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Duodenal CD8+ T resident memory cell apoptosis contributes to gut barrier dysfunction and microbial translocation in early alcohol-associated liver disease in humans

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Summary

Background: Intestinal T cells are key in gut barrier function. Their role in early stages of alcohol-associated liver disease (ALD) remain unknown.

Aim: To explore the links between intestinal T cells, microbial translocation and ALD.

Methods: Patients with alcohol use disorder (AUD) following a rehabilitation programme were compared to subjects with non-alcoholic fatty liver disease (NAFLD) and healthy controls. Clinical and laboratory data (liver stiffness, controlled attenuation parameter, AST, ALT, K18-M65) served to identify AUD patients with isolated steatosis (minimal liver disease) or steatohepatitis/fibrosis (ALD). Serum microbial translocation markers were measured by ELISA, duodenal and plasma levels of sphingolipids by targeted LC–MS. T lymphocytes in duodenal biopsies were characterised by immunohistochemistry, flow cytometry and RNA sequencing on FACS-sorted cells. Mechanisms for T-cell alterations were assessed *in vitro*.

Results: Patients with ALD, but not those with minimal liver disease, showed reduced numbers of duodenal CD8+ T resident memory (TRM) cells compared to controls or patients with NAFLD. TRM transcriptomic analysis, *in vitro* analyses and pharmacological inhibition of cathepsin B confirmed TRM apoptosis driven by lysosomal membrane permeabilisation and cathepsin B release into the cytosol. Altered lipid metabolism and increased duodenal and plasma sphingolipids correlated with apoptosis. Dihydroceramide dose-dependently reduced viability of TRM. Duodenal

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TRM phenotypic changes, apoptosis and transcriptomic alterations correlated with increased levels of microbial translocation markers. Short-term abstinence did not reverse TRM cell death in patients with ALD.

Conclusions: Duodenal CD8+ TRM apoptosis related to functional changes in lysosomes and lipid metabolism points to impaired gut adaptive immunity specifically in patients with AUD who developed early ALD.

1 | INTRODUCTION

Chronic alcohol consumption is one of the leading causes of chronic liver disease and liver-related deaths worldwide. Although most patients with alcohol use disorders (AUD) develop steatosis, only a minority progresses to the more severe forms of alcohol-associated liver disease (ALD) and the mechanisms involved in disease progression are not completely understood.

Emerging evidence suggests a contribution of intestinal barrier dysfunctions in ALD.[1,2] Increased intestinal permeability, gut microbiota changes and elevated translocation of microbes and/or their products in the portal and systemic circulation (a process called microbial translocation) have been linked to ALD development.[3,4] Changes in gut barrier functions (e.g. intestinal permeability,[5] nutrient malabsorption[6]) observed in heavy drinkers mainly occur in the proximal small intestine, the principal site of alcohol absorption.[7]

We recently demonstrated that bacterial and fungal microbial translocation in AUD patients can occur independently from paracellular and vascular intestinal permeability.[5] This implies that failure of other compartments of the intestinal barrier is needed to allow microbes to reach the blood circulation. One component, the intestinal-associated immune system, also contributes to a functional gut barrier.[8] To date, little is known about the gut immune system in intestinal barrier dysfunction at early stages of ALD in humans as studies on gut (dys-) immunity in AUDs generally focused on decompensated cirrhosis.[9] Interestingly, we observed a reduced number of T cells in the duodenal mucosa of AUD patients linked to elevated microbial translocation,[5] pointing to a potential contribution of T-cell changes to altered against microbes.

Intestinal mucosal T cells act as local gate keepers to protect against microbial invasion[10] thanks to their T-cell receptor (TCR) repertoire.[11] Intestinal T resident memory (TRM) lymphocytes are the main resident population retained at mucosal sites due to CD103 and high CD69 expression.[12] They are long-lived cells[13] that originate from killer cell lectin-like receptor G1 (KLRG1) negative precursors.[14,15] TRM is central in host defence in mucosal tissues allowing fast and targeted adaptive immune responses to invading pathobionts.[12] Their role is increasingly recognised in the development of chronic diseases especially in the intestine.[10]

The principal aim of this study was to characterise functional alterations in the duodenal T-cell compartment in AUD patients in relation to ALD. As a secondary aim, we also investigate potential factors implicated in duodenal T-cell alterations.

2 | METHODS

2.1 | Patients

AUD patients (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition criteria) admitted for elective alcohol withdrawal to a dedicated alcohol withdrawal unit were prospectively recruited from a highly standardised and controlled 3-week detoxification and rehabilitation programme (Figure S1). All patients reported long-term (>1 year) alcohol consumption (>60 g/day) and were actively drinking until the day of admission. Patients with known cirrhosis or previous episodes or signs of liver decompensation were excluded. Additional inclusion and exclusion criteria are available in Data S1. Clinical and baseline biochemical data as well as transient elastography with controlled attenuation parameter (CAP) (Fibroscan®) were collected at admission. The presence of steatosis was further confirmed by Doppler ultrasound. They were compared to healthy controls (social drinkers consuming <20 g of alcohol/day) matched for gender, age and BMI, to subjects with non-alcoholic fatty liver disease (NAFLD) in whom duodenal biopsies were obtained as a routine during work-up for bariatric surgery (details in Material S1) as well as to abstinent AUD patients who had completed the detoxification programme.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee (B403201422657; 2014/15OCT/514). Written informed consent was obtained from all patients and healthy controls.

2.2 | Clinical classification of AUD patients according to severity of liver disease

We defined liver disease severity in AUD patients based on clinically available data including the presence/absence of elevated transaminases, presence of steatosis (CAP ≥250 dB/m further confirmed by Doppler ultrasound) and the suspicion of significant fibrosis (liver stiffness ≥7.6 kPa). The pattern of liver disease in AUD patients was classified into minimal liver disease and those that have likely progressed to early stages of ALD (ALD). The minimal liver disease group was defined as normal transaminases, with or without the presence of steatosis but normal liver stiffness (kPa <7.6). The ALD group was defined as either clinical 'steatohepatitis' (increased

levels of either AST or ALT and presence of steatosis) or presence of fibrosis with or without clinical 'steatohepatitis' (Table S2). Serum Cytokeratin 18 levels (CK18-M65 ELISA kit; TECOmedical AG, Sissach, Switzerland), a liver-specific cell damage marker,[16] were used to adjust the clinical classification of the two groups (internally validated cut-off 270 U/L).

2.3 | Duodenal T-cell isolation and immunophenotyping by flow cytometry (FACS sorting)

CD8+ KLRG1(low) CD103+ T cells were isolated from distal duodenal biopsies using a modified version of the method previously described[17] and FACS sorted using a BD FACSAria™ III sorter equipped with BD FACSDiva™ software. Briefly, duodenal biopsies were collected in 10% RPMI and the epithelial layer containing intra-epithelial lymphocytes was stripped by incubation with 1 mmol/L dithiothreitol and 5 mmol/L EDTA in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) for 15 min at 37°C. Single-cell suspension from the epithelium was obtained by treatment with DNase I for 30 min at 37°C. The remaining lamina propria were washed with HBSS and digested with 1 mg/ml collagenase A (Roche Diagnostics Ltd.) in HBSS supplemented with calcium, magnesium and 1 mg/ml DNase I (Roche Diagnostics Ltd.) for 30 min at 37°C and mechanically disrupted by gentle pipetting until dissociation was complete. Mucosal cells released from the tissue samples were passed through a 70 μm cell strainer and washed with complete medium. Cell viability was assessed by trypan blue exclusion. Recovered mucosal cells were stained with the antibodies listed in Material S1.

2.4 | Assessment of cell death and lysosomal membrane permeabilisation

Apoptosis was assessed in duodenal CD8+ and CD4+ T cells using the eBioscience™ Annexin V Apoptosis Detection Kit APC by flow cytometry with a BD FACSCanto™ II equipped with BD FACSDiva™ software and further confirmed in cryosections with the terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) assay (in situ cell death detection kit, Roche). Lysosomal membrane permeabilisation was measured with LysoTracker™ Deep Red by flow cytometry with a BD FACSCanto™ II equipped with BD FACSDiva™ software (all Thermofisher). All assays were performed following the manufacturer's instructions.

2.5 | RNA sequencing

RNA sequencing from sorted duodenal CD8+ TRM cells was isolated using QIAGEN RNeasy Plus Mini Kit and sequenced using the HiSeq4000 instrument. Count-based differential expression analysis was done with R-based Bioconductor package DESeq2. GSEA was performed with clusterProfiler (v.3.14.3) Bioconductor package and gene sets from Molecular Signature Database (msigdbr v.7.1.1). p-values were adjusted for multiple testing with the Benjamini–Hochberg procedure, which controls the false discovery rate (FDR) (details in Material S1).

2.6 | LC–MS/MS analysis of sphingolipids

Plasma and duodenal sphingolipids were analysed by LC–MS/MS by using an Agilent 1290 HPLC system coupled with a high-resolution mass spectrometer system (Agilent 6550 ion funnel MS-qTOF) (see Material S1).

2.7 | Cell culture

Cell viability and apoptosis were evaluated in FACS sorted CD8+ TRM upon stimulation with different inhibitors and sphingolipids by using WST-1 and Caspase 3/7 assay respectively (see Material S1).

