Functionally Diverse Inflammatory Responses in Peripheral and Liver Monocytes in Alcohol-Associated Hepatitis

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Alcohol-associated hepatitis (AH) is an acute inflammatory disease in which gut-microbial byproducts enter circulation and peripheral immune cells infiltrate the liver, leading to nonresolving inflammation and injury. Single-cell RNA sequencing of peripheral blood mononuclear cells isolated from patients with AH and healthy controls paired with lipopolysaccharide (LPS) challenge revealed how diverse monocyte responses are divided among individual cells and change in disease. After LPS challenge, one monocyte subtype expressed pro-inflammatory genes in both disease and healthy controls, while another monocyte subtype was anti-inflammatory in healthy controls but switched to pro-inflammatory in AH. Numerous immune genes are clustered within genomic cassettes, including chemokines and C-type lectin receptors (CTRs). CTRs sense byproducts of diverse microbial and host origin. Single-cell data revealed correlated expression of genes within cassettes, thus further diversifying different monocyte responses to individual cells. Monocyte up-regulation of CTRs in response to LPS caused hypersensitivity to diverse microbial and host-derived byproducts, indicating a secondary immune surveillance pathway up-regulated in a subset of cells by a closely associated genomic cassette. Finally, expression of CTR genes was higher in livers of patients with severe AH, but not other chronic liver diseases, implicating secondary immune surveillance in nonresolving inflammation in severe AH. (*Hepatology Communications* 2020;4:1459-1476).

Activation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and C-type lectin receptors (CTRs), leads to a diverse, innate immune response including pro-inflammatory and anti-inflammatory gene expression. CTRs and TLRs participate in immune surveillance, sensing a broad range of potentially harmful, microbially derived pattern-associated molecular patterns (PAMPs) and host-derived damage-associated molecular patterns (DAMPs).[1,2]

Although PAMPs and DAMPs were first recognized as important in response to infection, growing evidence suggests other disorders, including metabolic diseases, can lead to PAMP or DAMP accumulation.[3] The liver immune system is crucial in the response to PAMPs and DAMPs, and dysregulation of liver immune cells can lead to pathologies such as chronic hepatitis C virus (HCV) infection, sterile inflammation in non-alcoholic fatty liver disease (NAFLD), and alcohol-associated liver disease (ALD).[4] For example, alcohol

Abbreviations: AH, alcohol-associated hepatitis; AHL, AH with liver failure; ALD, alcohol-associated liver disease; CCL, chemokine (C-C motif) ligand; CD, clusters of differentiation; CTR, C-type lectin receptor; CXCL, chemokine (C-X-C motif) ligand; DAMP, damage-associated molecular pattern; ExAH, explant tissue from patients with severe AH; HCV, hepatitis C virus; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; NKC, natural killer receptor gene complex; PAMP, pattern-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PRR, pattern recognition receptor; scRNA-seq, single-cell RNA sequencing; TDB, trehalose-6,6-dibehenate; TLR, toll-like receptor.

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consumption causes gut-barrier dysfunction and leakage of microbial products such as bacterial lipopolysaccharide (LPS) and *C. albicans*-derived β-glucans, as well as hepatocyte cell death, leading to release of hepatocyte-derived DAMPs.[5-8] Alcohol-associated hepatitis (AH) is an acute inflammatory condition that can lead to severe complications and short-term mortality among patients with ALD.[9] Patients with AH are chronically exposed to bacterial LPS at concentrations similar to those observed after alcohol consumption.[10] One hypothesis for the pathogenicity of AH is that inflammation driven by PAMPs and DAMPs leads to nonresolving inflammation, infiltration of peripheral immune cells, exacerbated liver damage, and a failure to develop endotoxin tolerance.[11-14] In AH, inflammatory pathways are simultaneously hypersensitive to PAMPs and DAMPs, despite a paradoxical increased risk for infection.[15]

Myeloid-derived cells, such as tissue-resident macrophages and peripheral monocytes, respond to LPS and β-glucans by secreting pro-inflammatory cytokines and phagocytosis of the foreign material.[6,16] In rodent models of ALD, Kupffer cells, the resident macrophage of the liver, become hypersensitive to LPS and respond with exacerbated pro-inflammatory cytokine and chemokine expression.[17,18] Peripheral blood mononuclear cells (PBMCs) isolated from patients with AH are similarly hypersensitive to LPS, implicating circulating monocytes, which highly infiltrate the liver in AH, and resident macrophages in disease severity.[19-21] Monocytes and macrophages are highly heterogeneous and have important pro-inflammatory and anti-inflammatory roles depending on the immune and metabolic environment.[21-24] This cellular diversity creates dynamically responsive cells important for all stages of inflammation, from initial response to infection/injury to resolution/healing.[22] A common feature of AH is increased circulating monocytes, implicating these cells as a major source of systemic inflammation.[25]

CTRs along with TLRs and other PRRs are critical for innate immune function, but the role and regulation of CTRs during alcohol consumption and after gut-barrier dysfunction lags behind TLRs. Challenge of bone marrow–derived macrophages with low-dose LPS, modeling the low-level endotoxemia observed during alcohol consumption, up-regulates the expression of *Mincle*, a CTR required for recognition and response to tuberculosis infection, fungal pathogens, and a hepatocyte-derived DAMP, Sap130.[7,26] *Mincle* up-regulation increases sensitivity of BMDMs to certain PAMPs and DAMPs.[26] Deficiency of either Mincle or Dectin-1, a β-glucan sensitive CTR, protects mice from ethanol-induced liver damage, implicating CTRs as pro-inflammatory receptors that exacerbate systemic and liver inflammation after gut-barrier dysfunction.[6,8] If CTRs are up-regulated in response to LPS, then they would increase the diversity of PAMPs and DAMPs sensed by the immune system, creating a secondary immune surveillance pathway capable of responding to a greater variety of microbial byproducts from the gut or cell-derived DAMPS. CTRs are organized into 14 groups based on similar domain structures. Group II and group V CTR genes are clustered in a cassette on chromosome 12 within a region termed the natural killer receptor gene complex (NKC).

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Group II and many group V CTRs are expressed in myeloid-derived cells and not natural killer (NK) cells, despite the name of the gene complex.[27]

Many pro-inflammatory genes are localized in cassettes within the genome, including C-X-C-type and C-C-type chemokines.[28,29] Little is known about co-regulation of functionally related genes that reside in genome cassettes, or how their regulation might contribute to health or disease. If all genes within a cassette are activated in a single cell, then a typical immune response would include subsets of specialized pro-inflammatory cells, such as high chemokine expressing cells; on the other hand, if each cytokine or chemokine within a cassette is independently regulated, then all cells would contribute to inflammation. Because CTRs are in a cassette in the genome, they might be coordinately expressed in specific, specialized immune cells geared toward sensing a greater variety of PAMPs and DAMPs.

Using single-cell RNA sequencing (scRNA-seq), we sought to understand the diversity of peripheral immune responses to low-dose LPS and how these are altered in AH, a severe inflammatory stage of ALD.[30] scRNA-seq allows us to study the diversity of individual cells in the peripheral immune system and how each cell type responds to stimulus, thus revealing the diversity of cell types and their functional responses simultaneously. From these data, we examine how individual genes and whole cassettes of genes (cytokines, chemokines, CTRs) are up-regulated in single cells, distinguishing whether they are regulated in a coordinated way, similar to bacterial operons, or with a diverse array of monocytes expressing different pro-inflammatory genes. If CTR genes are coordinately expressed after LPS, then these genes would act as a secondary immune surveillance system to detect and respond to other PAMPs and DAMPs that enter circulation from the gut after injury. Our data reveal extensive coordinated regulation of gene expression based on chromosomal proximity, and future studies will focus on developing therapies to reduce expression of entire gene cassettes, to reduce inflammation in response to harmful microbial- and host-derived molecular patterns. Finally, we observe higher expression of CTR genes in peripheral monocytes and in the livers of patients with severe AH, but not in patients with other liver disease. These data implicate CTRs and secondary immune surveillance in exacerbating liver inflammation in AH.

Materials and Methods

ALCOHOL-RELATED HEPATITIS AND HEALTHY CONTROL PATIENT SELECTION

Enrolled patients had confirmed diagnosis of AH by clinicians at the Cleveland Clinic based on medical history, physical examination, and laboratory results, according to the guidelines of the American College of Gastroenterology (https://gi.org/clinical-guidelines/) (Supporting Table S5). Healthy controls were recruited from the Clinical Research Unit at the Cleveland Clinic.

