPM, log counts per million; LPS, lipopolysaccharide; LR, liver regeneration; LSEC, liver sinusoidal endothelial cell; MFI, mean fluorescence intensity; MPO, myeloperoxidase; NASH, non-alcoholic steatohepatitis; PCA, principal component analysis; POD1, 1 day after liver resection; POD5, 5 days after liver resection; pTPM, protein-coding transcripts per million; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TMM, trimmed mean of *M* values; TNF, tumour necrosis factor.

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Conflicts of interest

The authors have no conflict of interest to declare related to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conception and design: PS, RS, AA. Acquisition of data: PS, LB, DP, JS, MH, SS, PH, TG, RS, AA. Analysis and interpretation of data: PS, LB, CM, DP, JS, JSt, MH, SS, HH, RG, DO, RK, PH, GG, CW, TG, RS, AA. Participated in drafting the article or revising it critically for important intellectual content: PS, LB, CM, DP, JS, JSt, MH, SS, HH, RG, DO, RK, PH, GG, CW, TG, RS, AA.

Data availability statement

Raw data have been made publicly available on GEO (Gene Expression Omnibus; Record ID GSE208413).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100683.

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