Transcriptional Analysis of Liver Tissue Identifies Distinct Phenotypes of Indeterminate Pediatric Acute Liver Failure

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Many patients with indeterminate pediatric acute liver failure (PALF) have evidence of T-cell driven immune injury; however, the precise inflammatory pathways are not well defined. We have characterized the hepatic cytokine and transcriptional signatures of patients with PALF. A retrospective review was performed on 22 children presenting with indeterminate (IND-PALF; n = 17) or other known diagnoses (DX-PALF; n = 6) with available archived liver tissue. Specimens were stained for clusters of differentiation 8 (CD8) T cells and scored as dense, moderate, or minimal. Measurement of immune analytes and RNA sequencing (RNA-seq) was performed on whole-liver tissue. Immune analyte data were analyzed by principal component analysis, and RNA-seq was analyzed by unsupervised hierarchical clustering, differential gene expression, and gene-set enrichment analysis. Most patients with IND-PALF (94%) had dense/moderate CD8 staining and were characterized by Th1 immune analytes including tumor necrosis factor α, interferon γ (IFN-γ), interleukin (IL) 1β, IL-12, C-X-C motif chemokine ligand (CXCL) 9, and CXCL12. Transcriptional analyses identified two transcriptional PALF phenotypes. Most patients in group 1 (91%) had IND-PALF and dense/moderate CD8 staining. This group was characterized by increased expression of genes and cell subset–specific signatures related to innate inflammation, T-cell activation, and antigen stimulation. Group 1 expressed significantly higher levels of gene signatures for regulatory T cells, macrophages, Th1 cells, T effector memory cells, cytotoxic T cells, and activated dendritic cells (adjusted $P < 0.05$). In contrast, patients in group 2 exhibited increased expression for genes involved in metabolic processes. *Conclusion:* Patients with IND-PALF have evidence of a Th1-mediated inflammatory response driven by IFN-γ. Transcriptional analyses suggest that a complex immune network may regulate an immune-driven PALF phenotype with less evidence of metabolic processes. These findings provide insight into mechanisms of hepatic injury in PALF, areas for future research, and potential therapeutic targets. (*Hepatology Communications* 2021;5:1373-1384).

Pediatric acute liver failure (PALF) is a rare condition in which a child with no prior history of liver disease rapidly develops severe hepatic dysfunction. There are several known causes; however, in up to 40% of cases no etiology is identified, and the diagnosis is indeterminate (IND-PALF).[1,2] Despite

Abbreviations: AA, aplastic anemia; CCL, C-C motif chemokine ligand; CD8, clusters of differentiation 8; CXCL, C-X-C motif chemokine ligand; DX-PALF, other diagnoses; FC, log2-fold change; FDR, false discovery rate; GO, Gene Ontology; GSEA, gene-set enrichment analysis; GSVA, gene-set variation analysis; HLA, human leukocyte antigen; IFN, interferon; IHC, immunohistochemical; IL, interleukin; IL-2R, soluble interleukin-2 receptor; IND-PALF, Indeterminate PALF; IRAK3, IL-1 receptor-associated kinase 3; MIP, macrophage inflammatory protein; NLRP1, family pyrin domain containing-1; PALF, pediatric acute liver failure; PC, principal component; PCA, principal component analysis; RNA-seq, RNA sequencing; TNF, tumor necrosis factor.

Received February 24, 2021; accepted March 1, 2021.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1726/suppinfo.

Supported by the Scoby Family and the Northwestern Medicine Dr. Michael M. Abecassis Transplant Innovation Endowment Grant.

efforts to adjudicate cases and improve the diagnostic workup over the past 15 years, the prevalence of IND-PALF has remained relatively stable.[1-5] Studies suggest that the liver injury in patients with IND-PALF is immune-mediated, driven by untethered inflammatory responses.[6,7] Many patients with IND-PALF have features of immune activation, including elevated serum soluble interleukin-2 receptor (IL-2R) levels, peripheral blood cytopenias or development of aplastic anemia (AA), and improvement with immunosuppressive therapy.[6,8-10] Furthermore, patients with IND-PALF have increased numbers of T cells and markers of T-cell immune activation in peripheral blood, and liver tissue specimens are characterized by a dense clusters of differentiation 8–positive ($CD8^+$) perforin-positive T-cell infiltrate.[11-13] Prominent hepatic CD8 T-cell staining is a biomarker of patients with PALF with activated CD8 T-cell hepatitis and may be used to help identify this group. In addition, we found increased T-cell clonality in liver specimens from these patients, supporting a role for antigen-driven CD8 T-cell expansion.[11,13] Although studies have measured serum inflammatory mediators in patients with PALF and identified patterns that correlate with outcome, the precise mechanisms driving activated CD8 T-cell hepatitis are poorly understood.[14,15] We aimed to determine the inflammatory pathways and gene-expression profiles in liver tissue from patients with PALF with activated CD8 T-cell hepatitis. We characterized hepatic cytokine levels and transcriptional signatures that were similar and different between activated CD8 T-cell hepatitis and alternate etiologies of PALF. Our results indicate that the liver injury in most patients with IND-PALF is driven by a CD8 T-cell and Th1-mediated inflammatory process, while also supporting the presence of distinct sub-phenotypes within this group. Future research is needed to understand how these patients may differ in their response to injury and to potential future therapies.

Materials and Methods

PATIENTS

Medical records of patients who presented to Ann & Robert H. Lurie Children's Hospital of Chicago from 2000-2015 were retrospectively reviewed for PALF cases, identified by International Classification of Diseases, Ninth Revision, database code search for acute liver failure and hepatic encephalopathy. Patients were included if they met PALF diagnostic criteria as defined by the PALF Study Group,[1] were between the ages of 1 and 17 years, and had archived frozen liver tissue available. Patients with PALF due to ischemia, rheumatologic disease, or malignancy were excluded. This study was approved by the Lurie Children's Institutional Review Board, and the requirement for informed consent was waived. No

DOI 10.1002/hep4.1726

Potential conflict of interest: Dr. Loomes consults and received grants from Albireo and Mirum. She consults for Travere Therapeutics.