2.8 | Statistics

Data were analysed using Graph Pad Prism 9 (GraphPad Software) and presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Normality was assessed by the Kolmogorov–Smirnov test followed by t-tests or the Wilcoxon test as appropriate. Data were compared by one-way ANOVA for multiple groups, followed by Bonferroni's post hoc test for pairwise comparisons. Pearson's or Spearman's correlation tests were used for correlations between data sets. A $p < 0.05$ was considered as statistically significant. Flow cytometry data were analysed using FlowJo (TreeStar).

3 | RESULTS

3.1 | Study population

The study population consists of a cohort of 121, middle-aged, predominantly male actively drinking AUD patients, 38 healthy controls, 10 NAFLD patients and 23 abstinent AUD patients (Table 1, Table S1). Active drinkers reported alcohol consumption until the evening prior to admission. Thirty-six percent (44/121) had detectable blood alcohol concentrations at admission (median 0.5 g/L; range 0.1–2.9 g/L). Fifty-five (55) patients were classified as having minimal liver disease. As by definition transaminase levels were within normal range and all had CK18-M65 levels below 270 U/L. Seventy-five percent of those patients had liver stiffness values below 6 kPa ($n = 42$), whereas the remaining ones ranged between 6 kPa and the predefined cut-off of 7.6 kPa. Sixty-one AUD patients were considered as having ALD (Table S2). Patients with ALD had high CK18-M65 levels. Among

TABLE 1 Baseline demographic and biochemical data of the study population

Demographics	Healthy controls (n = 38)	AUD patients (n = 121)	NAFLD (n = 10)
Gender (female/male)	18 (47%)/20 (53%)	36 (30%)/85 (70%)	6 (60%)/4 (40%)
Mean ± SEM			
Age (years)	46.1 ± 9.5	48.1 ± 9.1	39.3 ± 12
Height (m)	1.73 ± 0.09	1.73 ± 0.06	1.68 ± 10
Weight (kg)	73.8 ± 12	73.1 ± 10.4	134 ± 19.5
BMI	24.6 ± 2.8	24.2 ± 3.1	45 ± 3
Biological and imaging data			
Mean ± SEM (normal range)			
AST (IU/L)	17.8 ± 3.5	78.9 ± 57 (<40)	57.1 ± 24.7
ALT (IU/L)	10.9 ± 2.9	65.2 ± 46.3 (<40)	94.4 ± 49.4
γ-GT (IU/L)	22.4 ± 8.6	258.4 ± 258.9 (<40)	68.9 ± 24.8
Bilirubin (mg/dl)	0.21 ± 0.13	0.58 ± 0.26 (0.3–1.2)	0.74 ± 0.24
Albumin (g/L)	44 ± 0.8	46.4 ± 3.1 (35–52)	45.6 ± 2.1
CAP (dB/m)	ND	282 ± 50	350 ± 36
kPa	ND	8.4 ± 4.8	12.5 ± 3.5
CK18-M65 (U/L)	173.6 ± 43.3	436.4 ± 321	765.9 ± 175.9
SAF score	ND	ND	S2A3F2

Abbreviations: AST, Aspartate transaminase; ALP, alkaline phosphatase; ALT, Alanine transaminase; BMI, body mass index; γ-GT, gamma-glutamyltransferase.

ALD patients with Fibroscan readings suggestive of significant fibrosis (≥7.6 kPa) only 10 patients had values compatible with cirrhosis (>19.6 kPa). Thus, our ALD cohort is indeed primarily composed of patients with early forms of ALD. Five AUD patients did not fit into our clinical classification. All patients had a preserved synthetic liver function and no clinical signs of liver decompensation. Doppler ultrasound ruled out significant vascular or biliary problems.

NAFLD patients had active NASH (A3) and significant fibrosis (F2) on histology together with very high CK-M65 levels which situates them at the upper limit of the spectrum of liver disease observed in AUD patients.

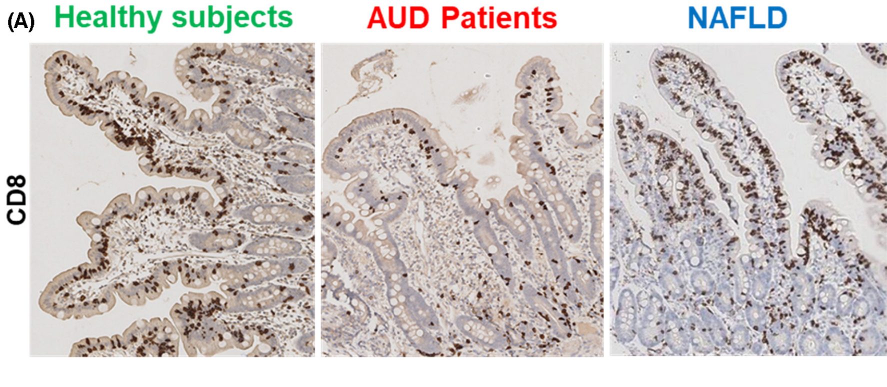
For methodological reasons and limited amounts of tissue available, the different analyses were performed in subsets of patients.

3.2 | Reduced duodenal CD8+ T resident memory lymphocytes characterises AUD patients with ALD

The intestinal immune system plays an important role in defending the host against invading pathogens. T cells constitute an important part of the intestinal immune machinery. Recent data indicate that in particular T resident memory cells act as immune sentinels against invading pathogens.[18] We, therefore, evaluated the intestinal T-cell compartment in duodenal biopsies from healthy controls and in actively drinking AUD patients. Immunohistochemistry revealed that the number of CD8+ T lymphocytes was lower in actively drinking AUD patients compared with healthy controls (Figure 1A,B). Reduction of CD8+ T cells occurred in both the epithelium and the lamina propria (Figure S2). Further analysis by flow cytometry showed that in healthy controls, 55% of T cells were CD8+ and 33% were CD4+ (Figure 2A), while the remaining cells expressed both CD8 and CD4 (not shown). Mucosal CD8+ CD103+ T cells with low KLRG1 expression are considered as long-lived T resident memory cells.[19,20] Almost all CD8+ cells showed low expression of KLRG1 (Figure 2B,C) indicating that they are likely T resident lymphocytes (TRM). CD8+KLRG1low cells further expressed CD103, a marker of intestinal T resident memory lymphocytes in humans,[15] thus confirming that the CD8+ cell pool is principally composed of TRMs (Figure 2B,C).

As initially found by immunohistochemistry, flow cytometry quantification also revealed a pronounced reduction of CD8+ T cells in actively drinking AUD patients (Figure 2A). Moreover, this reduction was related to a reduced proportion of CD8+ long-lived TRMs that was primarily observed in AUD patients with ALD but not in AUD patients with minimal liver disease (Figure 2B,C). In addition, TRM in AUD patients retained their ability to express CD69+, which is required for retention and local activation of the cells in the tissue, compared with healthy controls (Figure 2D). By contrast to CD8+ T cells, no significant changes were found in the CD4+ T cell pool in actively drinking AUD patients compared with controls (Figure 2A, Figure S3). Strikingly, in NAFLD patients with at least a similar disease stage on biopsy than ALD patients, no reduction of CD8+ T cells was found on immunohistochemistry (Figure 1A,B).

These observations indicate that actively drinking AUD patients with ALD are characterised by a specific reduction of duodenal CD8+ TRMs which is not observed in NAFLD. Furthermore,



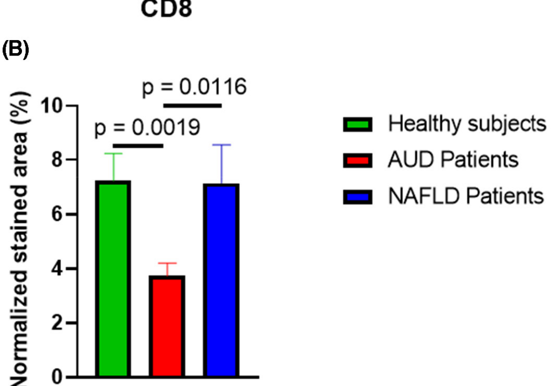


FIGURE 1 Evaluation of number of CD8+ T lymphocytes in the duodenum of alcohol use disorder (AUD) patients, non-alcoholic fatty liver disease (NAFLD) patients and controls. (A) Representative immunohistochemistry staining of duodenal CD8+T lymphocytes showing a significant reduction of CD8+ in the duodenum of AUD patients (n = 15) compared to controls (n = 9) and NAFLD patients (*n* = 10) (B).

this reduction was associated with ALD but not with isolated steatosis.

