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Epigenetic basis for monocyte dysfunction in patients with severe alcoholic hepatitis

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TG gives advice to Promethera Biosciences and Martin Pharmaceuticals. CM received grant from Gilead and gives advice to Gilead, Abbvie and Intercept. Other authors have no competing interests.

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Author Contributions

LW conducted most of the experiments. KKS contributed to the design of immunophenotype experiments; AL, CM, JS, TS, ET and TG performed the patient recruitment. AA, MS and FL performed bioinformatics analysis. LW and AA analyzed the data and prepared the figures. JD provided input for data analysis and interpretation. TG and SG supervised the work and wrote the manuscript. All authors were involved in critically revising the

manuscript for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

Abstract

Background & Aims: Severe forms of alcohol-related liver disease are associated with increased susceptibility to infections contributing to their poor prognosis. The cellular and molecular mechanisms responsible for this altered host defense are incompletely understood.

Methods: We performed whole blood phenotypic analysis and ex vivo stimulation with various pathogen-associated molecular patterns (PAMPs). We included 34 patients with alcohol-related cirrhosis (18 of which had biopsy-proven severe alcoholic hepatitis [sAH]), 12 healthy controls and 11 patients with chronic alcohol consumption without significant liver disease. We also evaluated the transcriptomic (RNA-seq) and chromatin accessibility (ATAC-seq) profiles of CD14⁺ monocytes from a subset of patients.

Results: Circulating monocytes and conventional dendritic cells ([DCs](#)) from patients with sAH displayed complex alterations characterized by increase of activation and inhibitory surface markers and impaired pro-inflammatory response upon stimulation with PAMPs representative of gram-negative bacteria (LPS, Pam3CSK4) or fungal pathogens (Zymosan). Their decreased ability to produce more than one cytokine (polyfunctionality) upon PAMPs stimulation was correlated with the risk of developing infection at 28 days or of mortality at 90 days. [The presence of Acute-on-Chronic Liver Failure \(ACLF\) in patients with sAH did not significantly modify the immune profile of monocytes and DCs.](#) Moreover, CD14⁺ monocytes of patients with sAH displayed altered transcriptional and epigenomic profiles characterized by downregulation of key innate immune and metabolic pathways and upregulation of important immunomodulatory factors.

Conclusions: The altered transcriptional program and functional properties of monocytes from sAH patients contributing to their susceptibility to infection have strong epigenetic determinants.

Lay summary:

Severe alcoholic hepatitis is associated with a complex monocyte dysfunction characterized by modified chromatin access inducing a transcriptomic profile associated with inappropriate response to bacterial and fungal products. These observations suggest some mechanisms underlying the susceptibility to infectious complications.

Introduction

Increased susceptibility to infections is a hallmark of severe forms of alcoholic liver disease (ALD), like severe alcoholic hepatitis (sAH) and acute-on-chronic liver failure (ACLF), and contributes largely to their poor prognosis [1]. This susceptibility is not limited to bacterial infections but also observed for viral and fungal infections [2, 3]. The main clinical challenge still remains their early diagnosis and prompt adequate management.

The causative mechanisms of this susceptibility remain elusive. sAH and/or alcohol-related ACLF is associated with an immune dysfunction characterized by the coexistence of systemic inflammation [4] and impaired response of immune cells to pathogen and their products [5]. Several studies demonstrated a reduced pro-inflammatory cytokine production by lipopolysaccharide (LPS)-stimulated monocytes and a decrease in their capacities to kill microbes. Mechanisms responsible for this monocyte dysfunction are supposed to be multifactorial: reduced expression of HLA-DR [6], increased expression of MER tyrosine kinase (MERTK) [7], increased exposure to prostaglandin-E2 [8] and alteration in monocyte glutamine metabolism [9]. The function of other mononuclear phagocytes, namely conventional and plasmacytoid dendritic cells (DC), that play a major role in the orchestration of immune responses, remains unexplored in ALD.

Recently, it has been described that myeloid cells from innate immune system can be trained through epigenetic and metabolic programming by diverse mediators resulting in hyper- or hypo-responsiveness upon re-stimulation [10]. In sepsis, the current paradigm suggests that pathogen associated molecular patterns (PAMPs) induce innate immune cells tolerance by epigenetic silencing of several host defense genes thus contributing to the higher risk of secondary infections [11].