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donor organs were obtained from executed prisoners or other institutionalized persons.

CLINICAL, LABORATORY, AND PATHOLOGY DATA

Charts were reviewed to collect demographic, laboratory, and clinical data including development of AA and 21-day outcome of liver transplantation, spontaneous recovery with native liver, or death. Patients were classified as IND-PALF if no known cause for their liver disease was identified despite an age-appropriate diagnostic evaluation, as previously described.[2,11] All non-indeterminate etiologies for PALF that met inclusion criteria comprised the other diagnoses (DX-PALF) group. As part of a previous pathology study, formalin-fixed paraffin-embedded liver-tissue specimens were stained with hematoxylin and eosin and antibodies to immune cells of interest, including CD8 and perforin (a marker of lymphocyte cytolytic activity), as previously described.[11] Immunohistochemical (IHC) staining was performed by the clinical pathology laboratory. Results were reviewed by study investigators, and CD8 and perforin-staining patterns were subjectively scored as dense, moderate, or minimal.[11] Descriptive statistics were used to describe the patients, including median and interquartile range, and counts with percentages for categorical data. For comparison of continuous variables between groups, the Mann-Whitney U test was used. The Fisher's exact test was used for comparisons of categorical data between groups.

IMMUNE ANALYTE ANALYSIS IN PALF LIVER TISSUE

Immune analyte assays were performed on liver-tissue samples from 15 patients with IND-PALF and 5 patients with DX-PALF. Frozen liver-tissue samples were weighed, and approximately 75 mg of tissue per patient was placed into 0.5 mL of ice-cold Complete Lysis Buffer (MesoScale Discovery, Gaithersburg, MD). Samples were homogenized, rocked on ice for 30 minutes at 4°C, and centrifuged at 10,000*g* for 10 minutes at 4°C. The supernatant was collected and the protein concentration was calculated for each sample homogenate using the Bradford colorimetric method.[16] To determine the concentration of immune analytes in each sample, we used the MesoScale Discovery assay for 39 human cytokines, chemokines, and growth factors: eotaxin-1, eotaxin-3, granulocyte-macrophage colony-stimulating factor, interferon-γ (IFN-γ), interferon-inducible T-cell alpha chemoattractant/C-X-C motif chemokine ligand (CXCL) 11, interleukin (IL) 1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-γ inducible protein 10/CXCL10, monocyte chemoattractant protein (MCP) 1/C-C motif chemokine ligand (CCL) 2, MCP2/CCL8, macrophage derived chemokine/CCL22, monokine induced by IFN-γ/CXCL9, macrophage inflammatory protein (MIP) 1α/CCL3, MIP1β/CCL4, MIP3α/CCL20, stromal cell-derived factor 1/CXCL12, thymus and activation regulated chemokine/CCL17, transforming growth factor β, tumor necrosis factor (TNF) α, TNF-β, and vascular endothelial growth factor A (MesoScale Discovery, Gaithersburg, MD). Samples were assayed in duplicate following the manufacturer's instructions, and immune analytes were measured using an electrochemiluminescent detection method (MesoScale Discovery). Principal component analysis (PCA) was used to reduce dimensionality of the data, and meaningful principal components (PCs) were used in univariate logistic regression models to predict outcomes of interest. Unsupervised hierarchical clustering analysis was used to partition patients into homogenous groups. Wilcoxon rank-sum test with Bonferroni correction for multiple comparisons was used to compare cytokine and chemokine levels between patient groups. A P value < 0.05 was considered statistically significant.

TRANSCRIPTIONAL PROFILING OF PALF

Total RNA was extracted from archived frozen liver tissue using a TissueLyser LT and the RNeasy Mini Kit protocol (QIAGEN, Hilden, Germany). RNA purity and concentration were confirmed using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA sequencing (RNA-seq) was conducted in the Northwestern University NUSeq Core Facility. RNA quantity was determined with a Qubit fluorometer (Invitrogen, Carlsbad, CA). Total RNA samples were checked for quality based on fragment size using an Agilent Bioanalyzer 2100 (Santa Clara, CA). RNA of sufficient quality for RNA-seq was extracted from liver

TABLE 1. CHARACTERISTICS AND IHC STAINING PATTERNS FOR PATIENTS WITH PALF

Variable	Total Cases n = 23	IND-PALF n = 17	DX-PALF n = 6	*P* Value
Median age, years (IQR)	4 (2-8)	3 (2-5)	6 (4.3-13.8)	0.208
Male gender, n (%)	14 (61)	10 (59)	4 (67)	0.999
Developed aplastic anemia, n (%)	4 (17)	4 (24)	0	0.539
21-day outcomes, n (%)				0.999
Liver transplantation	21 (91)	15 (88)	6 (100)	
Alive with native liver	2 (9)	2 (12)	0	
CD8 staining pattern, n (%)	23	17	6	0.008
Dense/moderate	18 (78)	16 (94)	2 (34)	
Minimal	5 (22)	1 (6)	4 (66)	
Perforin staining pattern, n (%)	20	15	5	0.033
Dense/moderate	10 (50)	10 (66)	0	
Minimal	10 (50)	5 (33)	5 (100)	

Abbreviation: IQR, interquartile range.

tissue from 22 patients with PALF with median RNA integrity number of 7.3 (interquartile range 6.9-8.2). RNA-seq was performed on liver-tissue samples from 16 patients with IND-PALF and 6 patients with DX-PALF. The Illumina TruSeq Stranded Total RNA Library Preparation Kit (San Diego, CA) was used to prepare sequencing libraries from 200 ng of total RNA samples. This procedure includes ribosomal RNA (rRNA) depletion with the Ribo-Zero rRNA Removal Kit, complementary DNA synthesis, 3' end adenylation, adapter ligation, and library polymerase chain reaction amplification and validation (Illumina). The lllumina HiSeq 4000 Sequencer was used to sequence the libraries with the production of single-end, 50 bp reads. Sequencing reads were aligned to the human genome, and annotated transcripts were obtained from the Ensembl database. Hierarchical clustering, PCA, and identification of differentially expressed genes between groups of interest was performed using DESeq2.[17] Differentially expressed genes were considered significant based on an adjusted *P* value < 0.05 and log2-fold change (FC) > 1. Ranked gene lists were generated with edgeR and uploaded into the GSEABase package for gene-set enrichment analysis (GSEA) using the Gene Ontology (GO) database and immunologic signatures database.[18-20] Enrichment of GO processes was visualized with Cytoscape 3.8[21] using the EnrichmentMap application.[22] To account for heterogeneity between human liver samples, we also performed gene-set variation analysis (GSVA) using the GSVA R package[23] and characterized the expression of previously published immune cell subset signatures.[24,25] The accession number for the RNA-seq data reported in this paper is in the Gene Expression Omnibus database: GSE16 4397 (https://www.ncbi.nlm.nih.gov/geo/).