SINGLE-CELL RNA-Seq WITH LPS STIMULATION

Cryopreserved PBMCs from 4 patients with AH and 4 age-matched healthy controls were thawed following the 10× protocol for cryopreserved PBMCs. Cryopreserved PBMCs have highly correlated expression patterns to fresh cells.[31] Additionally, LPS responses were similar between cryopreserved and fresh cells in AH and healthy control PBMCs.[19,21] Cells were then plated and stimulated with 100 pg/mL LPS for 24 hours. To resuspend, cells were washed one time with phosphate-buffered saline PBS, then incubated on ice with PBS + 2.5 mM ethylene diamine tetraacetic acid for 15 minutes. After gently resuspending cells by pipetting, cells were spun in eppendorf tubes at 300× *g* for 5 minutes. Cells were washed with PBS + 0.04% bovine serum albumin, resuspended, and counted for 10× sequencing. Gel beads in emulsion and libraries were performed according to manufacturer's instructions (Chromium v3.1). Libraries were quantified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), then pooled and sequenced using a NextSeq550v2.5.

STUDY APPROVAL

The study protocol was approved by the institutional review board for the Protection of Human Subjects in Research at the Cleveland Clinic and University Hospitals, Cleveland. All methods were performed in accordance with the internal review board's guidelines and regulations, and written, informed consent was obtained from all subjects.

DATA AND CODE AVAILABILITY

The scRNA-seq data for this study can be found at National Center for Biotechnology Information Gene Expression Omnibus under accession number PRJNA596980. All scripts used for analyses and differential expression results for all cell types can be found at the author's github (github/atomadam2).

Results

SINGLE-CELL RNA-Seq OF LOW-DOSE LPS-STIMULATED PBMCs REVEALS INCREASED MONOCYTE POPULATIONS IN AH

To understand how diverse cellular functions are regulated in single cells, we developed a protocol for scRNA-seq of PBMCs isolated from patients with AH then challenged for 24 hours with and without low-dose LPS (100 pg/mL), to model pathophysiological concentrations in patients with AH.[7,10,32] PBMCs from 4 patients with AH and 4 age-matched healthy controls were isolated, sequenced and analyzed, resulting in 18,433 single-cell transcriptomes (Supporting Tables S1 and S2).

For clustering analyses, all data sets were first normalized using the SCTransform algorithm, which was found to accurately combine and cluster single-cell data from different platforms.[33-35] Our data were then combined with a publicly available data set that included PBMCs sequenced from eight different scRNA-seq platforms.[33,36] Thus, we were able to use data generated by other groups to identify cell types and ensure our data clustered accurately and reproducibly. Because our data set represents four separate conditions (healthy basal, healthy LPS, AH basal, and AH LPS), clustering analyses were performed by first computationally determining anchor genes to identify common cell types in all samples and conditions without the effect of differentially expressed genes.[37] This enabled us to cluster and identify all PBMC cell types, such that individual clusters can easily be compared across different conditions and to other PBMC data sets (Supporting Fig. S1).

Through the clustering analyses, major PBMC cell types were clearly defined (Fig. 1A). As has been shown previously,[25] patients with AH had a four-fold increase in monocytes compared with healthy controls ($P < 0.05$, Fig. 1B,C). LPS challenge decreased the proportion of monocytes by about 50% in all samples ($P < 0.05$). We focused our transcriptomic analyses on monocytes, because increased numbers of peripheral monocytes is commonly observed in patients with AH and ALD, and monocytes from patients with AH have exacerbated responses to LPS compared with healthy controls.[18,19,25]

MONOCYTES ARE DIVERSE AND EXPRESS DIFFERENT MARKERS IN PATIENTS WITH AH

The results of our clustering analysis found four clusters of monocytes in our data and the publicly available data: three clusters of differentiation (CD) 14^+ monocyte clusters and a $CD16^+$ monocyte cluster (Fig. 1A and Supporting Figs. S2 and S3). The three $CD14^+$ monocyte clusters correspond to classical $CD14^+$ monocytes, based on co-expression of CD11b in both our cells and cells from the publicly available data (Supporting Fig. S2). In healthy controls, CD16 was restricted to $CD16^+$ monocytes as expected, but in AH, CD16 was expressed in all clusters. The $CD16^+$ monocytes are likely a combination of nonclassical $CD16^+$ monocytes and intermediate $C14^+/CD16^+$ monocytes, given the expression of CD14 within this cluster. Interestingly, $CD16^+$ monocytes from patients with AH expressed CD14 at higher levels (Fig. 1D). Using Seurat, we determined the genes uniquely expressed in each of these monocytes (Supporting Fig. S3). $CD14^+$ cluster 3 was notably high in human leukocyte antigen-DR expression, whereas $CD14^+$ cluster 2 highly expressed CD14 and calprotectin (*S100A8* and *S100A9*). Interestingly, $CD14^+$ cluster 1 did not have a single strongly expressed marker, possibly due to increased diversity within this cluster. One possible marker, SLC7A11, is a cysteine/glutamate transporter expressed on more activated monocytes,[38] whereas CD9, a tetraspanin, was also highly expressed in some of the cells in cluster 1. Next, we performed differential expression testing, comparing clusters to each other, in healthy controls, and in patients with AH at baseline and after LPS. Among the top genes differentially expressed between clusters were a number of PRRs, including *TLR2*, *TLR8*, *MDL* (*CLEC5A*), *Dectin-1* (*CLEC7A*), and *Mincle* (*CLEC4E*) (Supporting Tables S3 and S4).

To illustrate differences between these clusters, we selected candidate genes that represent various monocyte functions, including PRRs, complement receptors, and macrophage polarization. In general, each monocyte cluster expressed marker genes differently, at baseline and in response to LPS (Fig. 1D). TLR expression was diverse, with low basal expression of TLR4 and TLR8 in healthy controls, but high TLR2 expression in CD14^{+} cluster 2. In AH, almost all monocyte clusters had higher