In this study, we explored phenotypic profiles of the main subsets of the mononuclear phagocyte system in different stages of ALD. We also assessed their responses to diverse PAMPs and their ability to produce pro-inflammatory cytokines. We compared these immunological parameters with clinical outcomes (mortality and risk of infection). Finally, we studied transcriptional and chromatin accessibility profiles of CD14⁺ monocytes.

Materials and Methods

Patient recruitment

For this study, we prospectively recruited 34 patients with alcoholic cirrhosis diagnosed based on liver biopsy or classical clinical, biochemical and radiological criteria, 11 chronic alcohol abusers without signs of liver fibrosis (Fibroscan < 7 kPa and Fibrotest < 0.3), and 12 healthy control participants at C.U.B. Erasme Hospital between May 2016 and October 2017. Patients with cirrhosis were divided into two groups based on histology and severity scores: alcohol-related cirrhosis alone or severe (modified discriminant function higher than 32 [12]) biopsy-proven AH. The sAH group was further divided into patients with or without ACLF according the EASL-CLIF definition [13] for subgroup analyses. Inclusion and exclusion criteria used are detailed in supplementary methods.

Whole blood assays

Ex vivo phenotypic analysis, whole blood stimulation, cytokine measurements and preparation of the samples for flow cytometric analysis was performed as described previously [14] and is detailed in supplementary methods and Supplementary CTAT Table.

RNA-Seq and ATAC-Seq on CD14⁺ monocytes

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences) and were cryopreserved. CD14⁺ monocytes were isolated by fluorescence-activated cells sorting (FACS) on a BD FACS Aria III (>90% purity) and processed for RNA isolation or ATAC-Seq as previously described [15] and detailed in supplementary methods.

Results

Characteristics of the enrolled individuals

Based on clinical, histological and biochemical parameters (Table S1), we defined 4 groups: healthy controls (HC), chronic alcohol abusers without signs of hepatic disease (CAA), patients with alcohol-related cirrhosis (C) and patients with biopsy-proven severe alcoholic hepatitis (sAH). Overall, we had a majority of male individuals and cirrhotic patients tended to be older than other groups. Seven of the 18 sAH patients had ACLF according to the EASL-CLIF definition (Table S2) [13]. Occurrence of infection, liver transplant or death was monitored for up to 90 days after recruitment (Table S1).

Skewed pattern of PAMPs-elicited cytokine production in whole blood cells from sAH patients

In order to gain further insight into basic features of innate immune cells in ALD, we used a highly standardized and controlled protocol of ex vivo whole blood culture assay [16]. We assessed the production of cytokines in culture supernatants with medium alone or upon stimulation with representative PAMPs: bacterial LPS, a TLR4 ligand; Zymosan, a component of cell wall from yeast that signals through TLR2 and Dectin-1 and R848, a synthetic ligand for TLR7/8. For almost all the conditions tested, no significant changes in cytokine levels in the supernatants were observed between HC, CAA and cirrhotic patients. In response to LPS (and to a lesser extent to Zymosan), we noted a general trend for reduced production of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β and IL-12/23p40 in sAH patients compared to the other groups (Fig. 1a). IL-10 levels in this group were also decreased compared to the cirrhotic group. However, this was not associated with global hypo-responsiveness in these patients since, in response to R848, production of these cytokines was maintained or even increased in the case of IL-6. Remarkably, we observed hyperproduction of IL-8 in sAH patients compared to the other groups, irrespective of the

stimulus. Conversely, induction of the IFN-dependent chemokine CXCL10 was consistently decreased in these patients. Complementing this finding, levels of Th1 supporting cytokines IL-12p70 and IFN γ elicited by R848 were also drastically reduced in sAH samples. In stark contrast, production of R848-induced type I IFNs (mostly produced by plasmacytoid DCs) was comparable among the groups (Fig. 1b). These results indicate that innate immune circulating cells of sAH patients exhibit a distinct profile of cytokine production characterized by strongly compromised IL-12p70/IFN γ /CXCL10 axis. The capacity to produce TNF α , IL-6 and IL-1 β is also reduced but in response to certain PAMPs only. In contrast, production of other cytokines such as IFN α was maintained or even increased in the case of IL-8.