Results

Twenty-two patients, 17 with IND-PALF and 6 with DX-PALF, met the inclusion criteria. All patients with IND-PALF had a negative age-appropriate workup for known causes of PALF. The group with DX-PALF was comprised of 3 patients with drug-induced liver injury (acetaminophen, valproic acid, and trimethoprim-sulfamethoxazole), 2 patients with autoimmune hepatitis, and 1 patient with Wilson disease. Patient clinical characteristics and IHC staining results are listed in Table 1. There was no difference in median age or gender between patient groups with IND-PALF and DX-PALF. Four (24%) patients with IND-PALF developed AA compared with none of the patients with DX-PALF ($P = 0.54$), and of these, 3 ultimately had their AA treated with hematopoietic stem cell transplant. Two patients with IND-PALF received 3-4 days of intravenous methylprednisolone before collection of the liver-tissue sample used in this study; no other patients were treated with immunosuppressive therapy before liver transplantation. Most (91%) patients had the 21-day outcome of liver transplantation and explant liver-tissue sample used for this study. Patients with IND-PALF were significantly more likely to have dense/moderate CD8

staining (94%, n = 16) compared to patients with DX-PALF (34%, n = 2) (P = 0.008). Perforin staining was dense/moderate in 67% (n = 10) of patients with IND-PALF compared to minimal staining in 100% (n = 5) of patients with DX-PALF (P = 0.03). Figure 1 displays representative CD8, perforin, and CD103 IHC staining patterns from an IND-PALF and a DX-PALF case. Additional details of individual patient data, including diagnosis and staining results, are reported in Supporting Table S1.

Th1 CYTOKINE AND CHEMOKINE PROFILE CHARACTERIZES IND-PALF

Unsupervised hierarchical clustering of 20 patients with PALF by inflammatory mediators identified two main patient groups (Fig. 2A). The cytokine A group contained only patients with IND-PALF (n = 8) with dense/moderate CD8 staining, whereas the cytokine B group included patients with DX-PALF and minimal CD8 staining as well as 6 patients with IND-PALF patients with dense/moderate CD8 staining. There were no significant differences in demographic or clinical data between patients in the two groups (Supporting Table S2). Unsupervised PCA found that PC1 reflected a Th1 cytokine and chemokine profile with key mediators including TNF-α, IFN-γ, IL-1β, IL-12, IL-8, IL-27, CXCL9, CXCL10, CXCL11, CXCL12, and CCL4. Patients with greater positive loading for PC1 were significantly more likely to have IND-PALF, dense/moderate CD8 staining, and dense/moderate CD8 and perforin staining (P = 0.04 for all models). After adjusting for multiple