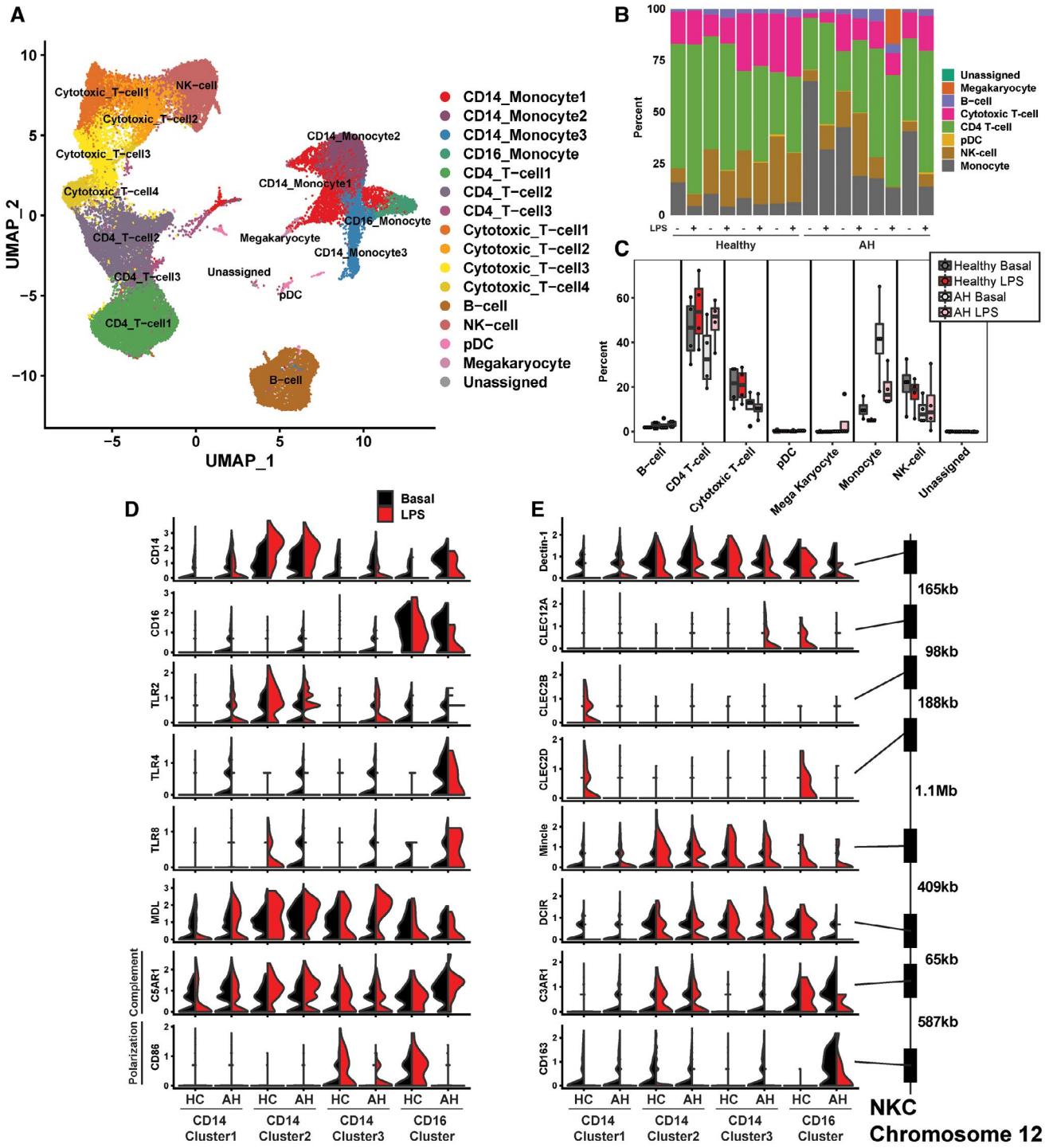


FIG. 1. Single-cell RNA-seq reveals increased monocytes in AH. Patient PBMCs from 4 patients with AH and 4 healthy controls were cultured ex vivo ± low-dose LPS (100 pg/mL) for 24 hours before scRNA-seq. Transcriptomes from 18,433 cells were combined with publicly available PBMC data, and analyses were performed on the complete data set.[33,34] (A) Dimensionality reduction using uniform manifold approximation and projection after normalization and combined in Seurat to ensure common cell types clustered without influence of disease or treatment-specific gene-expression differences (see Materials and Methods and Supporting Fig. S1). (B) Composition of each sample by cell type (clusters combined into broader cell types). (C) Average percentage of each cell type; error bars indicate SD. (D,E) Violin plots showing expression of monocyte genes separated by healthy control and AH. These data include basal (black) and LPS (red) treated cells. (D) Selected genes consisting of different monocyte markers, including classical monocyte markers (CD14, CD16), toll-like receptors (TLR2, TLR4, and TLR8), CTR (MDL), complement receptor (C5AR1), and polarization gene (CD86). (E) Selected genes from the NKC organized by position on chromosome 12. Abbreviations: PDC, plasmacytoid dendritic cell; HC, healthy control; LPS, Lipopolysaccharide; UMAP, uniform manifold approximation and projection.

TABLE 1. NUMBER OF DIFFERENTIALLY EXPRESSED GENES FOR EACH OF THE FOLLOWING COMPARISONS: HEALTHY CONTROL BASAL VERSUS AH BASAL, HEALTHY CONTROL ± LPS, AH ± LPS, AND HEALTHY CONTROL + LPS VERSUS AH + LPS

	HC B vs. AH B	HC ± LPS	AH ± LPS	HC LPS vs. AH LPS
B cell	10	5	12	47
CD14 Monocyte 1	355	363	313	396
CD14 Monocyte 2	347	88	280	26
CD14 Monocyte 3	255	15	323	6
CD4 T-cell 1	191	77	29	107
CD4 T-cell 2	145	95	20	65
CD4 T-cell 3	532	181	152	15
Cytotoxic T-cell 1	14	7	54	96
Cytotoxic T-cell 2	41	18	26	65
Cytotoxic T-cell 3	11	28	6	16
Cytotoxic T cell 4	15	3	1	21
NK cell	39	22	93	63

Note: False discovery rate less than 0.01. Clusters not included had too few cells in one or more conditions.
Abbreviations: AH, Alcohol-associate Hepatitis; B, basal; HC, Healthy Control; LPS, Lipopolysaccharide.

expression of all TLRs at baseline, but not after LPS. In contrast, some genes were ubiquitously expressed in all cells, including *CD14*, *MDL* (CTR), and *C5AR1* (complement receptor) (Fig. 1D). *CD86*, a marker of macrophage polarization, was restricted to $CD14^+$ cluster 3 and $CD16^+$ monocytes and repressed in AH.

Expression of genes that originate from the NKC was diverse across monocyte clusters. Similar to *MDL*, *Dectin-1* and *DCIR* were ubiquitously expressed (Fig. 1E). After LPS challenge, CTR expression increased: *CLEC2B* and *CLEC2D* in $CD14^+$ cluster 1, and *Mincle* in $CD14^+$ cluster 2. The NKC also contains other innate immune genes. *C3AR1*, a complement receptor, is restricted to $CD14^+$ cluster 2 and $CD16^+$ cells (Fig. 1D,E). *CD163*, a marker of macrophage polarization, had the opposite expression pattern to *CD86*; *CD163* was expressed at baseline and repressed by LPS. These data indicate diversity in gene expression among monocyte subtypes, with the NKC contributing to monocyte diversity.

MONOCYTES HAVE THE MOST DIFFERENTIALLY EXPRESSED GENES IN AH AND IN RESPONSE TO LPS

We carried out differential gene-expression measurements for each cell cluster for the following comparisons: basal gene expression differences (healthy control vs. AH), LPS challenge of healthy control cells, LPS challenge of AH cells, and responses to LPS (healthy control vs. AH) (Table 1). In all comparisons, the monocyte clusters had the highest numbers of differentially expressed genes, whereas the fewest were in B cells. Among the three $CD14^+$ clusters, each monocyte cluster differed in gene expression both at baseline (healthy vs. AH) and in response to LPS. Patients with

AH, who have chronic levels of endotoxin, responded to low-dose LPS challenge with exacerbated pro-inflammatory gene expression, consistent with a failure to develop endotoxin tolerance.

$CD14^+$ cluster 2 up-regulated similar genes in response to LPS in both AH and healthy controls (Fig. 2A). On the other hand, $CD14^+$ cluster 1 from healthy controls up-regulated very different genes in response to LPS compared with the AH (Table 1). Moreover, in AH, $CD14^+$ cluster 1 and $CD14^+$ cluster 3 up-regulated many of the same genes as $CD14^+$ cluster 2 after LPS challenge. Forty-nine common genes were up-regulated in $CD14^+$ cluster 2 (AH and healthy controls) and $CD14^+$ cluster 1 from AH, which include pro-inflammatory cytokines, chemokines, metallothioneins, and number of other important immune factors (*C3*, *SOD2*, *SERPINA1*, and *NRP1*) (Fig. 2B). Combined pathway analyses of genes up-regulated in response to LPS revealed the most similarities between healthy control and AH $CD14^+$ cluster 2 and AH $CD14^+$ cluster 1, including up-regulation of genes involved in myeloid leukocyte activation, IL-10 signaling and wound healing, whereas healthy control $CD14^+$ cluster 1 up-regulated interferon-related genes (Fig. 2C).

Following up on the pathway analyses, expression of specific genes revealed a clear phenotypic change in certain AH monocyte clusters. In healthy controls, $CD14^+$ clusters 1 and 3 had little to no *IL-1β* expression, whereas in AH these clusters responded with high expression of *IL-1β* (Fig. 3A). In contrast, $CD14^+$ cluster 2 highly expressed *IL-1β* after LPS in both healthy controls and AH. Additionally, chemokine (C-X-C motif) ligand 2 (CCL2) and CXC-chemokines, which are grouped in a cassette in the genome, were also up-regulated by LPS in $CD14^+$ cluster 2 and AH $CD14^+$ cluster 1 (Fig. 3B).

Because $CD14^+$ cluster 1 gains a pro-inflammatory function in AH, we analyzed the normal LPS response in healthy controls. After LPS challenge, $CD14^+$ cluster 1 expressed antiviral and interferon-regulated genes, including *IFIT* and *OAS* genes, in healthy controls but not in AH (Fig. 3A). *IFIT* and *OAS* are antiviral genes up-regulated by interferon, LPS, and PAMP signaling, and *IFIT1* negatively regulates pro-inflammatory gene expression.[39-41] In healthy controls, calprotectin (*S100A9*), a TLR4 signaling DAMP, is repressed in response to LPS in $CD14^+$ cluster 1 but not in AH or in $CD14^+$ cluster 2.[42] These data indicate a shift in phenotype for $CD14^+$ cluster 1 in AH. In AH, these cells have lost their more diverse, antiviral, and interferon-associated functions and are now pro-inflammatory (Fig. 3C). Expression of pro-inflammatory genes from AH monocytes that do not typically express these genes may explain some of the increased LPS sensitivity and exacerbated inflammation that has been previously observed in AH PBMCs.[19,21]

CLUSTERED CTR AND CHEMOKINE GENES ARE HIGHLY CO-REGULATED IN MONOCYTES

If LPS stimulates expression of genes within a small genomic cassette, then single-cell data can reveal how individual genes within the cassette are regulated. If gene expression within the cassette is highly correlated among cells, then these genes are regulated like an operon. Alternatively, if there is no correlation, then individual cells regulate each gene separately. Because gene correlation analyses are difficult to perform from single-cell data due to the low coverage, we used the bigSCale2 algorithm, which calculates correlation coefficients from iterative differential expression calculations between small groups of cells.[43]

Among healthy-control monocytes, there is some correlation between CTR gene expression at baseline among *Mincle* (*CLEC4E*), *Dectin-3* (*CLEC4D*), and *LINC00937* (Fig. 4A,B, bottom left). After LPS challenge, these genes in the cassette are highly correlated, along with *DCIR* (*CLEC4A*) and *M6PR* (Fig. 4C, bottom left). In AH, at baseline and after LPS, *Mincle*, *Dectin-3*, *Dectin-2*, *LINC00937*, and *DCIR* are highly correlated (Fig. 4D,E, bottom left). Interestingly in AH cells, LPS challenge revealed another nearby cluster of genes with high correlation (*TMEM52B*, *OLR1*, *Dectin-1* [*CLEC7A*], and *CLEC12A*). This cassette is also correlated with the other CTR cassette (Fig. 4E, bottom left).