Increased severity of ALD is accompanied by alterations in the frequency and phenotype of circulating mononuclear phagocytes

In order to establish the cellular basis for altered PRR responsiveness in sAH patients, we analyzed the main circulating immune cell populations by multi-color flow cytometry. We did not observe major changes in lymphocytes, NK cells or granulocytes although there was a trend toward a decrease in the absolute count of these cells with the severity of the disease, except for neutrophils (Fig 2a). Next, we evaluated the three main subsets of monocytes based on CD14 and CD16 expression (Fig. 2b&c). Intermediate (CD14^{hi}CD16⁺) and to a lesser extent classical (CD14^{hi}CD16⁻) monocytes were increased in sAH patients compared to CAA. This was associated with a clear decrease in the non-classical (CD14^{lo}CD16⁺) monocyte subset. In addition, we observed that sAH patients displayed reduced counts of conventional (cDCs) and plasmacytoid (pDCs) dendritic cell subsets (Fig. 2b&c and Fig S1a). These data indicate that increased severity of ALD is associated with important changes in the relative proportions of circulating mononuclear phagocytes.

To gain further insight into the activation status of these cells, we analyzed the expression of several markers at the surface of CD14⁺ monocytes, cDCs and pDCs. As previously demonstrated in ACLF [6, 7], CD14⁺ monocytes from sAH patients displayed reduced HLA-DR expression (Fig. 3), mainly driven by a reduced expression on classical monocytes (data not shown). HLA-DR expression by pDCs was significantly reduced in ALD compared to HC, even in CAA. Although there was a trend for decreased HLA-DR expression on cDCs, it did not reach statistical significance due to high heterogeneity. Steady-state expression of CD86, a costimulatory protein, and CD69, an early activation marker, was stable across the groups. In contrast, for the three cellular populations, we observed a decrease of the maturation marker CD83 in stable cirrhotic patients. In comparison to this latter group, expression of CD83 was significantly upregulated in sAH patients. A similar profile was observed for the inhibitory protein PDL-1. Finally, expression of the FcγRI receptor CD64 by monocytes, generally associated with systemic inflammation, was progressively enhanced with the severity of the disease (Fig. 3). Of note, the phenotype of monocytes from sAH patients was not modified by the presence of ACLF with the exception of lower CD64 expression (Fig S2a). Taken together, these data indicate complex and dynamic alterations of the proportion and phenotype of the different subsets of the mononuclear phagocyte system during ALD. Chronic alcohol abuse in itself induces slight changes in the phenotype of mononuclear phagocytes with increased expression of CD64 on monocytes and decreased absolute numbers and HLA-DR expression of pDCs. Cirrhotic patients display decreased expression of both CD83 and PDL1 across all mononuclear phagocytes. Yet, the expression of these markers increases on the cells of sAH patients, thus conferring them unique features of both increased activation and inhibitory markers compared to cirrhotic patients.

Global alteration of the capacity of monocytes of sAH patients to produce cytokines

We identified distinctive patterns of whole blood cytokine production and changes in the frequency and phenotype of circulating mononuclear phagocytes, which led us to assess cytokine production at the single-cell level by flow cytometry in response to various stimuli in the same experimental settings [16]. We evaluated the expression of IL-6, IL-1 β , TNF- α and IL-12/23p40 by CD14⁺ monocytes in response to PAMPs. We observed that for LPS, Pam3CSK4 and Zymosan, the proportion of monocytes expressing each of these cytokines was consistently decreased in sAH patients compared to the other groups. For R848, the proportions of IL-12/23p40⁺ and to a lesser extent of TNF- α ⁺ and IL-6⁺ cells were decreased but their capacity to produce IL-1 β was maintained. For PGN, we did not observe any statistical differences between the groups (Fig. 4a). There were no major differences in the ability of monocytes to respond to PAMP stimulation between sAH patients with or without ACLF (Fig. S2b). Furthermore, cDCs had a similar response profile to monocytes (Fig. S4a). These experiments demonstrate that the ability of monocytes and cDCs to mount an appropriate pro-inflammatory response is severely impaired in sAH patients upon stimulation with PAMPs associated with gram-negative bacteria (LPS, Pam3CSK4) or fungal pathogens (Zymosan). Yet, these cells are still able to either partially or globally respond to respectively endosomal (R848) or other bacterial (PGN via NOD2) PAMPs.