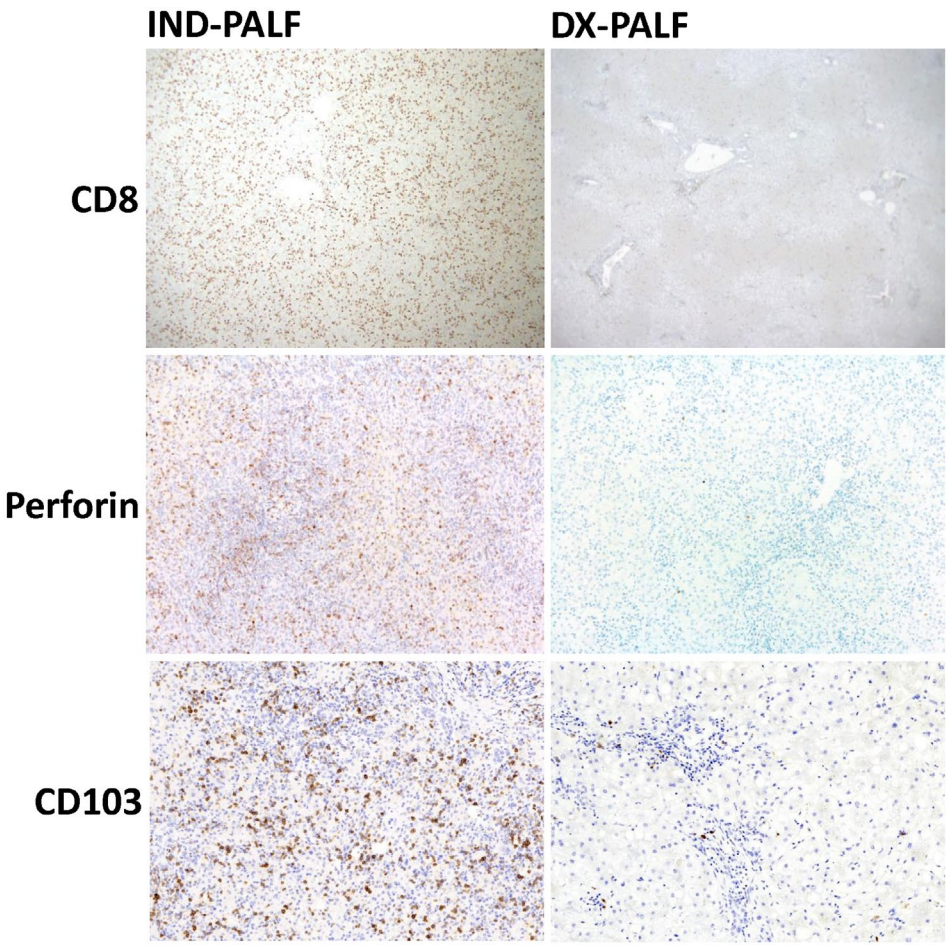
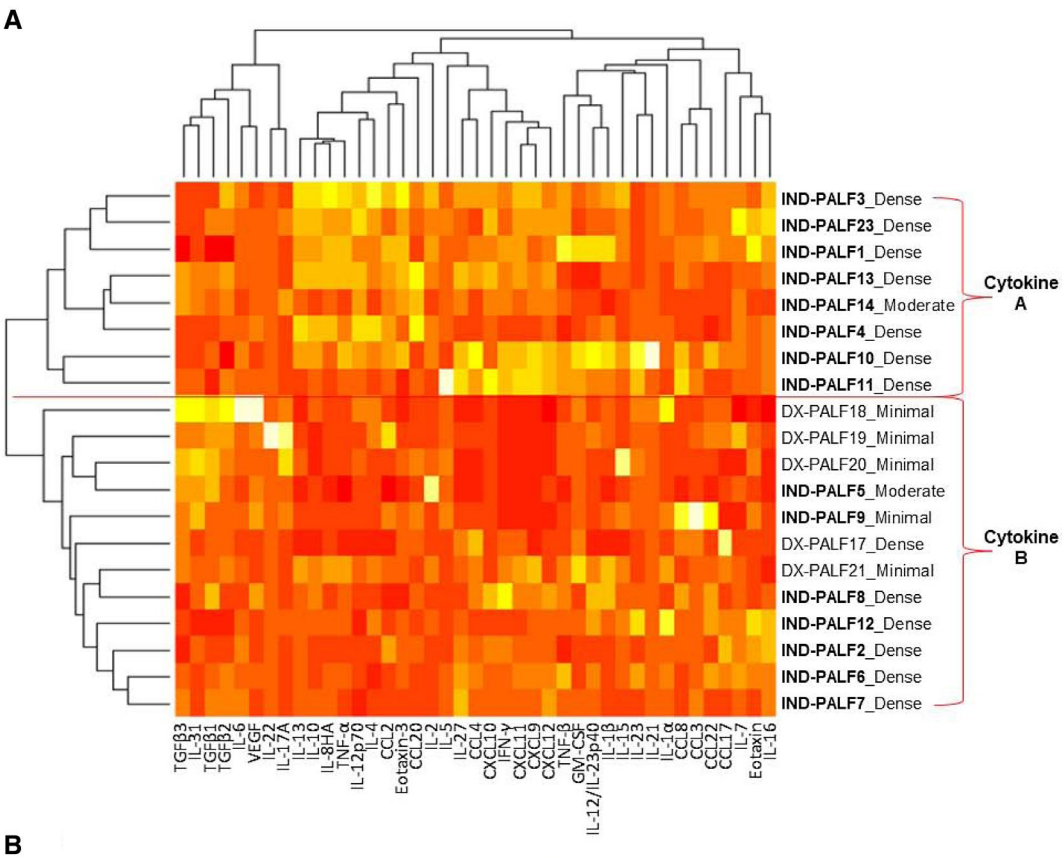


FIG. 1. Images at ×10 magnification of representative CD8, perforin, and CD103 IHC staining patterns from an IND-PALF case with dense CD8 staining and a DX-PALF case with minimal CD8 staining.

A



B

Cytokine/Chemokine	Dense/moderate CD8 staining (n=15)	Minimal CD8 staining (n=5)	P-value
CXCL10 pg/mg median (IQR)	988 (488-1489)	51 (49-59)	0.017
CXCL12 pg/mg median (IQR)	216 (180-238)	116 (112-133)	0.017
IL-16 pg/mg median (IQR)	4248 (3518-5595)	1205 (545-1408)	0.031

FIG. 2. (A) Unsupervised hierarchical clustering of immune analytes identifies two main patient groups: cytokine A and cytokine B. (B) Median levels of CXCL10, CXCL12, and IL-16 were significantly higher in patients with dense/moderate compared to minimal CD8 staining after adjusting for multiple comparisons.

comparisons, median levels of CXCL10, CXCL12, and IL-16 were significantly higher in patients with dense/moderate compared to minimal CD8 staining (Fig. 2B).

RNA-Seq ANALYSIS IDENTIFIES TWO TRANSCRIPTIONAL PROFILES OF PALF

PCA of RNA-seq libraries by sample demonstrated a group of patients with IND-PALF and DX-PALF clustering together by PC1 and identified one outlier that was excluded from additional analyses (Fig. 3A). Further analysis by unsupervised hierarchical clustering identified two transcriptional phenotypes of patients with PALF (Fig. 3B). Group 1 was comprised of 11 patients, most (n = 10) with IND-PALF and all with dense/moderate CD8 staining. Group 2 was comprised of 10 patients, including 4 patients with DX-PALF (3 with minimal and 1 with moderate CD8 staining), 1 patient with IND-PALF and minimal CD8 staining, and 5 patients with IND-PALF