3.3 | Changes in the transcriptomic profiles in TRM of patients with ALD

Intestinal CD8+ TRM cells receive multiple signals from their tissue microenvironment that integrate into a transcriptional programme driving differentiation, activation and maintenance.[21,22] To evaluate mechanisms implicated in TRM reduction in patients with ALD, we analysed transcriptional profiles by bulk RNA sequencing of sorted duodenal TRMs in AUD patients at different disease stages and healthy controls. Using principal component analysis (PCA; Figure 3A, Figure S4), we found a main divergence between transcriptomic profiles of TRMs in patients with ALD according to the second principal component (PC2), while those in the minimal liver disease group clustered together with controls. No down-regulated genes were observed. The differences in the transcriptomic profiles only consisted of a restricted number of 52 and 41 genes that were specifically up-regulated in TRM cells of patients with ALD compared to healthy controls or to AUD patients with minimal liver disease respectively (Figure 3B, Figure S5). Applying gene set enrichment analysis (GSEA) to search for enriched pathways, we found that significantly up-regulated gene sets in TRM cells of patients with ALD included important signatures related to apoptosis, lysosomes and lipid metabolism, antimicrobial and immune responses (Figure S6 and details in Material S1).

3.4 | Increased apoptosis of duodenal CD8+ TRM in patients with ALD

Since RNAseq analysis revealed a strong signature pointing to apoptosis, we further investigated whether programmed cell death could be involved in TRMs reduction in patients with ALD. Gene ontologies related to apoptosis were significantly enriched. The apoptosis transcriptional signature was further confirmed by additional KEGG analysis (Figure 4A, Figure S7). To investigate the presence of apoptotic duodenal CD8+ TRM in more detail, we used a TUNEL assay in duodenal cryosections and Annexin V (ANX5) in a combination with a cell-membrane impermeable dye (e.g. DAPI) by FACS, to allow to distinguish quantitatively late-apoptotic cells (ANX5+DAPI+) from 'alive' cells (ANX5-DAPI-).[23] The TUNEL assay revealed the presence of apoptotic duodenal CD8+ T cells in AUD patients compared to no or little apoptosis in healthy controls and NAFLD patients (Figure 4B). FACS analysis confirmed an increased proportion of late-apoptotic CD8+ duodenal T cells (ANX5+ DAPI+) in parallel to a reduction of 'alive' CD8+ T cells in in patients with ALD, whereas the levels were similar in minimal liver disease compared with controls (Figure 4C,D). Cell death was restricted to the CD8+ T cell pool since the proportions of live and late-apoptotic CD4+ T cells were similar in AUD patients and healthy controls (Figure S8). Increased proliferation could compensate the loss of cells due to apoptosis. Therefore, we evaluated the proliferation of CD8+ T cells using the proliferation marker Ki67 by immunofluorescence. The proliferation of CD8+ T lymphocytes was low with no difference between AUD patients and controls (Figure S9).

Taken into consideration that the duodenal CD8+ T cell pool is composed at >90% by TRM cells, our results indicate that apoptosis

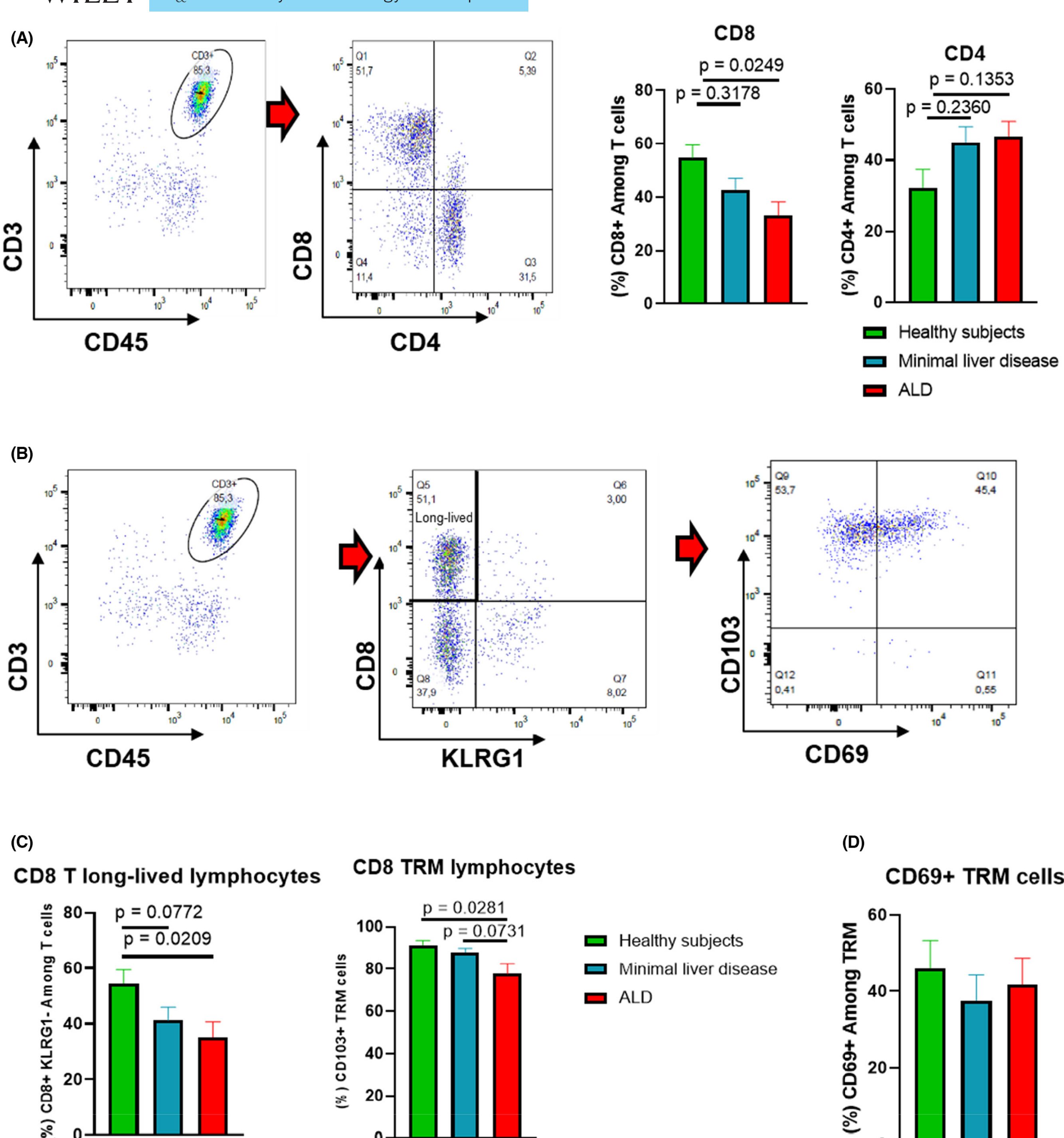
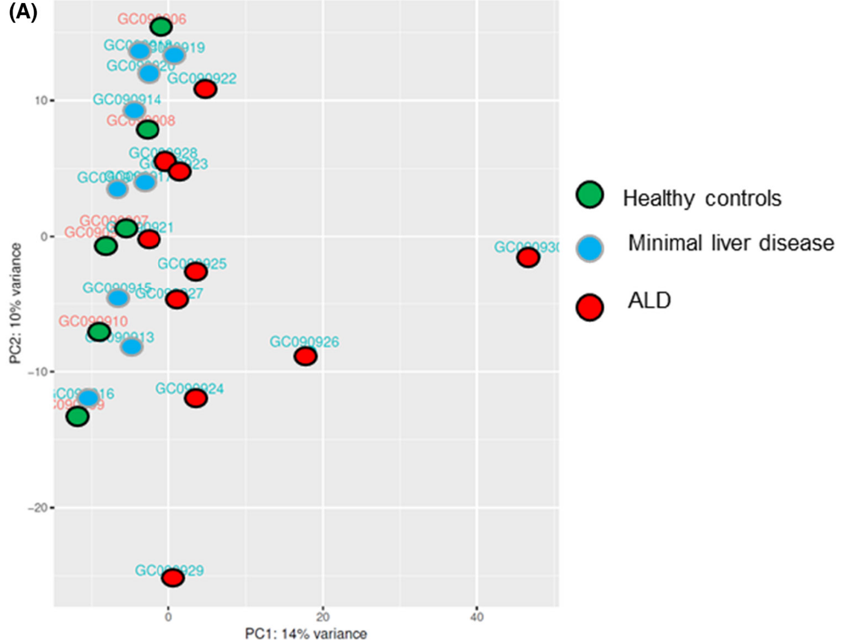


FIGURE 2 Assessment of duodenal T lymphocytes subsets in alcohol use disorder (AUD) patients according to alcohol-associated liver disease (ALD) stage. Gating strategy used to identify duodenal CD4+ and CD8+ T cells (left). Analysis (right) showed diminution of CD8+ T cells in patients with ALD (*n* = 10) compared to controls (*n* = 6) (A). (B) Gating strategy used to identify CD8+ T resident memory (TRM) lymphocytes. (C) Flow cytometry analysis showed diminution of long-lived CD8+ KLRG1low CD103+ TRM cells specifically in patients with ALD (*n* = 10) compared to patients with minimal liver disease (*n* = 9) and controls (*n* = 6). (D) Similar proportions of duodenal CD69+ TRM were found in AUD patients (*n* = 22) compared to controls (*n* = 6).

of duodenal CD8+ TRM cells is a process that specifically occurs in ALD but not in NAFLD at a similar disease stage nor in isolated alcohol-related steatosis. Guided by the results of the RNAseq, we next investigated what mechanisms could drive TRM apoptosis in ALD patients.