To visualize how all the different immune parameters globally vary across the clinical groups we used t-SNE, an analytical method that compares biological samples without taking into account sample classification. This analysis shows an approximate 3-way separation between the CAA/HC (cluster A), cirrhotic patients (cluster B) and the sAH group (cluster C), thus showing that immunological features of sAH patients are sufficient to distinguish them from other groups in an unsupervised manner (Fig. S3a). Next, using two different metrics, we identified the top ten immunological parameters that can discriminate these three

groups (Fig. S3b). They encompass predominantly the capacity of monocytes and cDCs to produce cytokines.

ALD patients with poor polyfunctionality are at higher risk of infection and death

Based on the global impairment of circulating phagocytes to respond to stimulation by various PAMPs, we hypothesized that the ability of a single cell to produce more than one cytokine simultaneously (i.e. polyfunctionality) could represent an important parameter to define the immune status of ALD patients. This approach revealed that monocytes from sAH patients were deficient in their capacity to produce 3 or 4 cytokines (Fig. 4b). We also performed these analyses on cDCs and reached similar conclusion (Fig. S4b). Based on these data for our 34 cirrhotic patients, we used an unsupervised approach to define whether this polyfunctionality parameter was correlated with clinical outcomes. Using a 2-dimension reduction, we identified a cluster of patients with higher risk of infection (Fig. 4c) or mortality (Fig. 4f) within respectively, 28 or 90 days after sampling. We generated a radar plot of the most discriminatory variables for the infectious and mortality outcome. As shown in Fig. 4d & 4g, a low degree of polyfunctionality is significantly associated with a higher risk of infection in the next 28 days or death in the next 90 days. Of note, the infection rate in the follow-up was not associated with the administration of corticosteroids (Table S1) and the ability to predict the occurrence of an infection was better, although not significant, using polyfunctionality than the most discriminative immunological parameters (Fig. 4e). In conclusion, low polyfunctionality of monocytes and cDCs can be used as a predictor of higher risk of infection or mortality during follow-up in patients with ALD.

Monocytes from sAH patients display an altered transcriptomic profile characterized by immunosuppressive features

To gain further insight into the molecular features of CD14⁺ monocytes in sAH, we performed global transcriptional profiling on a subset of patients. We observed a clear separation between samples from sAH and HC upon principal component analysis (PCA) (Fig. 5a) and identified statistically differentially expressed genes (610 up- and 111 downregulated genes compared to HC, with a fold change > 2 and a false discovery rate <0.05, Fig. 5b). Consistent with their altered phenotype and functional response, we observed decreased expression of genes related to key immune pathways such as innate immune responses, cytokines, response to IFNs and antigenic presentation in monocytes from sAH patients (Fig. 5c&d). Gene set enrichment analysis (GSEA) also revealed dysregulated expression of genes involved in key metabolic processes, including lipid metabolism, cellular respiration and translation (Fig. 5c). This was accompanied by increased expression of genes involved in cell homeostasis and ion transport (Fig. 5e). Furthermore, we confirmed previous results showing increased expression of *MERTK* [7]. Its ligand *GAS6*, was also strongly upregulated in patients. High expression of *THBS1* (encoding Thrombospondin 1), a multifunctional extracellular matrix protein, could contribute to immune-suppression and liver fibrosis as it controls latent TGF- β activation [17]. Expression of several semaphorins (*SEMA6B*, *SEMA4C*, *SEMA3F*) was also elevated in patients. These proteins play multiple roles in the control of cell migration, inflammation and angiogenesis [18]. Furthermore, important intracellular immunomodulatory proteins were also upregulated such as *JAK3* and genes encoding the transcription factors *PPARG* and *MAF* (Fig. 5f). *JAK3* was shown to dampen inflammatory cytokine production by human monocytes upon TLR stimulation [19]. Along the same line, treatment with PPAR- γ agonist reduces TLR-dependent stimulation of

DCs [20] and c-Maf is an important immunoregulatory factor that promotes IL-10 expression by macrophages [21].