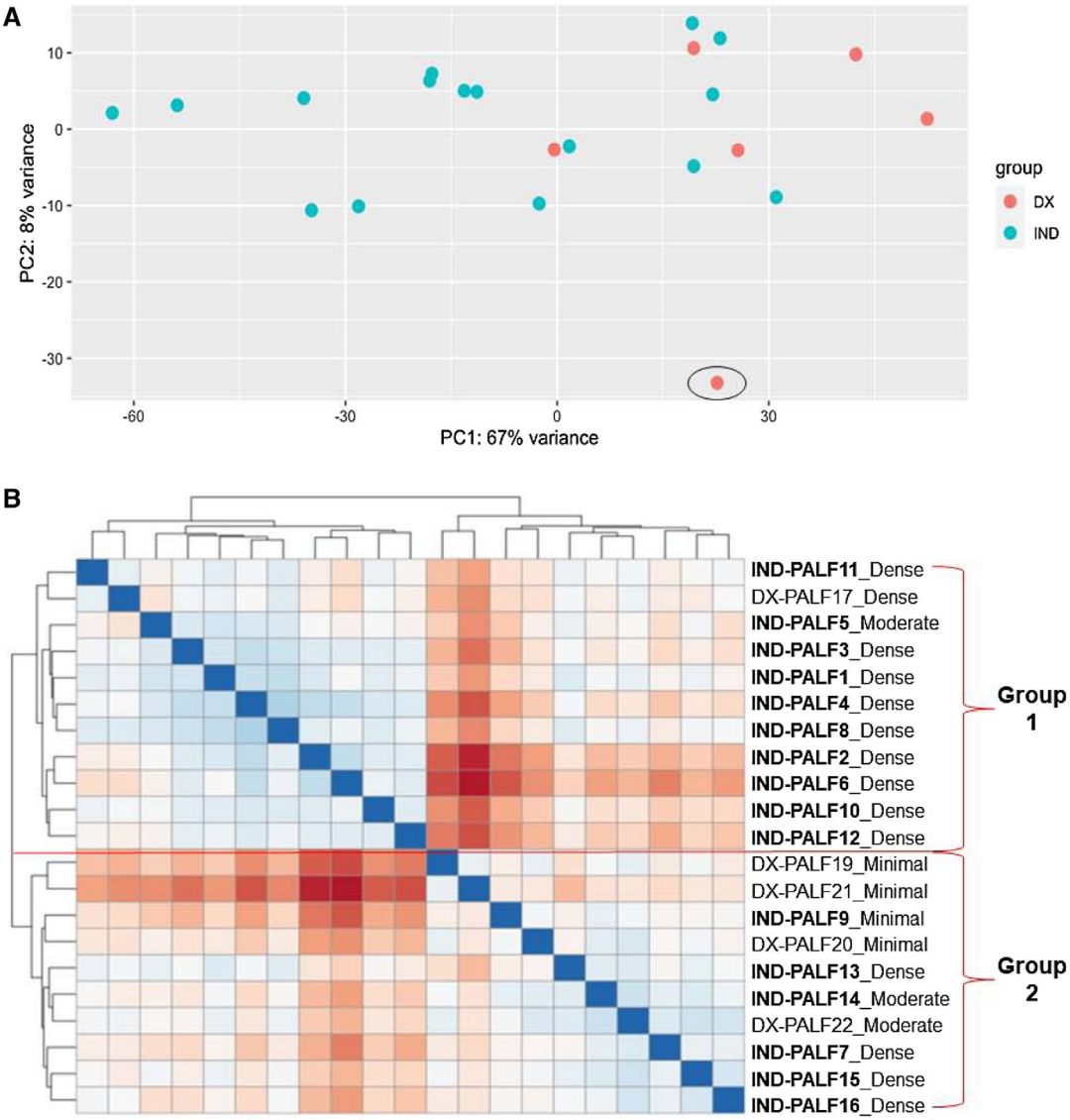


FIG. 3. (A) PCA of RNA-seq data reveal that patients partially cluster by diagnosis; one outlier (black circle) is identified. (B) Unsupervised hierarchical clustering of RNA-seq data identifies two main patient groups (group 1 and group 2).

and dense/moderate CD8 staining. Patients in group 1 were significantly more likely to have dense/moderate CD8 staining pattern on liver biopsy (P = 0.04), higher peak total bilirubin (P = 0.02), and higher peak international normalized ratio (INR) (P = 0.04) compared with patients in group 2 (Supporting Table S3). We identified 1,689 differentially expressed genes in group 1 and 1,616 in group 2, as defined by adjusted P value < 0.05 and $\log_2$FC > 1. Patients in group 1 had increased expression of genes related to innate immune inflammation, including NOD-like receptor family pyrin domain containing-1 (*NLRP1*), *NLRP2*, and IL-1 receptor-associated kinase 3 (*IRAK3*) as well as up-regulation of the gene for IL-2RA, which is expressed on activated T cells and involved in regulatory T-cell responses. GO enrichment analysis by GSEA identified 762 up-regulated gene sets in group 1 with a false discovery rate (FDR) < 25% and 450 gene sets with a nominal P value < 1%. Group 2 had 800 up-regulated gene sets enriched at FDR < 25% and 488 with a nominal P value < 1%. Overall, enriched gene sets in group 1 were related to immune-system processes including lymphocyte activation, myeloid cell activation, leukocyte activation and chemotaxis,

and cytokine production, whereas pathways related to metabolic processes including metabolism and transport, energy metabolic processes, metabolism and biosynthesis, and lipid metabolism were up-regulated in group 2 (Fig. 4A). Additional analysis with GSVA further supported enrichment for specific immune cell signatures in group 1 (Fig. 4B). Group 1 expressed significantly higher levels of previously reported gene signatures for regulatory T cells (20 genes) and macrophages (33 genes) from Charoentong et al. as well as signatures for Th1 cells (23 genes), T effector memory cells (15 genes), cytotoxic T cells (18 genes), and activated dendritic cells (5 genes) from Bindea et al. (adjusted P value < 0.05).[24,25] Expression of other immune cell signatures tested, including those for T central memory cells, Th2 cells, Th17 cells, B cells, and neutrophils, were not significantly higher in group 1. There was only slight agreement in patient clustering in the same group when comparing the cytokine analysis to the RNA-seq analysis with kappa statistic 0.15 (95% confidence interval –0.25-0.56).