3.4.1 | Lysosomal membrane permeabilisation induodenal CD8+ TRMcells

It has been shown that lysosomal membrane permeabilisation could contribute to programmed cell death.[24] Interestingly, several



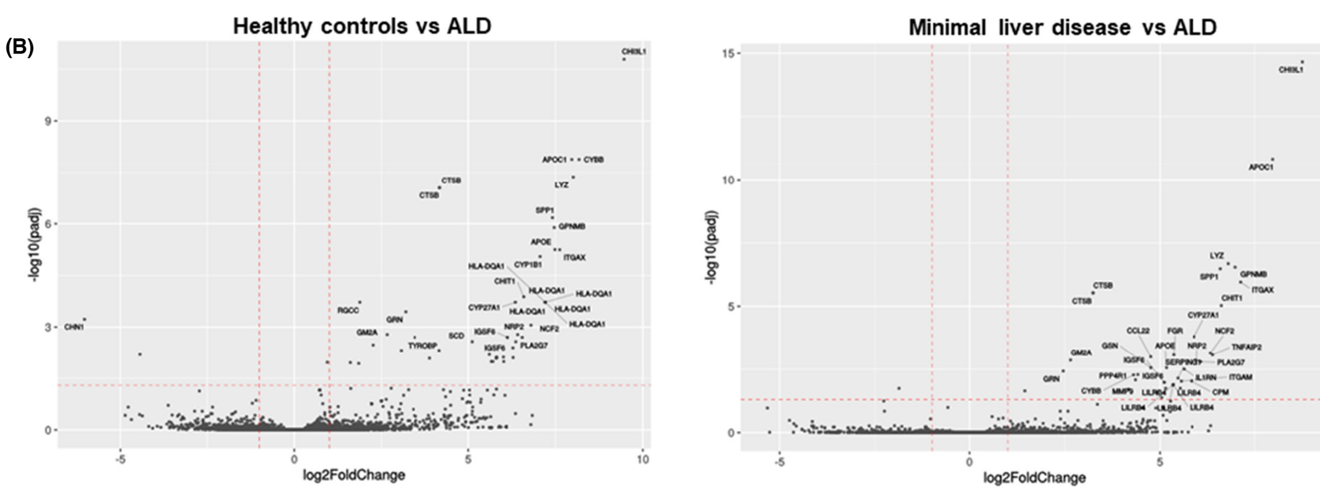


FIGURE 3 Transcriptomic analysis of duodenal T resident memory lymphocytes (TRM) from alcohol use disorder (AUD) patients at different alcohol-associated liver disease (ALD) stage and healthy controls. Principal component analysis of TRM from AUD patients (*n* = 19) compared to controls (*n* = 6) (A) indicated different transcriptomic profiles (principal component 2; PC2) in the sub-group of patients with ALD. (B) Significant enrichment of different genes in TRM of patients with ALD compared to healthy volunteers (left) and minimal liver disease (right).

significantly enriched genes were related to lysosomes (e.g. CTSB, CTSH, LMNA), with Cathepsin B (CTSB) being the most up-regulated gene (Figure 5A). KEGG pathway visualisation pointed to the intrinsic apoptotic pathway while genes associated with the extrinsic pathway of apoptosis were not induced (Figure S10). We, therefore, assessed lysosomal integrity and function using LysoTracker®. We found a significant reduction of mean fluorescence intensity in duodenal CD8+ T cells from patients with ALD as compared with minimal liver disease (Figure 5B), indicating an increase in pH of lysosomes, a hallmark of lysosomal membrane permeabilisation. By contrast to CD8+ T cells, duodenal CD4+ T cells in ALD patients showed no differences in lysosomal integrity compared to patients with minimal liver disease (Figure S11).

To test whether CTSB, an important lysosomal protease, is contributing to apoptosis, we inhibited CTSB using a specific inhibitor CA-074,[25] alone or in combination with ethanol in sorted duodenal CD8+ TRM and we measured Caspase 3/7 activity. Remarkably, inhibition of CTSB by CA-074 reduced Caspase 3/7 activity specifically in patients with ALD but not in duodenal CD8+ TRM cells from AUD patients with isolated steatosis (Figure 5C). The effect was independent of the presence of ethanol (Figure S12) thus indicating that lysosomes via Cathepsin B contribute to mucosal TRM apoptosis in AUD patients with liver disease.

3.4.2 | Contribution of sphingomyelins in duodenal CD8+ TRM cell death

RNA sequencing data also showed that specific lipid transcriptomic signatures were enriched in TRM from patients with ALD (Figure 6A,

Figure S13), with APOE, APOC1, SCD, LPL, IRS2, CYP1B1, CYP27A1 being the most significantly up-regulated genes together with transcripts of genes mediating transport and metabolism of long-chain fatty acids (see Material S1). Lipid metabolism and especially sphingolipids have been linked to lysosomal dysfunction, as shown, for example in human lysosomal storage disorders.[26] Sphingolipids also regulate major cellular processes such as apoptosis, proliferation and senescence. Interestingly, we have also shown that circulating saturated sphingomyelins (SM) are associated with the severity of ALD[27] in humans. These observations prompted us to investigate the potential involvement of sphingolipid species in TRM cell death.

Increased plasma sphingomyelins SM (d18:0/14:0) and SM (d20:0/16:0) correlate with up-regulated gene sets linked to lipid metabolism/lysosomes

We identified increased plasma levels of the sphingomyelins SM (d18:0/14:0) and SM (d20:0/16:0), two isomers of SM d32:0 and d36:0 previously identified by untargeted lipidomics approach (Figures S14 and S15A-C). Interestingly, increased plasma levels were specific of patients with ALD, whereas those levels remained close to control values in patients with minimal liver disease (Figure 6B). Circulating SM levels significantly correlated with top-ranked genes of lipid metabolism/lysosomes in the TRM transcriptomic profiles (Table S3), supporting a potential interaction between these lipids and duodenal TRMs.

Reduced TRM viability after in vitro stimulation with the SM (d18:0/14:0) cleavage product C14 dihydroceramide

In addition, SM (d18:0/14:0) inversely correlated with the proportion of duodenal TRM lymphocytes (Figure 6C) pointing to a potential toxicity of this sphingomyelin. To test this hypothesis, we assessed in vitro cell viability of sorted CD8+ TRM cells upon stimulation with C14 dihydroceramide, the cleavage product of sphingomyelin SM (d18:0/14:0) and found a reduced viability of CD8+ TRM lymphocytes in a dose-dependent manner (Figure 6D).

Overall, these data support a link between increased sphingomyelins and TRM cell death.

Up-regulation of duodenal genes implicated in de novo sphingolipid synthesis

In parallel to increased plasma levels of saturated sphingomyelin SM (d36:0), we also found significantly elevated levels of dihydroceramide (d36:0) (Figure 6E), a precursor of SM (d36:0), only in duodenal biopsies of ALD indicating that those SM might originate from the duodenum itself and eventually spill over into the circulation in patients with ALD.

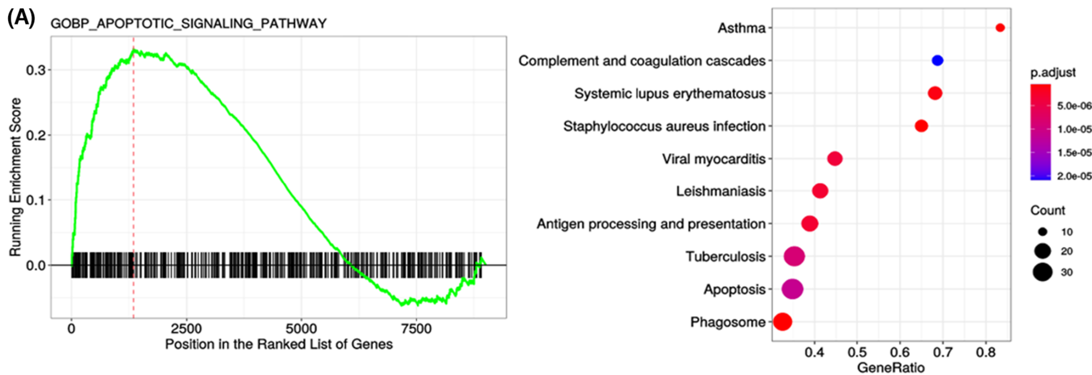
Higher sphingolipid levels could either result from increased absorption from the diet or be related to de novo synthesis in the duodenum. Absorption of dietary sphingomyelins requires hydrolysis to sphingosine by intestinal alkaline sphingomyelinase and a neutral ceramidase, encoded by the ENPP7 and ASAH2 genes respectively.[28] Assessment of these two enzymes in duodenal biopsies of AUD by qPCR revealed down-regulation of both ENPP7 and ASAH2 transcripts (Figure 6F). Therefore, increased levels of these specific sphingomyelins are unlikely a consequence of up-regulation of the uptake of dietary sphingomyelins. Ceramide synthase 6 (CERS6) is responsible for the synthesis of dihydroceramides with C14- and C16-N-fatty acids.[29] In addition, dihydroceramide desaturase 1 and 2 (DEGS1 and DEGS2), with the second being intestinal specific, are key enzymes involved in ceramides synthesis.[26] Duodenal CERS6 and DEGS2, but not DEGS1 (not shown), mRNA levels were up-regulated in AUD patients compared to healthy controls (Figure 6F). These observations indicate that higher tissue and plasma levels at least partially result from de novo synthesis in the tissue itself which in turn then contribute to intestinal CD8+ TRM lymphocytes death in patients with ALD.