Recently, *Korf et al.* also provided transcriptomic data for monocytes from ACLF patients [9]. We observed that genes that were up- or down-regulated in ACLF patients in their study were enriched in monocytes from sAH or HC individuals, respectively (Fig. 5g). This indicates that a core molecular signature could be identified in independent cohorts. We also analyzed publicly available gene sets from blood monocytes isolated during gram-negative sepsis or its resolution [22]. Inflammatory genes that were upregulated during sepsis tended to be downregulated in sAH patients compared to HC, indicating that monocytes during ALD do not display similarities with septic patients in the acute phase. However, genes that were modulated in the recovery phase (i.e. several weeks after the sepsis) were significantly up- or down-regulated in our patients, indicating potential common underlying mechanisms between these two clinical situations associated with heightened sensitivity to infections. Next, we evaluated the potential role of different circulating mediators in inducing the transcriptomic changes observed in our patients. Monocytes exposed to LPS display altered capacity to respond to rechallenge (the classical model of endotoxin tolerance) [23]. A similar process occurs when cells are primed by TNF α [24]. We observed that genes that were downregulated 24h after LPS or TNF α stimulation were significantly decreased in monocytes from sAH individuals. However, genes induced in these conditions were not enriched in patients, thus showing that LPS or TNF α cannot alone recapitulate the transcriptomic signature observed in sAH patients. No enrichment was observed for gene sets from β -glucan-trained monocytes; a situation that leads to increased responsiveness upon rechallenge [23]. IFN γ priming also leads to functional reprogramming of monocytes [25]. Here we observed that IFN γ -induced genes were significantly decreased in our patients. Immunosuppressive mediators such as ATP, PGE2 and IL-10 have a major impact on

myeloid cell functional state [26, 27]. Although PGE2 was shown to be increased in these patients [26], the gene signature it induces in myeloid cells was not significantly modified in our dataset. We reached similar conclusions with ATP-responsive genes. Yet, genes that are up- or down-regulated upon IL-10 treatment were significantly modulated in sAH samples.

Altogether, our data indicate that CD14⁺ monocytes from sAH patients display an altered profile with downregulation of key innate immune and metabolic pathways and upregulation of important immunomodulatory factors that could account for their altered responsiveness to PAMPs. This unique profile displays striking similarities with monocytes from patients that recovered from sepsis and is compatible with the effect of immunosuppressive factors such as IL-10.

Monocytes from sAH patients display altered patterns of chromatin accessibility

The functional status of monocytes is highly plastic in response to environmental cues. In the context of endotoxin tolerance, trained immunity or cytokine priming, epigenetic reprogramming has emerged as a critical determinant [23, 24]. To further determine whether similar underlying molecular processes were at play in the context of sAH, we analyzed the epigenomic landscapes of these monocytes by ATAC-seq. We observed extensive modifications in monocytes from sAH patients (Fig. 6a). We focused on the 4316 differentially accessible regions that allow us to segregate sAH vs HC samples in PCA (Fig. 6b). We then scanned for binding motifs at the center of ATAC peaks located in these enhancer regions. Analysis of putative TF site enrichment in sAH vs HC-specific enhancers indicated a significant enrichment for distinct motifs in both groups. For example, there was a clear overrepresentation of AP1 (Jun/Fos), CEBP and MAF binding sites in the sAH group (Fig 6c&d). In contrast, NF-κB, STATs or IRFs motifs were preferentially identified in enhancer regions that were less accessible in this group (Fig. 6c&e). Regulatory regions that

were more or less accessible were clearly associated with genes that were up or down-regulated in monocytes from sAH, respectively (Fig. 6f&g). This observation strongly suggests that epigenetic imprinting is responsible for sAH transcriptional signature. This approach also allowed us to infer genes that are potentially directly regulated by these differentially active elements (Fig. 6h). For example, less accessible regions were found in the loci of HLA genes, while more accessible regions were associated with important immunoregulatory genes such as *MERTK*, *GAS6* or *PPARG*. (Fig. 6i).