More specific analysis of immune processes using the Broad Institute Immunologic signatures database identified 1,653 gene sets significantly enriched at FDR < 25% and 904 with a nominal P value < 1% in group 1. Gene sets of interest in group 1 from both databases included enrichment for antigen signaling, IL-2 production, IFN-α-stimulated dendritic cells, and TLR-stimulated monocytes (Fig. 4C). In contrast, gene sets of interest in group 2 patients included enrichment for multipotent progenitor cells, steroid biosynthetic processes, and fatty acid, organic acid,

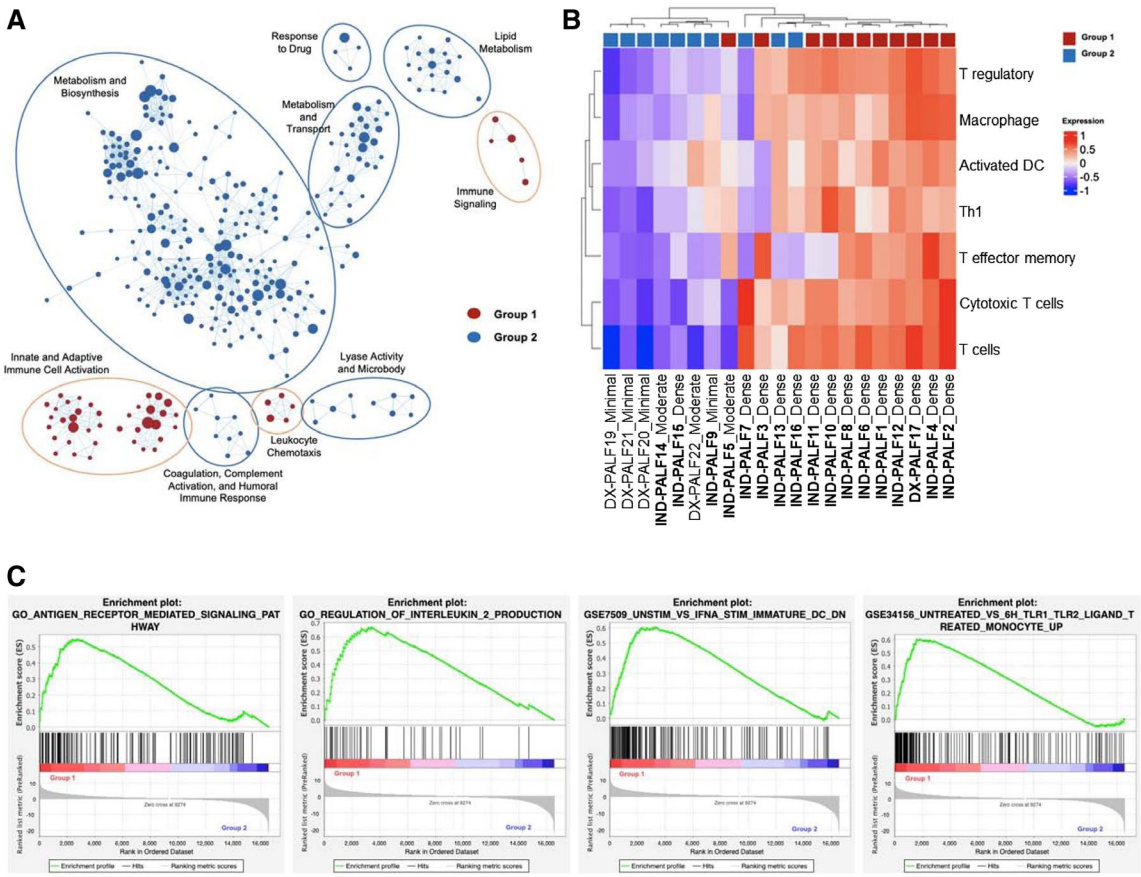


FIG. 4. (A) GO enrichment analysis by GSEA identified enriched gene sets in group 1 related to immune system processes, whereas pathways related to metabolic processes were up-regulated in group 2. (B) Gene-set variation analysis evaluated sample-to-sample heterogeneity and showed consistent enrichment in immune-cell signatures in group 1 (adjusted P value < 0.05 for all). (C) More granular analysis of specific immune processes enriched in group 1 identified up-regulation in antigen signaling and IL-2 production, as well as previously identified gene sets for IFN-α-stimulated dendritic cells and toll-like receptor stimulated monocytes. Abbreviations: TLR, toll-like receptor; Treg, regulatory T cell.

and lipid catabolic processes. Taken together, findings from our transcriptional analyses suggest that a complex immune network may regulate a PALF phenotype with greater immune-driven liver injury (group 1) in contrast to PALF cases with greater evidence of homeostatic and metabolic processes (group 2).

DENSE/MODERATE CD8 STAINING IN PATIENTS WITH PALF HAVE A DISTINCT TRANSCRIPTIONAL SIGNATURE

To gain insight into the CD8-driven transcriptional signature, we identified differentially expressed genes between patients with dense/moderate and those with minimal CD8 staining. Using cutoffs for adjusted $P < 0.05$ and $\log_2 FC > 1$, there were 1,335 differentially expressed genes in patients with dense/moderate CD8 staining and 1,066 genes in patients with minimal CD8 staining. Along with up-regulation of gene expression for CD8 antigen, as expected, the dense/moderate CD8 staining group had increased expression of genes encoding chemokine (C-C motif) receptor 5 (CCR5) and CXCR6, receptors on $CD8^+$ and Th1 T cells involved in lymphocyte recruitment and migration to the liver. Up-regulation for genes encoding chemokines induced by IFN-γ (*CXCL9*, *CXCL10*, and *CXCL11*) and IL-16 were also present in the dense/moderate CD8 staining group. Additional genes of interest with increased expression in patients with dense/moderate CD8 staining included genes for the inhibitory receptors T-cell immunoglobulin and ITIM domains (*TIGIT*), cytotoxic T lymphocyte-associated antigen-4 (*CTLA4*), lymphocyte activation gene-3 (*LAG3*), and human leukocyte antigen (HLA) class I genes including *HLA-A*, *HLA-B*, and *HLA-F*.