The C-X-C chemokine and C-C chemokine genes are also clustered in the genome and induced following LPS challenge, so we investigated possible coregulation. Healthy control monocytes have minimal correlated expression at baseline, but after LPS, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL1*, *CXCL8*, and nearby *EREG*, *MTHFD2L*, and *PPBP* are highly correlated (Fig. 4B,C, top right). In AH, chemokine (C-X-C motif) ligand (CXCL) expression was

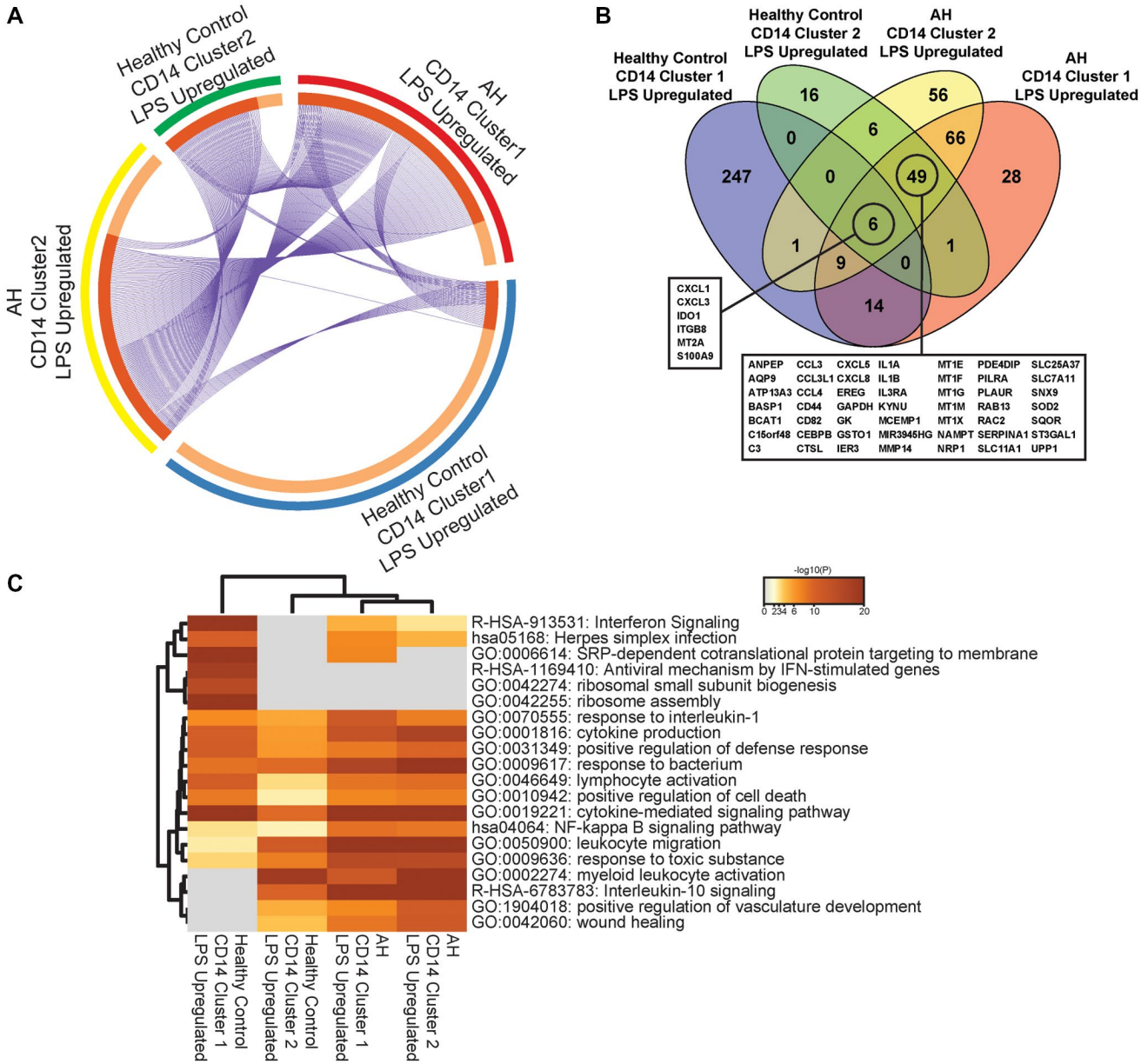
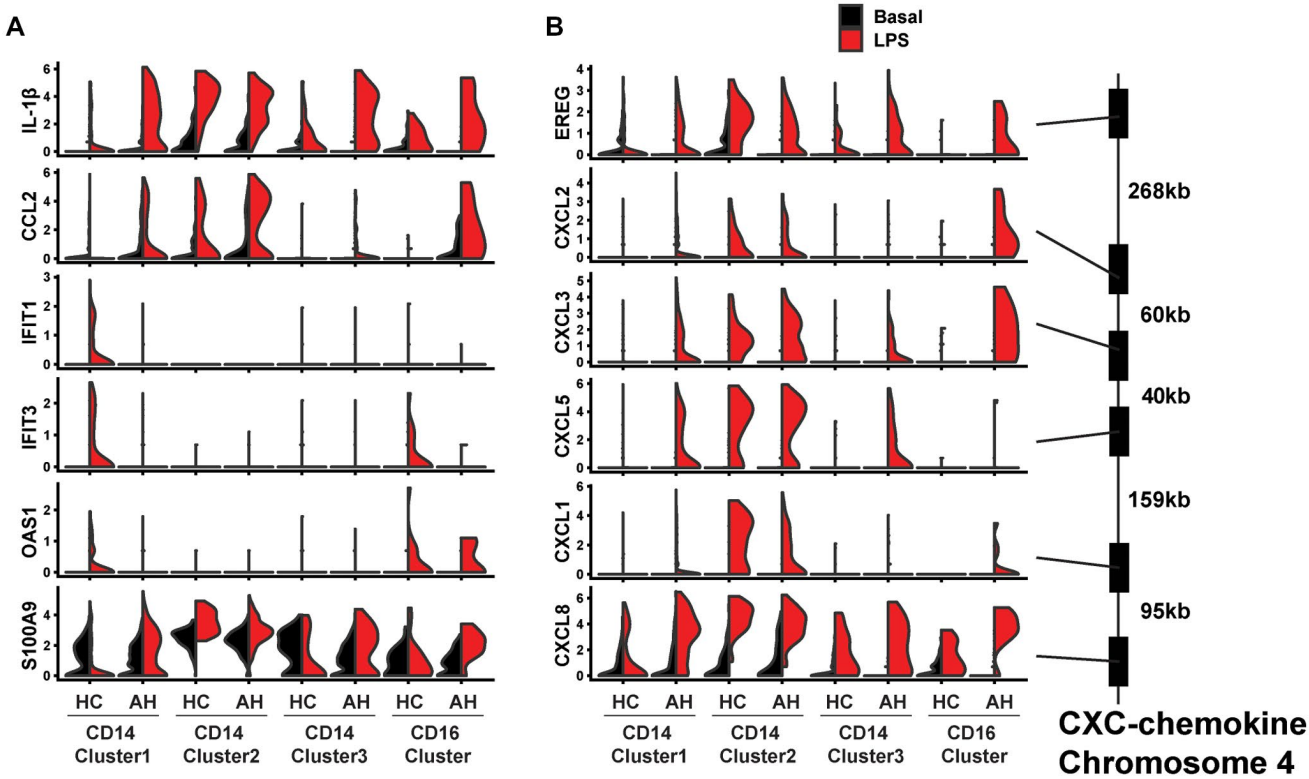


FIG. 2. Differential gene-expression analyses reveal the greatest effect of disease on CD14$^+$ monocyte cluster 1A. Differential expression of genes in response to LPS was measured in all CD14$^+$ monocyte clusters identified and characterized from the cells in Fig. 1; false discovery rate <0.01. Pathway analyses focused on genes up-regulated by LPS in each monocyte cluster. (A) Circos plot showing commonly up-regulated genes in response to LPS between CD14$^+$ cluster 1 and CD14$^+$ cluster 2 from healthy controls and patients with AH. (B) Venn diagram showing the number of overlapping genes up-regulated by LPS between CD14$^+$ clusters 1 and 2 in healthy controls and AH. Boxed are all genes contained in that subset. (C) Heatmap of pathway analyses of genes up-regulated by LPS In CD14$^+$ monocyte clusters 1 and 2. Abbreviations: AH, Alcohol-associated Hepatitis; LPS, Lipopolysaccharide.

correlated at baseline and after LPS (Fig. 4D,E, top right). The two CCL chemokine clusters (*CCL3*, *CCL4*, *CCL3L1*, and *CCL4L2*) and (*CCL2*, *CCL7*, and *CCL13*) are also highly correlated in all conditions (Supporting Fig. S4).