3.5 | Pathophysiological consequences: Elevated microbial translocation inversely correlated with alterations in CD8 TRM pool in ALD

Intestinal CD8+ TRM lymphocytes act as local gatekeepers to protect against microbial attachment and invasion and translocation of microbes and/or their products in the circulation. We, therefore, assessed whether increased serum levels of microbial translocation markers, characteristic of patients with ALD,[5] correlated with changes in mucosal lymphocytes. Serum microbial Gram marker soluble CD14 (sCD14) was specifically elevated in the ALD group (Figure 7A), while serum Gram+ marker Peptidoglycan Recognition Proteins (PGRPs) levels increased in actively drinking AUD, irrespective of ALD stage, compared to controls. Interestingly, we observed a significant inverse correlation between number of CD8+ T cells determined by IHC and sCD14 levels (Figure 7B) as well as between the proportions of TRMs determined by flow cytometry and PGRPs levels ($r = -0.5159$; $p = 0.0340$).

Intestinal TRM produce different immune mediators to protect against potential pathogenic microbes. We, therefore, looked

FIGURE 4 Evaluation of apoptosis in duodenal CD8+ T lymphocytes in alcohol use disorder (AUD) patients at different alcohol-associated liver disease (ALD) stage. Gene set enrichment analysis (GSEA) highlighted significant up-regulation of apoptosis gene ontology in CD8+ T resident memory (TRM) cells of patients with ALD compared to controls (A). (B) Representative immunofluorescence staining of terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL), showing apoptotic cells CD8+ T cells preferentially in the duodenum of actively drinking compared to few apoptotic CD8+ T cells in healthy controls and non-alcoholic fatty liver disease (NAFLD) patients. (C) Gating strategy used to identify late-apoptotic annexin 5 (ANX5)+DAPI+ CD8+ T cells. (D) Flow cytometry analysis showed reduced proportion of live CD8+ T cells and increased proportion of late-apoptotic CD8+ T cells in patients with ALD as compared to the other groups.


(A)
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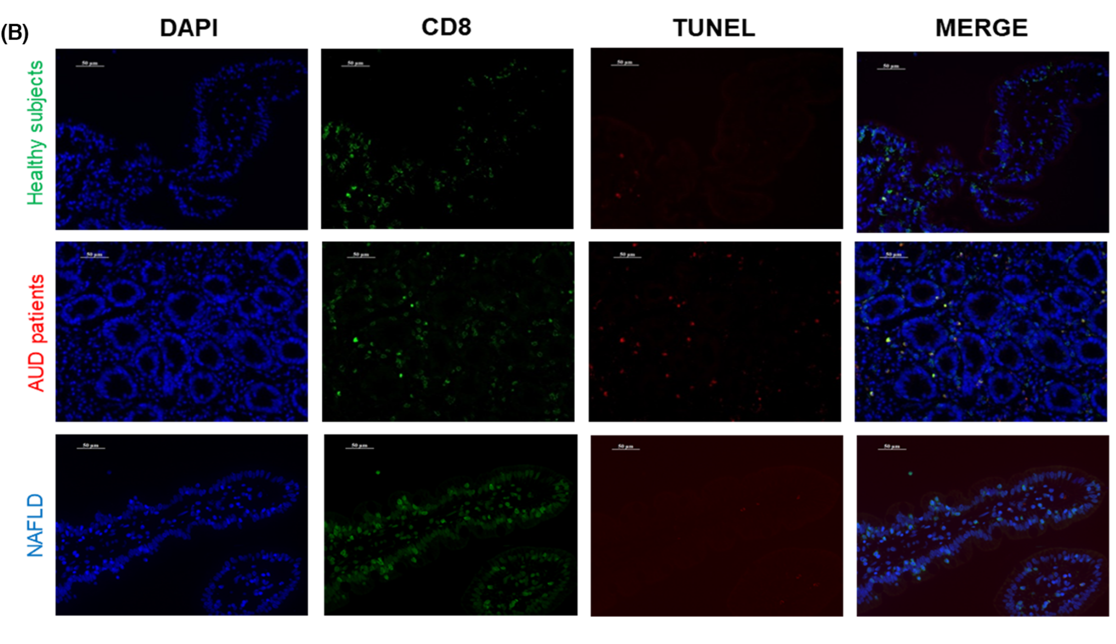
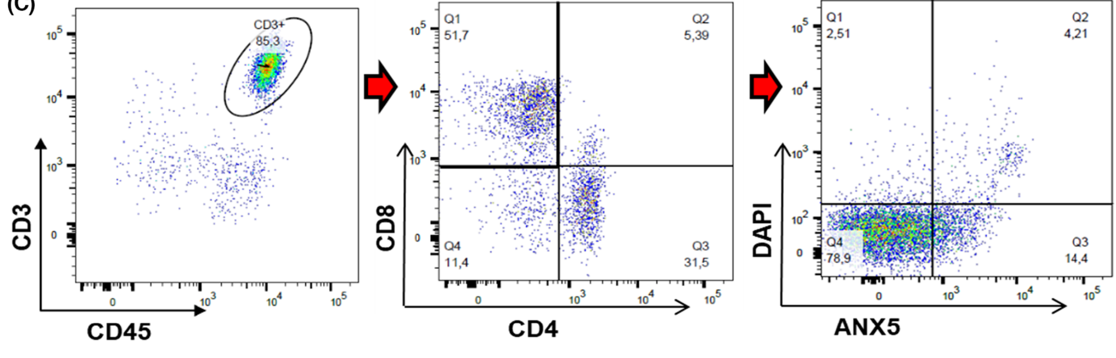
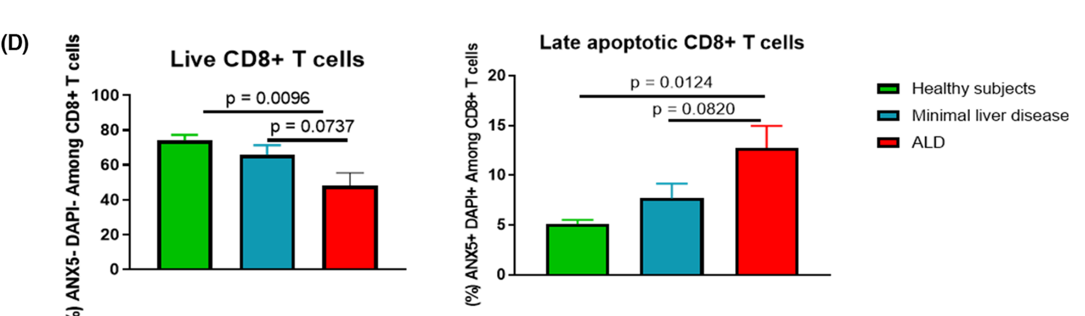
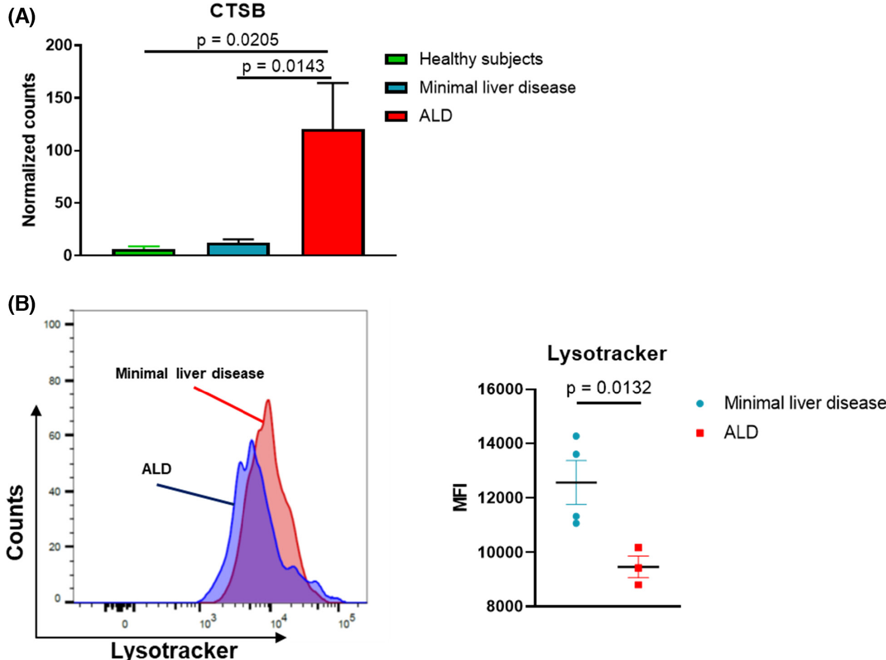
Position in the Ranked List of Genes
Asthma
Complement and coagulation cascades
Systemic lupus erythematosus
Staphylococcus aureus infection
Viral myocarditis
Leishmaniasis
Antigen processing and presentation
Tuberculosis
Apoptosis
Phagosome
GeneRatio
p.adjust
Count