GO enrichment analysis by GSEA identified up-regulated pathways related to immune-system processes in patients with dense/moderate CD8 staining similar to group 1, whereas patients with minimal CD8 staining showed enrichment for metabolic processes similar to group 2. To explore this relationship further, we examined the number of differentially expressed genes that overlapped between comparison groups. Dense/moderate CD8 staining and group 1 patients shared 857 genes, and minimal CD8 staining patients shared 881 genes with group 2, whereas there were only two genes that were common between group 2 and dense/moderate CD8 staining patients (Fig. 5A). Shared genes of interest included genes involved in the innate immune response (*NLRP1* and *IRAK3*) in dense/moderate CD8 staining and group 1 (Fig. 5B) as compared with genes involved in hepatic metabolic processes (cytochrome P450 family 7 subfamily A member 1 [*CYP7A1*] and *APOC1* [apolipoprotein C1]) in minimal CD8 staining and group 2 cases (Fig. 5C).

Finally, to explore whether two PALF transcriptional phenotypes are present in patients with IND-PALF only, we performed a subanalysis on this group. Similar to our previous findings, patients with IND-PALF also separated into two groups, of which one group demonstrated less enrichment for metabolic processes, similar to findings for group 1 and dense/moderate CD8 staining groups (Supporting Fig. S1). Overall, these data support the presence of two transcriptional phenotypes within IND-PALF, of which some appear more comparable to known diagnoses despite similar histologic evidence of hepatic CD8 T-cell infiltration.

Discussion

This study examines inflammatory mediators and performs transcriptome analysis using liver tissue from patients with PALF. Unsupervised hierarchical clustering by cytokine and chemokine levels found that patients with PALF with dense/moderate CD8 staining grouped together and reflected a Th1 type inflammatory response driven by IFN-γ. Clustering by transcriptional analysis similarly identified a group of patients with PALF with dense/moderate CD8 staining and up-regulation of immune pathways enriched for signatures related to Th1 T cells, effector memory T cells, and cytotoxic T cells (group 1). In contrast, group 2 also contained 5 patients with IND-PALF with dense/moderate CD8 staining but was characterized by up-regulation of metabolic pathways, suggesting subclasses of patients among IND-PALF diagnoses. In adults with acute liver failure, increased serum expression of microRNAs associated with successful liver regeneration are reported in patients who recovered with their native liver, and pathway analysis found that the microRNA changes in the spontaneous recovery group up-regulated metabolic processes.[26] In our study, most patients had the outcome of liver transplantation; however, it is

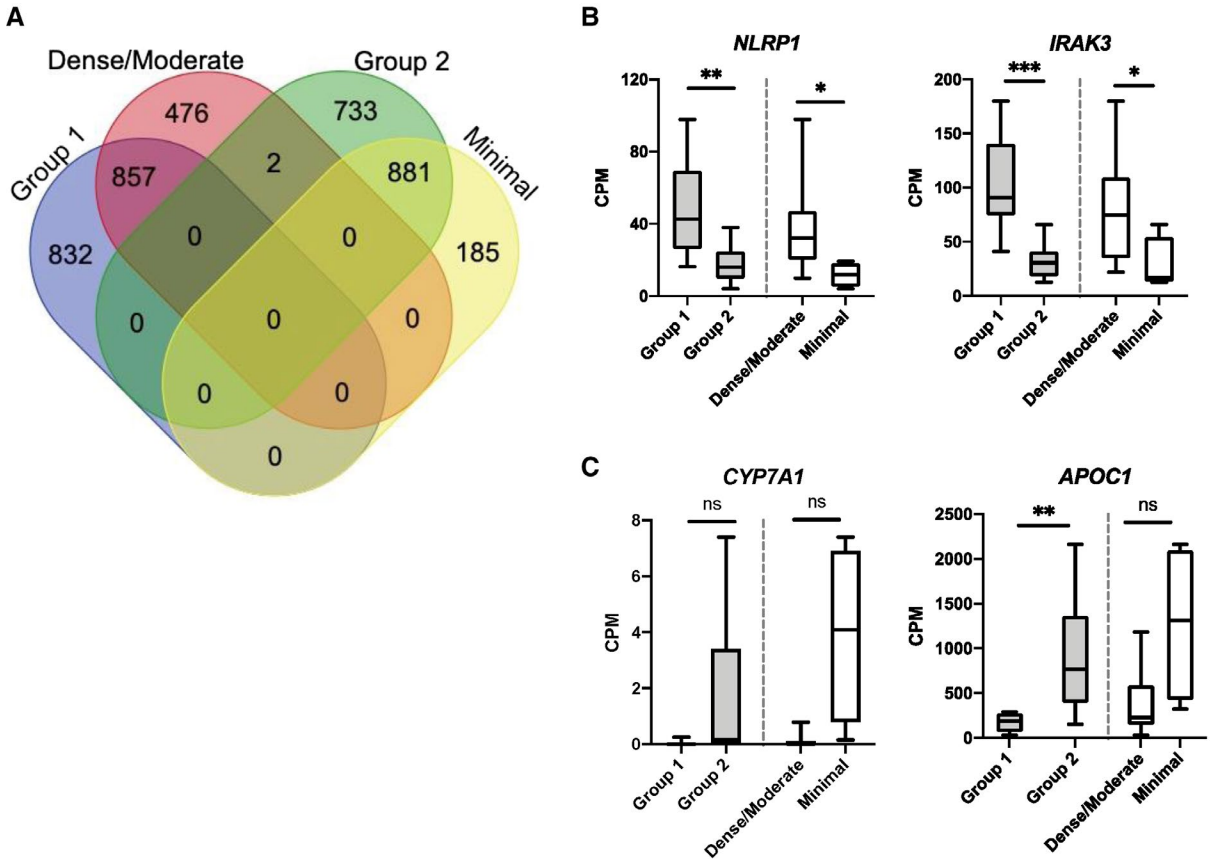


FIG. 5. (A) Venn diagram demonstrates overlap of differentially expressed genes between comparison groups of Dense/Moderate CD8 staining with group 1 and minimal CD8 staining with group 2. Visualization of counts-per-million reads are mapped for *NLRP1* and *IRAK3* shared in dense/moderate CD8 staining with group 1 (B) and *CYP7A1* and *APOC1* in minimal CD8 staining with group 2 (C). Paired two-tailed *t* test was performed and significance reported: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *NLRP1* and *IRAK3* were significantly different for each of the comparison groups. Differences in *CYP7A1* did not achieve statistical significance at $P = 0.09$ and $P = 0.1$ for group 1 versus group 2 and dense/moderate CD8 staining versus minimal CD8 staining comparisons, respectively. *APOC1* was significantly different for group 1 versus group 2 comparison but not for dense/moderate CD8 staining versus minimal CD8 staining ($P = 0.1$). Abbreviations: APOC1, apolipoprotein C1; CPM, counts per million; CYP7A1, cytochrome P450 family 7 subfamily A member 1; ns, not significant.