One possible interpretation of these results is that CD14$^+$ cluster 2, which has high chemokine and CTR expression, might bias the correlation analyses (Figs. 1D and 3A). If a cluster of high expressing cells were to bias the data, then these cassettes would be highly

correlated to each other. Looking at the correlation coefficients of the CXC chemokines in comparison to the CTR genes, there is no significant positive or negative correlation in expression between the cassettes (Fig. 4B-E, top left). Therefore, CXC chemokine and CTR gene cassettes are independently regulated in



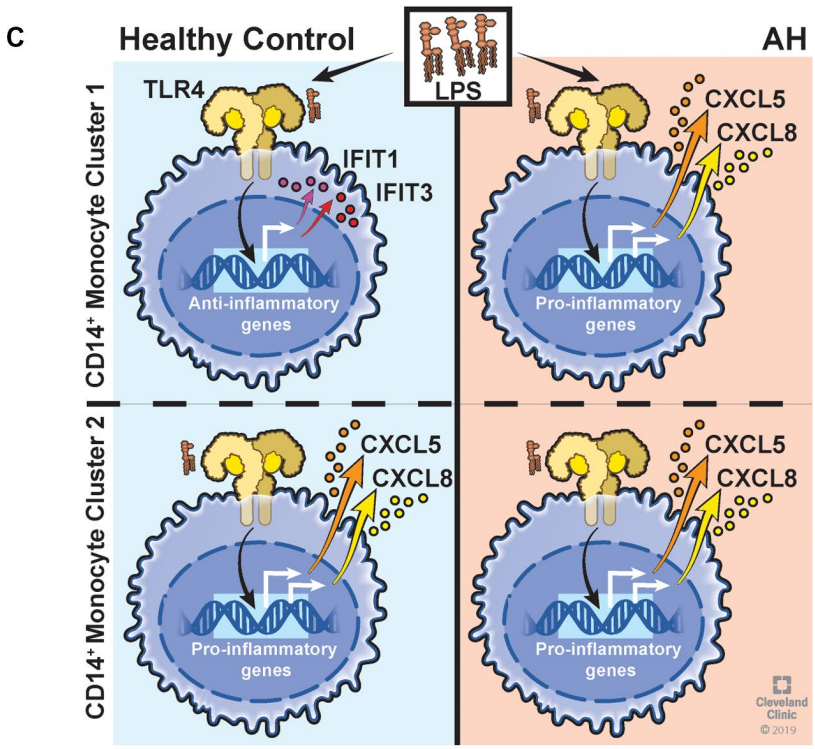


FIG. 3. $CD14^+$ monocyte cluster 1 shifts from anti-inflammatory to pro-inflammatory gene expression in AH. Violin plots showing expression of selected genes in different monocyte clusters with and without LPS in healthy controls and AH. These data include basal (black) and LPS (red) treated cells. (A) IL-1p and CCL2 are pro-inflammatory cytokines and chemokines not expressed in response to LPS in healthy control $CD14^+$ monocyte cluster 1, but activated by LPS in AH. IFIT1, IFIT3, and OAS1 are antiviral genes activated by LPS in healthy-control $CD14^+$ monocyte cluster 1, but not expressed in AH. S100A9 is a DAMP repressed by LPS in healthy-control $CD14^+$ monocyte cluster 1, but activated by LPS in AH. (B) Selected pro-inflammatory CXC-type chemokines from the CXC- chemokine cassette organized by position on chromosome 4. (C) Model for AH-induced changes in monocyte response to LPS. PBMCs have two distinct $CD14^+$ monocyte subpopulations. In response to LPS, $CD14^+$ monocyte cluster 1 in healthy controls expresses anti-inflammatory genes, such as IFIT genes, and represses DAMPs such as S100A9 (not shown). Alternatively, $CD14^+$ monocyte cluster 2 responds to LPS with pro-inflammatory chemokine (CXCLs) and cytokine expression. Healthy control cells have a highly diverse response to LPS. However, in AH, both $CD14^+$ clusters express pro-inflammatory chemokines and cytokines, thus exacerbating inflammation in response to low-dose LPS. $CD14^+$ monocyte cluster 3 and $CD16^+$ monocytes not shown. Abbreviations: AH, Alcohol-associated Hepatitis; HC, healthy control; LPS, Lipopolysaccharide.

individual cells. These results support the hypothesis that AH monocytes are poised at baseline for activation, but respond to LPS differently from healthy controls.

CTRs ARE UP-REGULATED BY LOW-DOSE LPS IN AH

To validate the single-cell data, we measured the expression of six CTRs based on position within the NKC from a larger number of patients with AH and healthy controls using quantitative polymerase chain reaction (PCR) (Fig. 5A). At baseline, *Dectin-1* (*CLEC7A*) was up-regulated in AH PBMCs (Fig. 5B). *Mincle* (*CLEC4E*) was up-regulated after low-dose LPS treatment in both healthy controls and AH PBMCs, similar to results observed using bone marrow–derived macrophages (BMDMs) from murine models of ethanol-induced liver injury.[26] Low-dose LPS up-regulated *Dectin-2*, *Dectin-3*, and *DCIR* (*CLEC4A*) in AH PBMCs (Fig. 5B). *MDL* was not significantly up-regulated. Together, these data reveal up-regulation of CTR genes in the peripheral immune system of patients with AH, and the close genomic cassette of *DCIR*, *Dectin-2*, *Dectin-3*, and *Mincle* have similar expression patterns in AH and in response to LPS (Fig. 5C).

EXPRESSION OF C-TYPE LECTIN RECEPTORS WAS HIGHER IN THE LIVERS OF PATIENTS WITH SEVERE AH COMPARED WITH OTHER LIVER DISEASES

Our data from PBMCs demonstrate that peripheral immune cells increase expression of CTRs in response to low-dose LPS. Monocyte infiltration is a key element of liver inflammation in AH.[44] Up-regulation of CTRs on monocytes would therefore contribute to enhanced immune surveillance in the liver and the periphery. A recent study performed bulk RNA-seq on liver tissues isolated from patients with early AH (n = 12), AH with liver failure (n = 18), explant tissue from patients with severe AH with emergency transplants (n = 10), NAFLD (n = 8), HCV (n = 9), HCV with cirrhosis (n = 9), and healthy controls (n = 10).[45] Patients with AH with liver failure and explant tissue from patients with severe AH with emergency transplants had the highest Model for End-Stage Liver Disease and Age, Serum Bilirubin, INR, and Serum Creatinine scores compared to patients with other liver diseases, which is indicative of severe AH, although all disease patients had elevated aspartate aminotransferase and alanine aminotransferase (Supporting Table S6). *DCIR*, *Dectin-3*, *Mincle*, *Dectin-1*, and *MDL* were all up-regulated in the patients with severe AH, whereas none of these genes were differentially expressed in NAFLD (Fig. 6A). Mincle expression was down-regulated in the patients with early AH, whereas DCIR was also up-regulated during HCV infection. To validate these studies, we performed quantitative PCR on human liver explant samples from patients with severe AH and controls. All CTRs measured were up-regulated in AH livers, including a robust up-regulation of *MDL* (Fig. 6B).

Expression of CTR genes in bulk RNA-seq data cannot directly distinguish the cell type expressing these genes. However, because CTR genes are primarily expressed in myeloid-derived cells,[1] expression is likely due to increased CTR expression by resident macrophages or infiltrating monocytes. Using scRNA-seq of healthy human liver tissue, MacParland et al

described two distinct macrophage populations: non-inflammatory and pro-inflammatory.[46] The non-inflammatory macrophages, characterized by expression of the gene *MARCO*, were long-lived Kupffer cells that primarily expressed immunoregulatory genes. On the other hand, the inflammatory cells, which were