(B)
DAPI
CD8
TUNEL
MERGE
Healthy subjects
AUD patients
NAFLD


(C)
CD3+
85,3
CD3
CD45
Q1 51,7
Q2 5,39
Q4 11,4
Q3 31,5
CD8
CD4
Q1 2,51
Q2 4,21
Q4 78,9
Q3 14,4
DAPI
ANX5


(D)
Live CD8+ T cells
p = 0.0096
p = 0.0737
(%) ANX5- DAPI- Among CD8+ T cells
Late apoptotic CD8+ T cells
p = 0.0124
p = 0.0820
(%) ANX5+ DAPI+ Among CD8+ T cells
Healthy subjects
Minimal liver disease
ALD



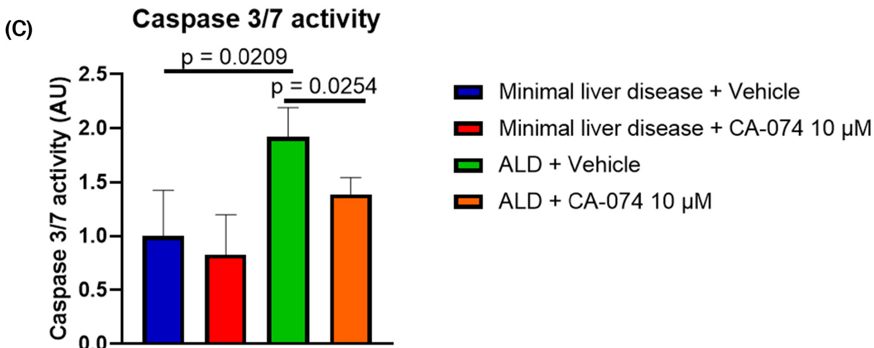


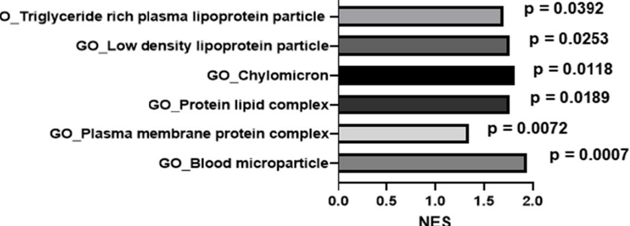
FIGURE 5 Evaluation of lysosomal function in T resident memory lymphocytes (TRM). (A) Significant up-regulation of Cathepsin B (CTSB) in TRM cells from patients with alcohol-associated liver disease (ALD) (n = 10) compared to minimal liver disease (n = 9) and controls (n = 6). (B) Lysotracker mean fluorescence intensity (MFI) on flow cytometry (left) significantly decreased in TRM lymphocytes from patients with alcohol-associated liver disease (ALD) compared to minimal liver disease (right, n = 3–4 / group). (C) Inhibition of Cathepsin B in sorted TRM lymphocytes with CTSB inhibitor CA-074 reduced elevated caspase 3/7 activity specifically in TRM lymphocytes from patients with ALD (n = 3-6/group).

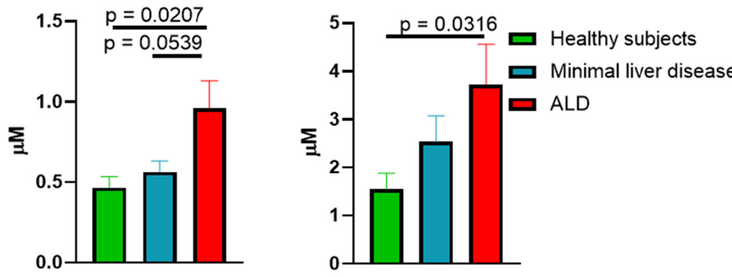
for alterations in the transcriptomic profile related to antimicrobial effects in TRMs. We found enrichment of gene sets mediating response to microbes, leucocyte activation and migration in TRM of patients with ALD (Figure 7C). Focusing on responses against microbes, the most up-regulated gene was the anti-fungal mediator CHI3L1, followed by CHIT1 and the antimicrobial molecule LYZ (see Material S1). Among cytokines, the most up-regulated gene was anti-inflammatory IL1 Receptor Antagonist (IL1RN) (Figure 7D), which was confirmed by quantitative PCR (Figure S16) in the mucosa of AUD patients compared to controls.

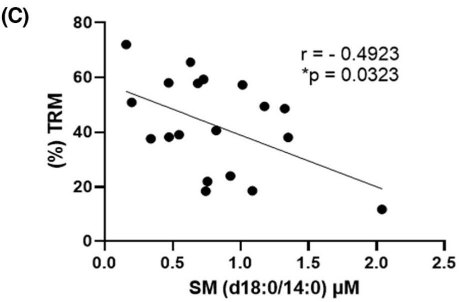
We finally assessed whether TRM in AUD patients were characterised by differential protein expression of surface markers associated with immune dysfunction. Proportions of duodenal TRM expressing programmed cell death 1 (PD1), highly expressed in TRM lymphocytes,[30] were reduced, while CD57+ TRM increased in patients with ALD (Figure 7E) which is compatible with an altered immune response.

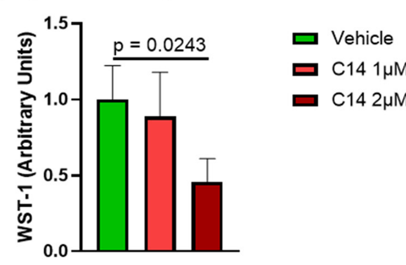
Taken together, our results support that alterations (e.g. cell death, changes in immune functions) in duodenal CD8+ TRM likely contribute to reduced immune surveillance against microbes thus facilitating

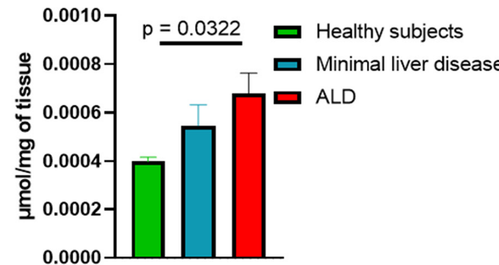
FIGURE 6 Sphingolipids linking transcriptomic alterations in T resident memory lymphocytes (TRM) and cell viability in patients with alcohol-associated liver disease (ALD). (A) Significant enrichment of gene sets related to lipid metabolism in TRM of patients with progressive ALD. (B) Targeted analysis by LC–MS/MS showed increased plasma levels of SM (d20:0/16:0) and SM (d18:0/14:0) in patients with ALD (n = 9/group). (C) Low percentages of duodenal TRM cells correlated with higher plasma levels of SM (d18:0/14:0). (D) Reduced viability of CD8+ TRM lymphocytes after stimulation with C14:0 ceramide at increasing concentrations assessed by the WST-1 assays (n = 3/group). (E) Duodenal dihydroceramide levels increased especially in patients with ALD. (F) Intestinal alkaline sphingomyelinase (ENPP7) and neutral ceramidase (ASAH2), enzymes involved in dietary sphingomyelin absorption, are down-regulated in distal duodenal biopsies from AUD patients (n = 46) compared to controls (n = 12). By contrast, dihydroceramide desaturase 2 (DEGS2) and Ceramide synthase 6 (CERS6), involved in de novo sphingolipid synthesis, are up-regulated in the duodenal mucosa from AUD patients (n = 46) compared to healthy volunteers (n = 12).

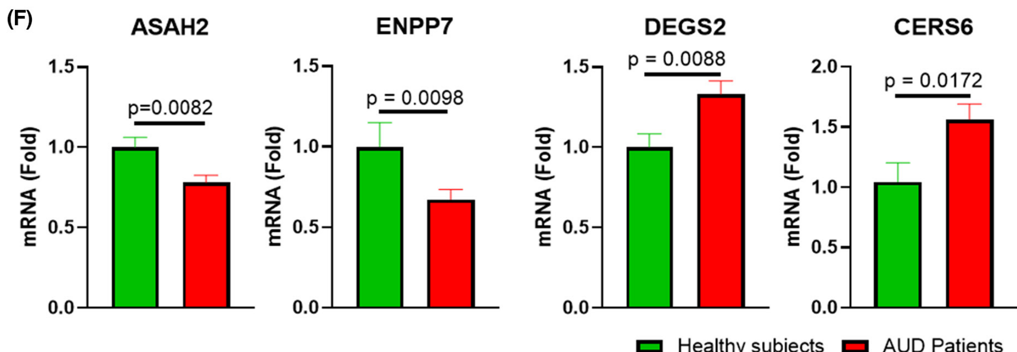

(A)
Lipid metabolism
GO_Triglyceride rich plasma lipoprotein particle
GO_Low density lipoprotein particle
GO_Chylomicron
GO_Protein lipid complex
GO_Plasma membrane protein complex
GO_Blood microparticle
p = 0.0392
p = 0.0253
p = 0.0118
p = 0.0189
p = 0.0072
p = 0.0007
NES