possible that up-regulation of metabolic pathways in group 2 represents a transcriptional signature of regeneration, and patients with IND-PALF in this group may have been more likely to spontaneously recover. Besides density of CD8 staining and peak total bilirubin and INR lab values, we did not identify any demographic or clinical features that differentiated the two groups, which may be related to the retrospective nature of our study, which was limited by available data and the common outcome of liver transplantation. Further studies are needed to better define how the transcriptional phenotype of IND-PALF subgroups may relate to outcomes, clinical biomarkers, and the degree of inflammatory injury in activated CD8 T-cell hepatitis.

Our results emphasize the central role of an activated Th1-driven CD8 T-cell response in the pathophysiology of PALF, while also highlighting innate immune pathways that may be involved. Patients with dense/moderate CD8 staining have increased expression of genes for the chemokines CXCL9, CXCL10 and CXCL11, which bind to the CXCR3 receptor on activated T cells and may function as key T-cell chemo-attractants in the activated CD8 T-cell hepatitis inflammatory network.[27,28] Similarly, the alarmin IL-16 was identified as a key cytokine in

both immune analyte and transcriptional analyses. IL-16 serves as a T-cell chemo-attractant and mediates cross-talk between T cells and dendritic cells.[29] Furthermore, enrichment for gene sets for IFN-α-stimulated dendritic cells in group 1 supports a role for dendritic cells in the cross-priming of CD8 T cells in response to inflammatory signals in PALF.[30] Group 1 also demonstrated enrichment in gene sets for activated monocytes that may be recruited to the liver in the setting of hepatic injury. Finally, we demonstrate evidence of common immune pathways in group 1 and dense/moderate CD8 staining patients, including up-regulation of *NLRP1* (encodes an inflammasome protein) and *IRAK3* (encodes a protein involved in toll-like receptor and IL-1R immune signal transduction pathways). Greater data re needed to identify the specific cell-to-cell signaling pathways as well as determine which immune pathways may be unique to specific PALF subgroups.

Our findings also support an antigen-driven mechanism for the activation and proliferation of cytotoxic T cells in IND-PALF. Group 1 demonstrated up-regulation of antigen stimulation, and patients with dense/moderate CD8 staining had up-regulation of genes for immune inhibitory receptors (*CTLA4* and *TIGIT*). Increased expression of these surface markers may be secondary to CD8 T-cell activation and an attempt to control an overactive inflammatory process, or may be related to T-cell exhaustion secondary to chronic antigen exposure. Similarly, a prominent IFN-γ response may lead to up-regulation of HLA class I gene expression and corresponding increase in antigen presentation and CD8 T-cell activation. These results, along with our previous findings of increased T-cell clonality in liver tissue from patients with IND-PALF,[11,13] suggest that the T cells are responding to antigen and clonally expanding. Future studies are needed to determine the nature of such a proposed antigen(s), including whether it is pathogen or self-derived, and to explore the role of HLA class I–restricted immunity in activated CD8 T-cell hepatitis.

Progenitor cells are critical to promote hepatic regeneration and compensate for severe liver injury; however, the role for specific progenitor subsets in PALF remains poorly defined. Interestingly, we showed significantly increased liver-tissue levels of CXCL12, a chemokine expressed by liver sinusoidal endothelial cells, in patients with CD8 dense/moderate versus minimal PALF. CXCL12 expression increases with acute or chronic liver injury and is involved in recruitment of bone marrow progenitors of liver sinusoidal cells for liver regeneration.[31,32] In the setting of PALF, CXCL12 may be up-regulated to mobilize bone marrow progenitors to the liver. Children with IND-PALF frequently present with bone marrow suppression, which may progress to AA, suggesting that a loss of bone marrow–derived progenitors may be a critical step in the disease mechanism of liver failure.[10,33] Future studies are needed to better determine the role for progenitor subsets in different etiologies of PALF and how CXCL12 and other markers of hepatic regeneration might help predict patient outcome.

We acknowledge several limitations in this study, largely secondary to known difficulties associated with obtaining human tissue samples in rare diseases. Subjects in this study were selected based on availability of archived liver tissue, and therefore biased toward those who underwent liver transplantation. As such, we were unable to analyze results by outcomes of interest. In addition, our findings reflect the immune analyte and transcriptional profile of liver tissue from patients at a single point in time, with heterogeneity in duration and degree of liver injury that may affect results. Finally, our analyses were limited by use of frozen whole-liver tissue and prevented cell subset–specific analyses. Future studies with RNA-seq of homogeneous immune cell subsets and single-cell RNA-seq are needed to more completely describe the transcriptome of immune cells in activated CD8 T-cell hepatitis.

In conclusion, our data support the presence of distinct transcriptional phenotypes within PALF. Enriched pathways related to innate and adaptive immune activation and signaling were present in patients with PALF with activated CD8 T-cell hepatitis, whereas a subgroup of these patients demonstrated increased expression for genes involved in metabolic pathways. The implications of these different subgroups of patients with PALF remains poorly defined, and how it may affect propagation of antigen stimulation and hepatic inflammatory injury in activated CD8 T-cell hepatitis is unknown. These results broaden the understanding of the complex processes involved in PALF disease pathogenesis, and suggest potential therapeutic targets including IFN-γ and Th1-driven pathways. Future research will focus on

prospective studies of the immune response in these patients, identifying the nature of a possible antigen trigger, and further exploring genetic factors that may be involved in disease pathogenesis.

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Supporting Information

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