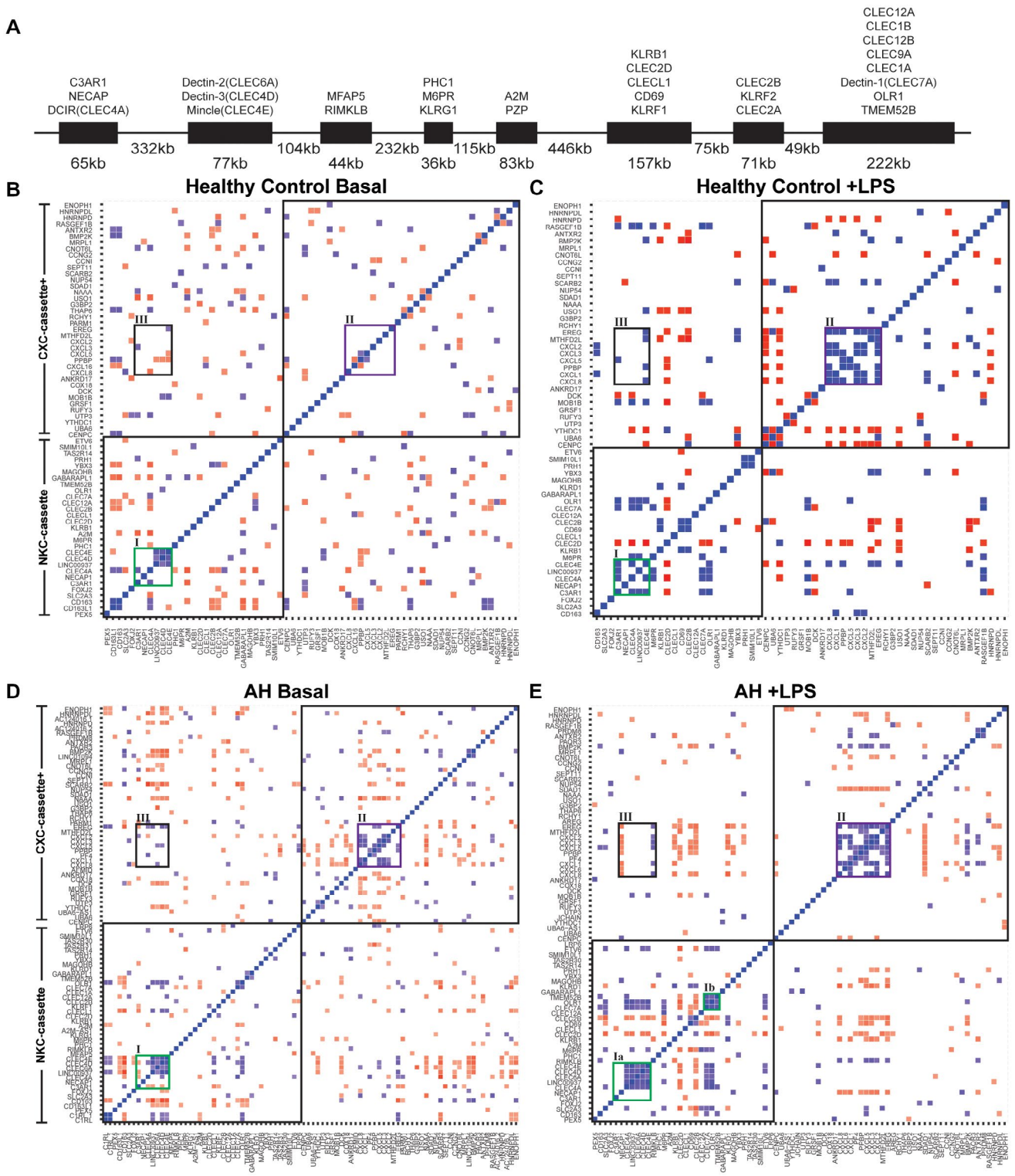


FIG. 4. Cassettes of innate immune genes are coordinately regulated. Correlation analysis for the expression of genes within and around the NKC and CXC-chemokine clusters using data for all monocytes identified. The regions of interest were ordered by chromosome position. Genes with low expression were removed. Only the upper and lower 2.5% of all correlation coefficients were plotted, representing the best correlations. In the data set, blue denotes significant positive correlations, and red denotes significant negative correlations. (A) Schematic of the entire NKC gene cluster, grouped as putative "mini-cassettes" based on proximity. (B-E) Combined heatmap of all pair-wise correlation coefficients for genes from the NKC and the CXC-chemokine cluster. NKC cluster is found at the bottom left and CXC-chemokine cluster is found at the top right of each figure (black boxes). I (green box) denotes the NKC cassettes (including Ia and Ib). II (purple box) denotes the CXC-chemokine cluster. III (black box) compares the NKC cassette with the CXC-chemokine cassette. (B) Healthy control basal. (C) Healthy control with LPS. (D) AH basal. (E) AH with LPS. Abbreviations: AH, Alcohol-associated Hepatitis; LPS, Lipopolysaccharide.

MARCO-negative, expressed pro-inflammatory genes in response to immune stimulus and were thought to be recently recruited macrophages or infiltrating monocytes. Using their data, we found that *Dectin-1* and *Mincle* were expressed on both $MARCO^+$ and $MARCO^-$ macrophage subsets, whereas *DCIR* was expressed only on $MARCO^-$ cells (*Dectin-2*, *Dectin-3*, and *MDL* were undetectable) (Fig. 6C). Interestingly, *MARCO* expression was reduced in severe AH livers, whereas the pro-inflammatory macrophage marker *LYZ* was higher, consistent with an increased pro-inflammatory environment in AH livers (Fig. 6D). Peripheral monocytes do not express *MARCO*, but do express high levels of *LYZ*, which supports the hypothesis that the liver inflammatory macrophages are recently recruited cells that originate from the periphery. This analysis implies that higher CTR expression in AH livers is due to increased expression of CTRs by pro-inflammatory, recently recruited myeloid cells.

MINCLE UP-REGULATION MAKES PBMCs SENSITIVE TO TREHALOSE-6,6-DIBEHENATE, A SYNTHETIC MINCLE AGONIST

If low-dose LPS induces expression of CTRs, then PBMCs should become more sensitive to the corresponding PAMPs, indicating functionality of the secondary immune surveillance pathway.

Mincle expression was higher in both healthy controls and AH PBMCs after low-dose LPS challenge; thus, these cells should be sensitive to the Mincle agonist Trehalose-6,6-dibehenate (TDB), a synthetic analog of *Mycobacterium tuberculosis* cord factor (Fig. 5C). To test this hypothesis, after 24 hours of low-dose LPS pretreatment, we challenged the cells for 2 hours with TDB. TDB alone, under the conditions used here, did not lead to expression of pro-inflammatory cytokines *IL-6* or *IL-1β* in either AH or healthy controls (Fig. 7A,B). However, PBMCs pretreated with low-dose LPS became sensitive to TDB, leading to increased expression of *IL-6* in both healthy control and AH cells, with AH cells having exacerbated expression of *IL-1β* (Fig.7A,B). Twenty-four-hour low-dose LPS alone also induced *IL-6* and *IL-1β*; *IL-1β* expression was higher in AH versus healthy controls (Fig. 7).

Discussion

Using scRNA-seq, we measured the transcriptomes of all PBMC types before and after LPS challenge to model innate immune responses that occur after gut-barrier dysfunction and leakage of microbial byproducts. From these data, we distinguished cell type–specific responses and how these responses were disrupted in AH, a severe inflammatory condition that can occur at any point in the spectrum of ALD pathologies. Our studies revealed extensive diversity in how monocytes can respond to LPS challenge. Not only were monocytes predifferentiated toward different immune functions, either poised to express highly pro-inflammatory or interferon-associated genes, but at a single-cell level, individual cells regulated gene cassettes, such as the CTR and chemokine cassettes, independently, further contributing to diversity in phenotypes. Additionally, in AH, we observed not only a greater proportion of monocytes, but subsets of these cells had lost anti-inflammatory functions and contributed to exacerbated inflammation. Importantly, bulk RNA-seq from human liver tissue from patients with different liver diseases revealed extensive infiltration of peripheral pro-inflammatory monocytes with high CTR expression in patients with severe AH, likely contributing to the severe hepatic inflammation characteristic of AH.