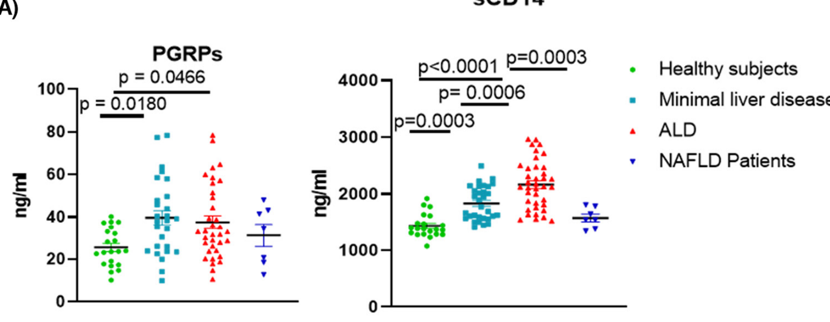

(B)
Plasma SM (d18:0/14:0)
Plasma SM (d20:0/16:0)
p = 0.0207
p = 0.0539
p = 0.0316
µM
Healthy subjects
Minimal liver disease
ALD

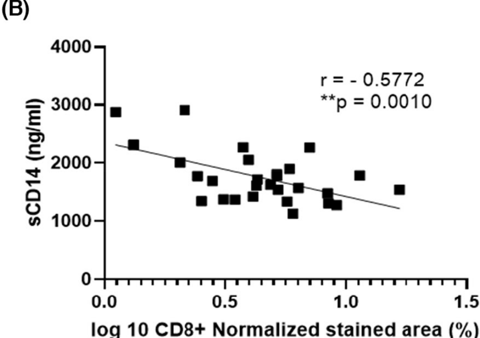

(C)
r = - 0.4923
*p = 0.0323
(%) TRM
SM (d18:0/14:0) µM


(D) Cell viability upon C14 dihydroceramide stimulation
p = 0.0243
WST-1 (Arbitrary Units)
Vehicle
C14 1µM
C14 2µM


(E) Duodenal dihydroceramide d36:0
p = 0.0322
µmol/mg of tissue
Healthy subjects
Minimal liver disease
ALD


Absorption
De novo synthesis
(F)
ASAH2
ENPP7
DEGS2
CERS6
p=0.0082
p = 0.0098
p = 0.0088
p = 0.0172
mRNA (Fold)
Healthy subjects
AUD Patients



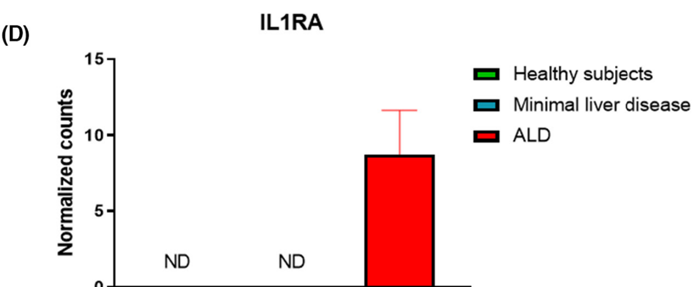


(C)

Immune responses

GO_Response to bacterium p = 0.0454
GO_Defense response to bacterium p = 0.0161
GO_Antimicrobial humoral response p = 0.0117
GO_Leukocyte chemotaxis p = 0.0076
GO_Positive regulation of leukocyte degranulation p = 0.0061
GO_Inflammatory response p = 0.0061
GO_Leukocyte mediated immunity p = 0.0048
GO_Cell activation involved in immune response p = 0.0048

0.0 0.5 1.0 1.5 2.0
NES



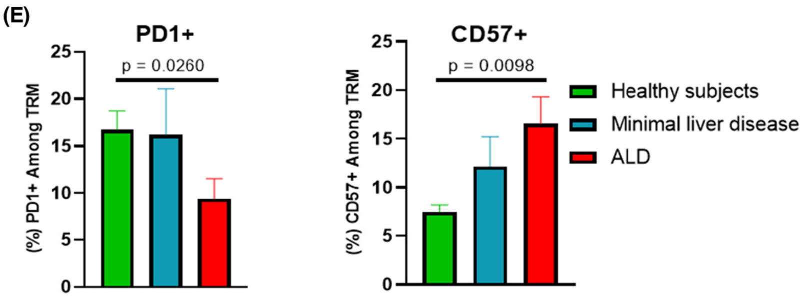


FIGURE 7 Evaluation of systemic microbial translocation and immunity in T resident memory (TRM) cells from alcohol use disorder (AUD) patients at different alcohol-associated liver disease (ALD) stage. (A) Serum levels of sCD14 microbial translocation marker increased significantly primarily in actively drinking patients with ALD, while PGRPs were elevated in AUD patients independently from ALD stage. (B) Inverse correlation between the number of mucosal CD8+ T cells and serum levels of microbial translocation marker sCD14. (C) Up-regulation of gene sets linked to inflammatory and antimicrobial responses in TRM cells from AUD patients with ALD. (D) Significant up-regulation of Interleukin 1 Receptor Antagonist (IL1RN) in TRM cells from patients with ALD. (E) Diminution in the proportion of PD1+ TRM and increased proportion of CD57+ TRM in the duodenum of patients with ALD compared to controls.

microbial translocation. These changes are strongly associated with ALD.

3.6 | Short-term abstinence does not reverse duodenal CD8+ T cell reduction, apoptosis and microbial translocation

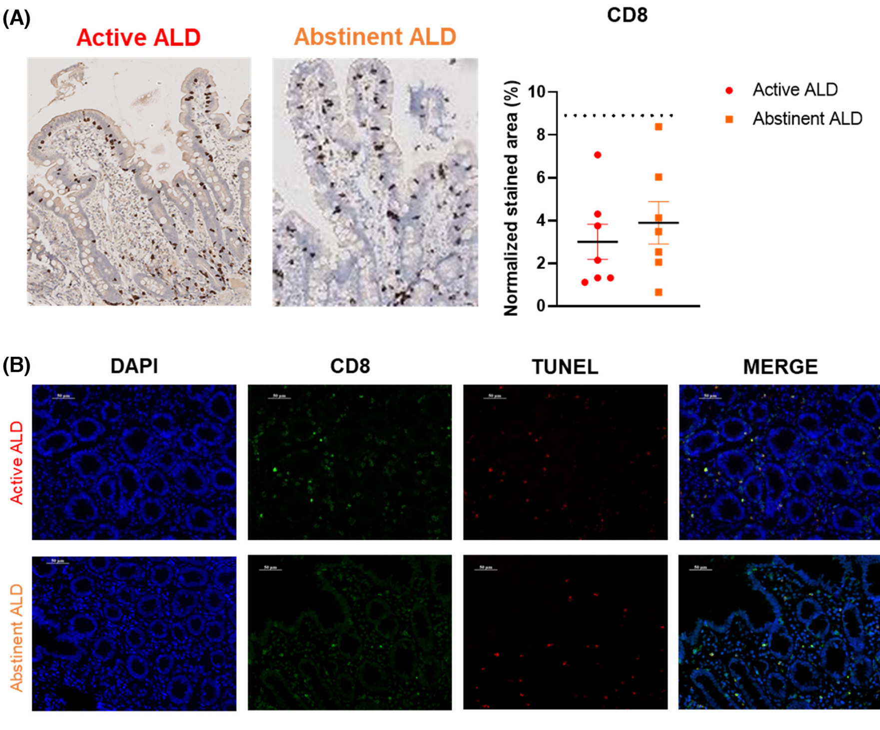
In absence of effective pharmacological therapy, abstinence represents the foundation of ALD treatment. We, therefore, assessed whether a short period of abstinence, often encountered in AUD patients, was able to reverse the diminution of duodenal CD8+ T resident memory cells and their apoptosis. We tested AUD patients with ALD (Table S1) who remained abstinent at the end of the 3-week detoxification programme and found similarly low numbers of duodenal CD8+ T cells in abstinent patients as compared to active drinkers (Figure 8A). We, therefore, evaluated whether apoptosis was still present in CD8+ T cells and found persistence CD8+ TUNEL+ apoptotic T lymphocytes in abstinent patients (Figure 8B), indicating still ongoing TRM cell death.

Finally, since we showed TRM correlated with microbial translocation markers, we evaluated whether a short time abstinence could normalise microbial translocation. Among the two serum microbial translocation markers, only sCD14 levels were reduced in abstinent patients but not PGRPs, the levels which remained high (Figure 8C). Although sCD14 levels decreased, they did not normalise in 60% of abstinent patients.

These observations suggest that short-term abstinence does not allow for reversal of TRM cell death and recovery of cell numbers close to controls which in turn is associated with persisting elevated microbial translocation in AUD patients with ALD.