Consistent with our transcriptomic data, we observed that regulatory regions that are less accessible in monocytes from sAH patients were associated with genes involved in antigen presentation, innate immune response and cytokine secretion (Fig. 6j, Fig. S5a). Regions that were more accessible in this group were associated with genes that were found to be non-tolerizable upon repeated exposure to LPS. This suggests that, as shown in the context of endotoxin tolerance, some LPS-responsive genes are epigenetically silenced while others are preserved or potentiated. Remarkably, these regulatory regions were also significantly associated with genes encoding components of alcohol, phospholipid and sterol metabolic pathways (Fig. 6k, Fig. S5b). Taken together, these data support the notion that the altered transcriptional program and functional properties of monocytes in this pathological context has strong epigenetic determinants.

DISCUSSION

In recent years, exploring immune dysfunction in advanced alcohol-related liver diseases has become of major interest since the principal event leading to acute decompensation of cirrhosis, ACLF and death is bacterial infection. The current paradigm suggests that this susceptibility to infections is driven by the dysfunction of immune circulating cells. We observed that the ability of monocytes and cDCs from sAH patients to produce inflammatory cytokines was severely impaired upon stimulation with PAMPs associated with gram-negative bacteria or fungal pathogens . Of note, IL-8 levels in whole blood cultures were actually increased in sAH patients and stimulation with R848 or PGN was less affected than with other ligands, indicating complex functional changes. Nevertheless, we observed a strong impairment of the IL-12p70/IFN γ /CXCL10 pathway, together with a downregulation of IFN-related genes at the transcriptional level in these patients. Polyfunctionality is recognized as a parameter to assess the quality of T cell responses [28]. Here, we showed that this parameter might also represent a useful marker for the functional status of innate immune cells as low polyfunctionality of monocytes and cDCs in ALD patients was associated with a higher risk of infection and mortality.

In our group of patients with sAH, the presence of ACLF did not significantly modify the phenotype and PAMPs-elicited cytokine production of circulating mononuclear phagocytes, suggesting that observed immune alterations are related to the presence of sAH and not ACLF. We did not explore the function of circulating mononuclear phagocytes of patients with ACLF without sAH.

Multiple molecular mechanisms may account for the unique phenotypic and functional features of monocytes and DCs in sAH patients. We confirmed previous findings showing that they display increased expression of the inhibitory receptor MERTK [7]. In addition, we observed important modifications in the expression of genes involved in cellular

respiration and metabolic pathways. This is in line with a previous report indicating that in patients with advanced cirrhosis, altered plasma amino acids levels interfered with the mitochondrial tricarboxylic acid cycle and ATP levels in DCs [29]. Furthermore, pharmacological inhibition of glutamine synthetase could partially restore the function of these cells [9]. In parallel, we observed that this distinct transcriptional status was associated with important changes in chromatin accessibility within enhancer regions, indicating that these cells are also reprogrammed at the epigenetic level. Exposure to IL-10 could contribute to this finding as this cytokine was shown to mediate epigenetic reprogramming leading to the repression of pro-inflammatory gene expression in intestinal macrophages [30]. Several reports indicate increased circulating levels of IL-10 in patients with sAH [31, 32]. Our results suggest that the transcriptional status of monocytes in sAH patients is distinct from the immune paralysis/tolerance state observed during acute sepsis. In contrast, we observed a strong enrichment for genes that are modulated in monocytes isolated several weeks/months after the septic event. This is quite striking as patients that experienced sepsis display a higher susceptibility to secondary infections that can persist for years [33]. It is therefore tempting to speculate that common mechanisms may account for long-term innate immune dysfunction post-sepsis and during ALD progression.

ALD is associated with dysbiosis [34], increased bacterial translocation and release of damage-associated molecular patterns (DAMPs) by dying liver cells. These circulating factors lead to systemic inflammation, which is highest in ACLF patients [35]. Thus, it is likely that chronic exposure to circulating bacterial products, PAMPs and DAMPs, plays a major role in the rewiring of monocytes and cDCs, preventing them from further responding to a new stimulation. Indeed, antibiotic prophylaxis has been shown to reduce the incidence of infections in ACLF patients [36].

In conclusion, we showed a profound epigenetic and transcriptomic reprogramming of circulating monocytes of sAH patients. This altered profile was associated with impaired response to PAMPs and decrease of polyfunctionality. The presence of ACLF did not modify the immune profile of patients with sAH. These immune features were linked to a higher risk of infections and mortality during follow-up. We provide new insights in the molecular basis of immune dysfunction observed in advanced ALD.