Gut-barrier dysfunction leads to low-dose endotoxemia in peripheral circulation, approximately

70-100pg/mL LPS in both healthy patients after one alcohol binge as well as at baseline in patients with AH.[7,10,32] Leakage of gut contents leads to more than just LPS in circulation, and in fact, *C. albicans*–derived β-glucans also enter the bloodstream during alcohol consumption.[6] We hypothesized that a secondary

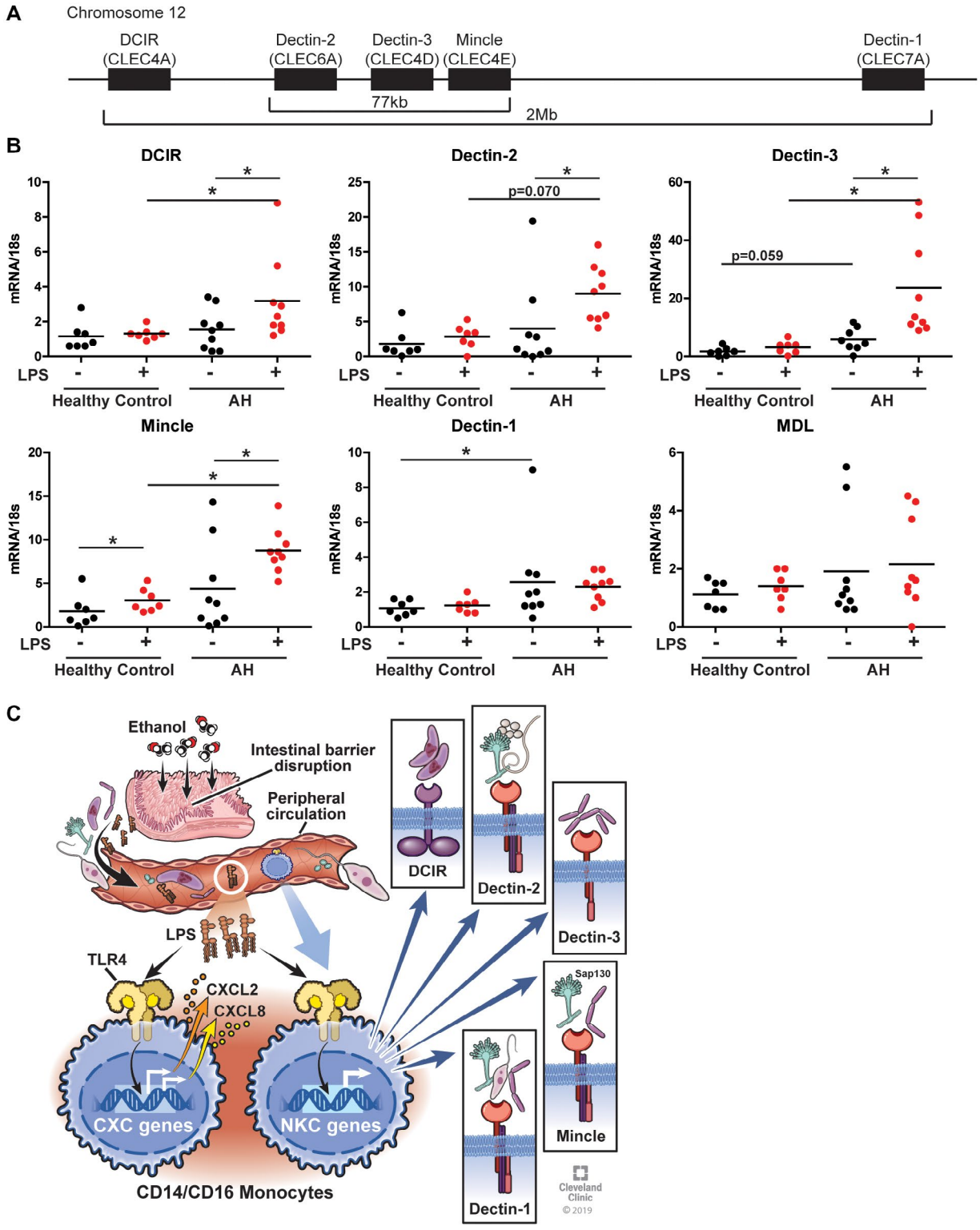


FIG. 5. Cytotoxic T lymphocytes are unregulated in PBMCs from patients with AH after low-dose LPS. (A) Schematic of the chromosomal organization of the CTR genes studied. MDL is on a separate chromosome. (B) PBMCs from patients with AH (n = 9) and age-matched healthy controls (n = 7) were challenged with and without low-dose LPS (100 pg/mL) for 24 hours, then messenger RNA expression was measured by quantitative PCR. Data were normalized to 18s ribosomal RNA. (C) Model for LPS-induced secondary immune surveillance. Ethanol consumption leads to leakage of gut contents, including bacterial LPS, into portal and peripheral circulation. Peripheral monocytes, through TLR4 signaling, respond to LPS with up-regulation of different innate immune gene cassettes. Some cells up-regulate CXC-chemokines, while other cells up-regulate the NKC cluster. These CTR genes sense other gut-derived microbial byproducts of fungal, viral, and bacterial origin. Coordinated expression of CTRs makes certain monocytes hypersensitive to these deleterious microbial byproducts. Abbreviations: AH, Alcohol-associated Hepatitis; CTR, C-type Lectin Receptor.

immune surveillance program senses the initial gut-derived LPS to trigger the expression of more PRRs, to sense other PAMPs and DAMPs, to ensure the clearance and removal of all foreign material. CTRs signal using several mechanisms to up-regulate pro-inflammatory pathways, although some may also function to inhibit these pathways.[47] The response to ligand is complex. For example, recognition of *C. albicans*, an invasive pathogenic fungi, requires a heterodimer of Dectin-2 and Dectin-3.[48] If CTRs are part of a secondary immune surveillance pathway and require heterodimers, then their expression would have to be coordinated, such that within a single cell, multiple CTRs would be up-regulated to form a functional complex.

In both the healthy control and AH monocytes, CTR genes were coordinately expressed after LPS challenge (Fig. 5D). Additionally, multiple pro-inflammatory chemokine clusters were also coordinately expressed, including CXC-type and CC- type chemokines. Expression of these clusters was already correlated at baseline in AH, consistent with the hypothesis that AH cells are constitutively activated or at least highly poised for activation. These results support a model in which there are a spectrum of pro-inflammatory cells that express differing levels of chemokines, as opposed to single cells expressing individual chemokines. Regulation of immune gene cassettes may be coordinated to exhibit a large immune response but from a small number of highly pro-inflammatory cells. By coordinately up-regulating the entire cassette of CTRs in a subset of cells, the secondary immune surveillance is active only in more specialized cells. Moreover, these gene cassettes are independently regulated from each other, which further diversifies single monocytes from each other during inflammation. In AH, some of the phenotypic diversity is lost, because monocytes involved in resolution have now shifted to a pro-inflammatory role, although the regulation of the gene cassettes is similar to healthy cells. However, a greater number of cells are poised for pro-inflammatory gene expression, contributing to LPS hypersensitivity in AH.

These CTR genes were highly up-regulated in the livers of patients with severe AH, implicating exacerbated secondary immune surveillance in the liver immune system. These specific CTRs are only expressed in myeloid-derived cells.[1] Although these genes are overexpressed, expression of *MARCO*, a marker for long-lived resident noninflammatory and immunoregulatory macrophages, was lower in the livers of patients with severe AH. In contrast, *LYZ* expression, a marker for recently recruited, pro-inflammatory macrophages, was higher in the livers of patients with severe AH. These results indicate that the balance of noninflammatory and pro-inflammatory hepatic macrophages in the liver has shifted to mostly pro-inflammatory cells in AH. We hypothesize that this shift is due to the recruitment of peripheral monocytes, in which *LYZ* and CTR expression is high. Loss of noninflammatory cells would explain the nonresolving inflammation in the AH. Moreover, increased expression of PRRs in the liver would contribute to liver inflammation and increased sensitivity to PAMPs and DAMPs.

The scRNA-seq of PBMCs after immune challenge is a powerful tool, because we can simultaneously ask how each cell type responded to the stimulus in both healthy controls and disease. Although we focused primarily on the monocytes for our analyses, every cell type had differentially expressed genes, including adaptive immune cells, which also express TLR4 and can respond to LPS.[49,50]. Interestingly, we find fewer differentially expressed genes among the adaptive immune cells at baseline and in response to LPS, as compared with monocytes and NK cells. In *Rhesus macaque* models of moderate and chronic heavy drinking, RNA-seq analyses reveal sensitivity to LPS, with genes related to innate immune function highly up-regulated, implicating innate immune dysfunction in both AH and chronic heavy drinkers.[51] One limitation to our experimental design is that we chose to use cryopreserved PBMCs,

rather than freshly isolated PBMCs. However, scRNA-seq data from cryopreserved PBMCs highly correlated with fresh cells.[31] Because our goal was to compare samples from patients with AH with sex-matched and age-matched healthy controls, we needed to perform the experiments simultaneously, to minimize possible