4 | DISCUSSION

We here addressed alterations in the duodenal adaptive immunity at early stages of ALD. We reveal for the first time that a specific reduction together with functional changes in duodenal CD8+ T resident memory lymphocytes in ALD correlates with elevated microbial translocation markers and liver disease severity in a large cohort of



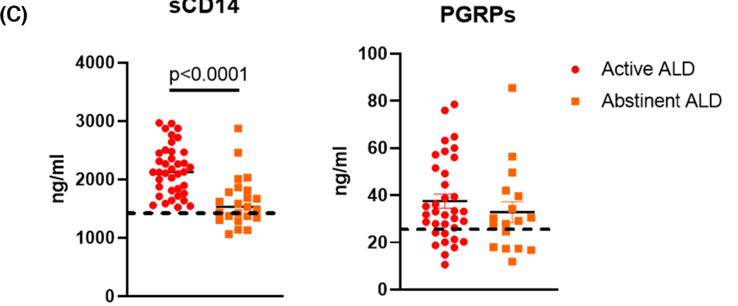


FIGURE 8 Evaluation of duodenal CD8+ T cell number and apoptosis in alcohol use disorder (AUD) patients with alcohol-associated liver disease (ALD) after a short period of abstinence. A short time abstinence does not reverse the number of duodenal CD8+ T lymphocytes at the level of the control, indicated with the dotted line, (A) and elevated apoptosis (TUNEL+ CD8+ cells) (B). Microbial translocation markers sCD14 and PGRPs did not normalise to the levels of the controls, indicated with the dotted line, in the majority of AUD patients with ALD upon a short time of abstinence (C).

patients. From the mechanistic point of view, TRM cells undergo apoptosis related to an altered lipid metabolism in association with lysosomal membrane permeabilisation.

The generation and long-term survival of pathogen-specific TRM lymphocytes are fundamental for local immunosurveillance and relies on consumption of exogenous-free fatty acids by these cells.[22] This metabolic substrate preference distinguishes subpopulations of T cells in mucosal barriers from their circulating counterparts.[22] In our study, expression of gene sets related to lipid metabolism were up-regulated in TRM and correlated with elevated plasma levels of several sphingomyelins, specifically in patients with ALD. These circulating bioactive lipids and/or their metabolic products have the potential to induce cell death in intestinal TRM. Ex vivo stimulation of TRM from AUD patients with these lipid species revealed a reduced viability in a dose-dependent manner, which is consistent with a causal role of these specific sphingolipids in TRM cell death. Lysosomal membrane permeabilisation was increased on TRM cells of patients with ALD. Inhibition of Cathepsin B, the most significant lysosomal gene revealed by RNA sequencing, reduces apoptosis thus improving survival of TRM. These observations indicate that lysosomal dysfunction contributes to TRM cell apoptosis. Sphingomyelins do not only reduce cell viability in vitro but sphingomyelin SM (d18:0/14:0) levels also correlate with up-regulated lysosomal genes such as CTSB indicating a potential link between their up-regulation and lysosomal dysfunction. Such a link between sphingolipids, lysosomes and disease in humans has already been described, for example in lysosomal storage disorders.[26]

Sphingomyelins are virtually synthetised in all the tissues but are especially abundant in the central nervous system.[31] Dietary sphingomyelins need to be hydrolysed by intestinal alkaline sphingomyelinase and neutral ceramidase to sphingosine, which is absorbed by enterocytes, converted to palmitic acid and integrated into chylomicron triglycerides.[28] Both enzymes were down-regulated in the mucosa of AUD patients indicating that increased levels of sphingomyelins likely do not derive from the diet. Alternatively, sphingomyelins can be synthetised by the intestine in pathological conditions involving Ceramide synthetase 6 and DEGS2.[29] The latter were up-regulated in the duodenum of AUD patients, supporting a possible role for local production of specific sphingolipids in the gut. Since lysosome stability is an important regulator of the maintenance of memory CD8+ T cells[32] in the duodenal mucosa, our result open the intriguing possibility that sphingomyelin-related lysosomal instability is an important contributor to TRM cell death in particular in AUD patients who develop early stages of ALD.

Perturbation of the TRM pool and their functional capacity likely play an important role in alcohol-induced gut barrier dysfunction in patients with ALD. Since mucosal TRM cells act as local gate keepers to protect against microbial invasion,[10] those cells consequently up-regulated gene sets associated with leucocyte activation and response to microbes. Notably, this enrichment was not linked to pro-inflammatory responses but to regulatory mediators such as IL1RN and antimicrobial molecules such as CHI3L1, CHIT1 and LYZ. However, this apparently good response is counteracted by increased cell death. As a consequence, the lower the proportion of TRM, the higher the levels of Gram− and Gram+ translocation markers in the blood. In addition, TRM cells also up-regulated surface markers that point to a phenotype related to immune dysfunction and this shift also correlated with sCD14 levels. As a net result, microbial immunosurveillance seems to be profoundly disturbed, allowing invading microbes from a severely dysbiotic microbiota in patients with ALD to reach the circulation. It is tempting to speculate that reduced TRM also translates into a reduced repertoire of specific (intestinal or gut derived) pathogens that will elicit an appropriate immune reaction, leaving the other ones unattended which might contribute to liver disease. It would be interesting to study the TCR repertoire to assess their heterogeneity and to identify whether they are directed against specific bacterial and/or fungal species. The exact mechanisms leading to more severe alterations of duodenal TRM in patients with ALD remain unknown. Our results are not in favour of direct alcohol toxicity but rather point to metabolic changes in TRMs. However, we did not assess potential toxicity of alcohol metabolites (e.g. acetaldehyde) that might reach the duodenum. Besides the observed changes in lipid metabolism affecting TRMs, additional alterations in host and/or microbial metabolites might disturb duodenal TRM metabolism and their functions, ultimately translating into cell death and/or reduced immunosurveillance against microbes. Microbial, tissue and serum metabolomic profiling should be helpful to further investigate those aspects. Given the complex organisation of the intestinal barrier, it is likely that several components of this barrier, including effectors of innate immune responses, need to fail concomitantly thus contributing directly or indirectly to activation of systemic and liver immune responses in ALD.

Finally, a reduced number of duodenal CD8+ T cells was not found in presence of metabolic steatohepatitis (NAFLD group) neither was an elevation of microbial translocation markers. This further highlights the importance of our findings in AUD patients with early stages of liver disease since the NASH patients ranged in the upper spectrum of liver disease severity observed in AUD patients with on average quite active NASH (A3) and F2 fibrosis. Increased endotoxaemia was also not reported in our NAFLD cohort of patients with active non-alcoholic steatohepatitis (NASH) which challenges some of the recent literature on the gut-liver axis in NAFLD.[33] It is tempting to consider the reduction in duodenal CD8+ TRM linked to increased systemic translocation of microbes and/or their products as a hallmark of alcohol consumption and ALD supporting that changes in microbial composition, leaky gut and alterations of gut adaptive immunity are drivers of early stages of ALD. Moreover, short-term alcohol abstinence did not restore mucosal CD8+ T cell alterations nor reverse markers of microbial translocation meaning that those changes are relatively long lasting. Therefore, they might be a reasonable target for new integrated multi-target therapeutic interventions aiming at preventing the occurrence of ALD as early as possible in the disease course.

An intrinsic limitation of this study is that data are mainly based on associations/correlations, which do not formally prove a cause-and-effect relationship. We can also not exclude that other effector immune

cells, such as macrophages and dendritic cells, might be defective in response to microbes. We have recently also shown a reduced number of macrophages in the duodenal mucosa of AUD patients.[5] The principal strength is the high number of patients included in the study who were recruited in a highly standardised clinical setting allowing to reduce as much as possible potential bias in patients' selection and collection of samples. Another strength is the inclusion of a NAFLD highlighting the relevance of our findings in AUD patients with liver disease. Given the difficulties in accessing tissues at early-stage disease, we believe our study adds significant insights into the role of the impairment of gut adaptive immunity in the early pathogenesis of ALD.

In conclusion, we reveal an intriguing diminution of CD8+ T resident memory lymphocytes specifically in the duodenal mucosa of patients with ALD and point to a contribution of lysosomal alterations to their cell death and a direct effect of duodenal sphingolipids on TRM cell survival. Our findings support an important role of CD8+ TRM in intestinal immune surveillance in humans abusing alcohol and contribute to our understanding of these lymphocytes in the development of ALD. Other studies have highlighted the protective properties of CD8 T resident memory cells in several infectious and inflammatory diseases.[34,35] Our observations may be relevant for clinical practice in the future since they suggest that impairment of the small intestinal adaptive immune response is linking the gut and the liver already at early stages in human ALD. Therefore, strategies aiming at reinforcing the intestinal immune system could prove beneficial in early human ALD.

AUTHOR CONTRIBUTIONS

Luca Maccioni: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); writing – original draft (lead); writing – review and editing (equal). **Axelle Loriot:** Formal analysis (equal); investigation (supporting); validation (equal); writing – review and editing (equal). **Joseph Dewulf:** Formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – review and editing (equal). **Guido Bommer:** Validation (supporting); writing – review and editing (supporting). **Yves Horsmans:** Validation (supporting); writing – review and editing (supporting). **Nicolas Lanthier:** Resources (supporting); validation (supporting); writing – review and editing (supporting). **Bernd Schnabl:** Validation (supporting); writing – review and editing (supporting). **Isabelle Leclercq:** Supervision (equal); validation (equal); writing – review and editing (equal). **Peter Stärkel:** Conceptualization (lead); funding acquisition (lead); supervision (lead); validation (equal); writing – original draft (equal); writing – review and editing (equal). All authors approved the final version of the mauscript.

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Declaration of personal interests: B.S. has been consulting for Ferring Research Institute, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals and Takeda. B.S.'s institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with B.S. as inventor related to this work. P.S. received grant support from Gilead Sciences Belgium.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.

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