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Figure legends

Figure 1: Skewed pattern of PAMPs-elicited cytokine production in whole blood cells from sAH patients

a. Dosage of cytokines and chemokines in the supernatant of whole blood culture after stimulation for 24h in HC (n=12), CAA (n=11), cirrhosis (n=16) and sAH (n=18). *b.* Dosage of Th1-related cytokines after stimulation of whole blood with R848 for 24h.

Tukey box and whiskers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. HC, healthy controls; CAA, chronic alcohol abusers; sAH, severe alcoholic hepatitis. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine or chemokine per group, followed by Dunn's correction for multiple testing.

Figure 2: Alterations of the frequency of circulating mononuclear phagocytes in advanced ALD

a. Cellular composition of whole blood in HC (n=12), CAA (n=11), cirrhosis (n=16) and sAH (n=18). *b.* Cellular composition of monocytes and DCs in whole blood of HC (n=12), CAA (n=11), cirrhosis (n=16) and sAH (n=18). *c.* Gating strategy and representative plots of monocytes and DCs for each group.

Tukey box and whiskers. * $p < 0.05$, ** $p < 0.01$. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine or chemokine per group, followed by Dunn's correction for multiple testing.

Figure 3: Alterations of the phenotype of circulating mononuclear phagocytes in advanced ALD

Median Fluorescence Intensity of surface markers on CD14⁺ monocytes, cDCs or pDCs in unstimulated whole blood of HC (n=12), CAA (n=11), cirrhosis (n=16) and sAH (n=18).

Tukey box and whiskers. *p<0.05, **p<0.01, ***p<0.001. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine or chemokine per group, followed by Dunn's correction for multiple testing.

Figure 4: Cytokine production impairment of CD14⁺ monocytes of sAH patients in response to various PAMPs

a. Flow cytometry analysis of intracellular production of cytokines by CD14⁺ monocytes upon 6h stimulation of whole blood from HC (n=12), CAA (n=11), cirrhosis (n=16) and sAH (n=18) patients. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine/monocytes per group, followed by Dunn's correction for multiple testing. *b.* Measure of the ability of monocytes to produce up to 4 cytokines among TNF- α , IL-6, IL-12p40 and IL-1 β simultaneously (polyfunctionality) upon stimulation with various ligands. Kruskal-Wallis test was performed to examine the statistical differences for the ability to produce 3 or 4 cytokines simultaneously per group, followed by Dunn's correction for multiple testing. *c.* t-SNE plot of polyfunctionality profiles in cirrhosis (n=16) and sAH(n=18). Occurrence of infection within 28 days of follow-up (green = no infection, red = infection). *d.* Radar plot showing the most discriminatory features stratifying patients according to their infection status at 28 days. Axes show the frequency (%) of monocytes or cDCs producing the indicated number of cytokines upon stimulation with LPS or R848. *e.* Histogram with AUROCs values (i.e. area under ROC curves) of the 4 most predictive individual and polyfunctionality markers of occurrence of infection within 28 days after recruitment. *f.* t-SNE plot of polyfunctionality profiles in cirrhosis (n=16) and sAH (n=18).

Occurrence of death within 90 days of follow-up (green = no death, red = death). **g.** Radar plot showing the most discriminatory features stratifying patients according to their mortality status at 90 days. Axes show the frequency (%) of monocytes or cDCs producing the indicated number of cytokines upon stimulation with LPS or PGN.

Boxplots are Tukey box and whiskers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to HC unless specified otherwise.

Figure 5: Transcriptomic profile of monocytes from sAH patients is characterized by immunosuppressive features

a. PCA plot representing the clustering of CD14⁺ monocytes from HC (n=6) and sAH (n=7) based on their transcriptional profile. **b.** Volcano plot showing gene expression changes in monocytes of HC versus sAH (in green: down- or up-regulated genes with a fold change > 2 and FDR < 0.05). **c.** Enrichment maps displaying gene sets significantly downregulated in monocytes of sAH patients compared to HC. **d.** Expression of genes involved in innate immune responses **e.** Enrichment maps displaying gene sets significantly upregulated in monocytes of sAH patients compared to HC. **f.** Expression of immunomodulatory genes in monocytes of HC and sAH patients **g.** BubbleGUM GSEA map established from available gene sets in monocytes or macrophages during various inflammatory settings or from monocytes as defined in **b**. For each gene set, origin of the data set and number of up- (red) and down- (blue) regulated genes are indicated. The panel summarizes the normalized enrichment score (NES) and false discovery rate (FDR) parameters.