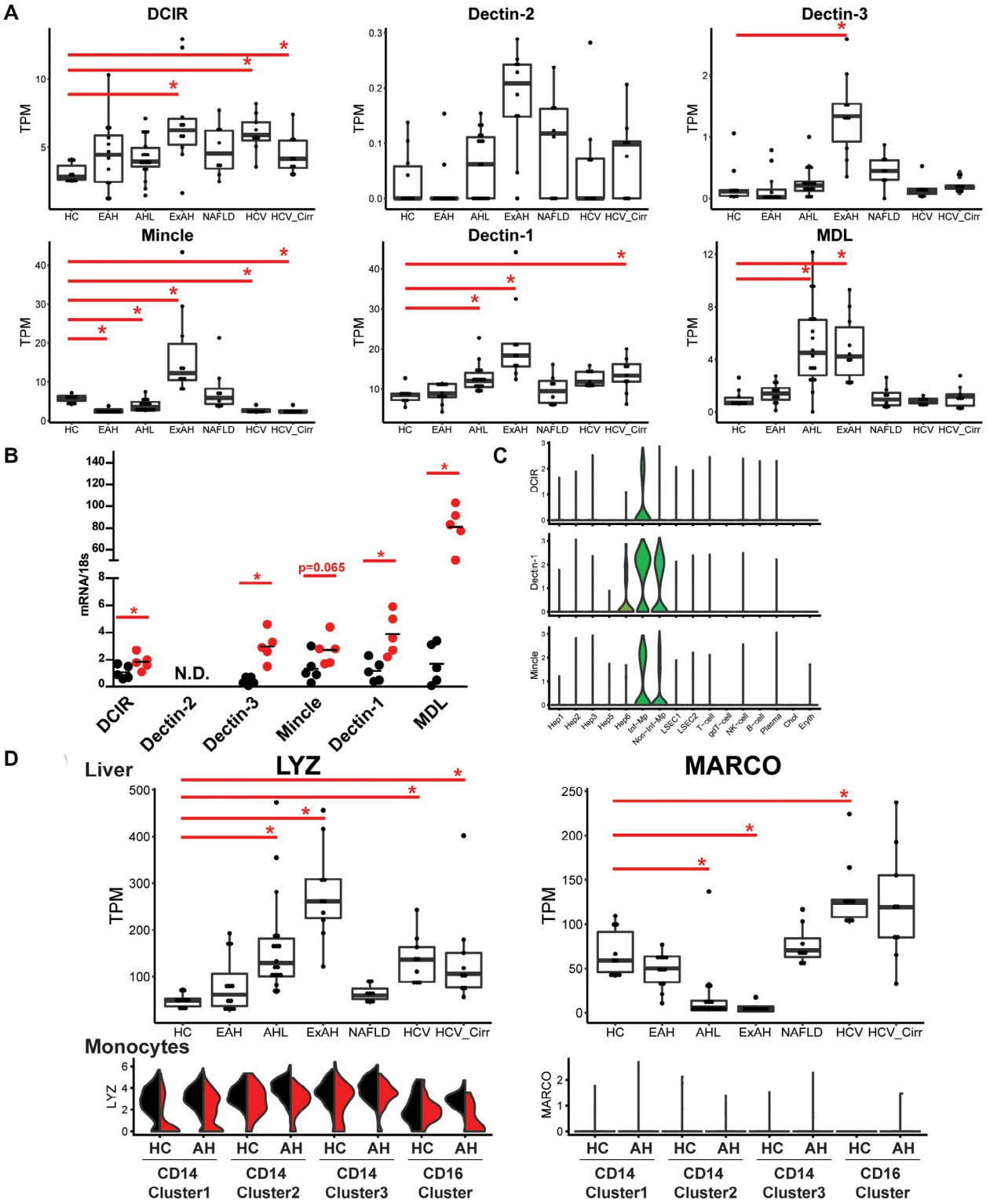


FIG. 6. Cytotoxic T lymphocytes are up-regulated in the livers of patients with severe AH. Comparison of gene-expression results from bulk RNA-seq of liver tissues from patients with different pathologies: healthy controls (HC, n = 10), early AH (EAH, n = 12), AH with liver failure (AHL, n = 18), explant tissue from patients with severe AH with emergency liver transplants (ExAH, n = 10), NAFLD (n = 8), HCV (n = 9), and HCV with cirrhosis (HCV Cirr, n = 9). Gene expression was measured by transcripts per million. (A) Boxplots of average expression for CTR genes in different disease groups; error bars indicate SD. Red stars indicate q <0.05 in comparison to healthy controls. (B) Quantitative PCR of CTR genes from liver-explant tissue from patients with AH and healthy donor tissue. $P < 0.05$. (C) Violin plots of scRNA-seq data from MacParland et al., showing the expression of CTRs in different liver cell types. (D) Top: Boxplots of average expression of different macrophage subset markers (LYZ, inflammatory macrophage; MARCO, noninflammatory macrophage) in different disease groups. Bottom: Expression of these markers in scRNA-seq data from peripheral monocytes. Statistical significance for RNA-seq was determined using the likelihood ratio test in Sleuth. Quantitative PCR was determined by least-square means. Abbreviations: Chol, cholangiocyte; CTL, cytotoxic T lymphocyte; HEP, hepatocyte; Inf-Mp, inflammatory macrophage; LSEC, liver sinusoidal endothelial cell; Non-Inf-Mp, noninflammatory macrophage; TPM, transcripts per million.

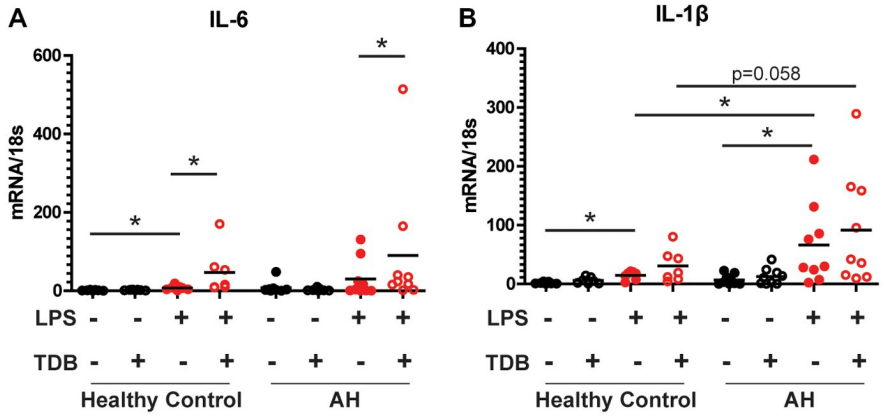


FIG. 7. Mincle up-regulation by low-dose LPS causes sensitivity to the synthetic Mincle agonist TDB. PBMCs from patients with AH (n = 9) and age-matched healthy controls (n = 7) were challenged with low-dose LPS (1 pg/mL) for 24 hours then subsequently treated with the synthetic Mincle agonist TDB. Messenger RNA expression was measured by quantitative PCR. IL-6 expression (A) and IL-1p (B) expression; $P < 0.05$. Statistical significance for quantitative PCR was determined by least-square means.

batch effects. The use of fresh cells in this manner would be impractical.

Differential expression analyses revealed a clear distinction between different groups of monocytes and how these cells were altered in AH. In AH, each monocyte cluster expressed very different genes at baseline, including PRR genes like *TLR4* and *TLR8*, showing a clear effect of alcohol on innate immunity. However, after LPS challenge, there was a major phenotypic shift in monocyte clusters 1/3 in AH compared with healthy controls (Fig. 3C). In healthy controls, these cells responded to LPS by activating genes involved in the resolution of inflammation, including IFIT genes, which are antiviral genes that repress pro-inflammatory cytokines and chemokines.[39,41,52] However, in AH, these cells responded to LPS with high pro-inflammatory gene expression, similar to monocyte cluster 2, which are always pro-inflammatory after LPS. Moreover, in AH, cells in monocyte cluster 1 lost their antiviral functions; they were unable to express the *IFIT* and *OAS* genes required for type-1 interferon responses to destroy viruses. This shift away from pro-resolution and antiviral responses may explain the anomaly in patients with AH in that they exhibit increased inflammatory responses, yet an increased risk of infection.[15]

Calprotectin (*S100A9*), a TLR4-signaling DAMP, was inhibited by LPS in $CD14^+$ monocyte cluster 1 in healthy controls, but up-regulated in AH. In contrast, in mouse models of NAFLD, *S100A9* is highly down-regulated in myeloid cells and not expressed in response to LPS.[53] These data suggest opposing inflammatory phenotypes between AH and NAFLD; AH immune cells are highly pro-inflammatory and lack resolution, while myeloid cells in NAFLD have a reduced capacity for inflammation. Moreover, in AH, expression of chemokines showed coordinated up-regulation in response to LPS, whereas in animals

fed a Western diet, certain chemokines are not highly expressed.[53]

In response to LPS challenge, healthy control monocytes were extremely diverse in function, including anti-inflammatory and interferon-responsive cells alongside pro-inflammatory cells. In AH, while pro-inflammatory cells became more pro-inflammatory, the anti-inflammatory monocytes switched to a pro-inflammatory response. These changes contribute to a reduced diversity in innate immune responses in AH, with all monocytes contributing to exacerbated inflammation. In severe AH, these highly pro-inflammatory monocytes infiltrate the liver, and increase nonresolving liver inflammation characteristic of AH, but not other severe liver diseases. Additionally, LPS activates expression of the CTR gene cassette, which make myeloid cells in the periphery and the liver more hypersensitive to other PAMPs and DAMPs. Future AH therapies will need to re-establish the inflammatory diversity in the immune system by not only decreasing pro-inflammatory responses, but also restoring the function of these interferon-associated cells. Because many innate immune genes originate from gene cassettes, beyond just chemokines and CTRs, these cassettes can be the focus of targeted therapies to modulate inflammation in many types of inflammatory disease.

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