Figure 6: Altered patterns of chromatin accessibility displayed in monocytes from sAH patients

a. MA plot of mean ATAC-seq counts per peaks showing the DOR (Differentially Open Regions) of CD14⁺ monocytes of HC (blue) and sAH patients (red) with the indicated number of regions. Histograms indicate the number of opening or closing regions in CD14⁺ monocytes of sAH (n= 8) compared to HC (n=6) at promoters and enhancers. *b.* PCA plot representing the clustering of monocytes of HC and sAH based on DORs. *c.* CiiiDER analysis for putative transcription factors motifs from DOR at enhancers. Transcription factors colored according to their gene coverage P-value and whether they are over (red) or under (blue) represented. The size of each point is proportional to the Log10 p-value. *d, e.* Motif enrichment analysis in more (*d*) or less (*e*) accessible enhancer regions in monocytes of sAH patients using AME. *f, g.* Cumulative distribution plot generated by BETA algorithm showing the predicted activating/repressive function of more (*f*) or less (*g*) accessible enhancer regions in monocytes of sAH patients with the indicated P-values determined by the Kolmogorov-Smirnov test (red: upregulated genes, blue: downregulated genes, dashed line: background) *h.* Expression of the genes associated with significant changes in chromatin accessibility as defined through BETA analysis. *i.* Representative ATAC-seq tracks of CD14⁺ monocytes for each patient group at enhancers of indicated genes. Significant different chromatin accessible regions are highlighted in grey. *j, k.* Selected Gene Ontology pathways enriched in genes associated with more accessible enhancer regions in CD14⁺ monocytes of HC (*j*) or sAH (*k*) using GREAT and presented as -Log10 of binomial FDR q-value.

Table 1: Clinical characteristics of patients

	Healthy controls (n = 12)	Chronic alcohol abusers (n = 11)	Cirrhosis (n = 16)	Severe alcoholic hepatitis (n = 18)	p - value
Age (yrs)	47.5 (41-52)	50 (39-62.5)	62 (59.5-65)	54.5 (43.25-61.75)	p = 0.013
Sex, Male (%)	7 (58%)	8 (72%)	13 (81%)	11 (61%)	p = 0.508
Total bilirubin (mg/dL)	-	-	1.55 (1.1-2.9)	11.25 (7.3-18.4)	p < 0.0001
INR	-	-	1.48 (1.2-1.6)	1.76 (1.56-1.98)	p = 0.0025
Creatinin (mg/dL)	-	-	0.85 (0.8-1.2)	0.95 (0.6-1.2)	p = 0.71
MELD score	-	-	14.5 (11.3-17.3)	24 (22-33)	p < 0.0001
Child-Pugh score	-	-	B8 (A6-B9)	C10 (C10-C11)	p = 0.0016
HVPG (mm Hg)	-	-	16 (13-18)	17 (14-20.5)	p = 0.62
Norfloxacin	-	-	2 (12%)	0	
mDF	-	-	-	61 (48-70)	
ACLF (%) -grades (1,2,3)	-	-	-	7 (39%) – (3, 3, 1)	
CLIF-C ALCF score *	-	-	-	49 (45–50)	
Corticosteroids (28 days after recruitment) **			0	8 (44.4%)	
Infection (28 days)	-	-	3 (19%)	9 (50%)	p = 0.057
Transplant-free mortality (28 days)	-	-	1 (6.7%)	3 (18.8%)	p = 0.31
Infection (90 days)	-	-	5 (31.3%)	11 (61.1%)	p = 0.08
Transplant-free mortality (90 days)			3 (20%)	6 (37.5%)	p = 0.28
Liver transplant (90 days)	-	-	1 (6.3%)	2 (11.1%)	p = 0.11

Data are presented as median (IQR) or number of patients (%)

* Score only calculated for the subgroup of sAH patients with ALCF (n=7)

** Corticosteroids use remains the same at 90 days

Figure 1

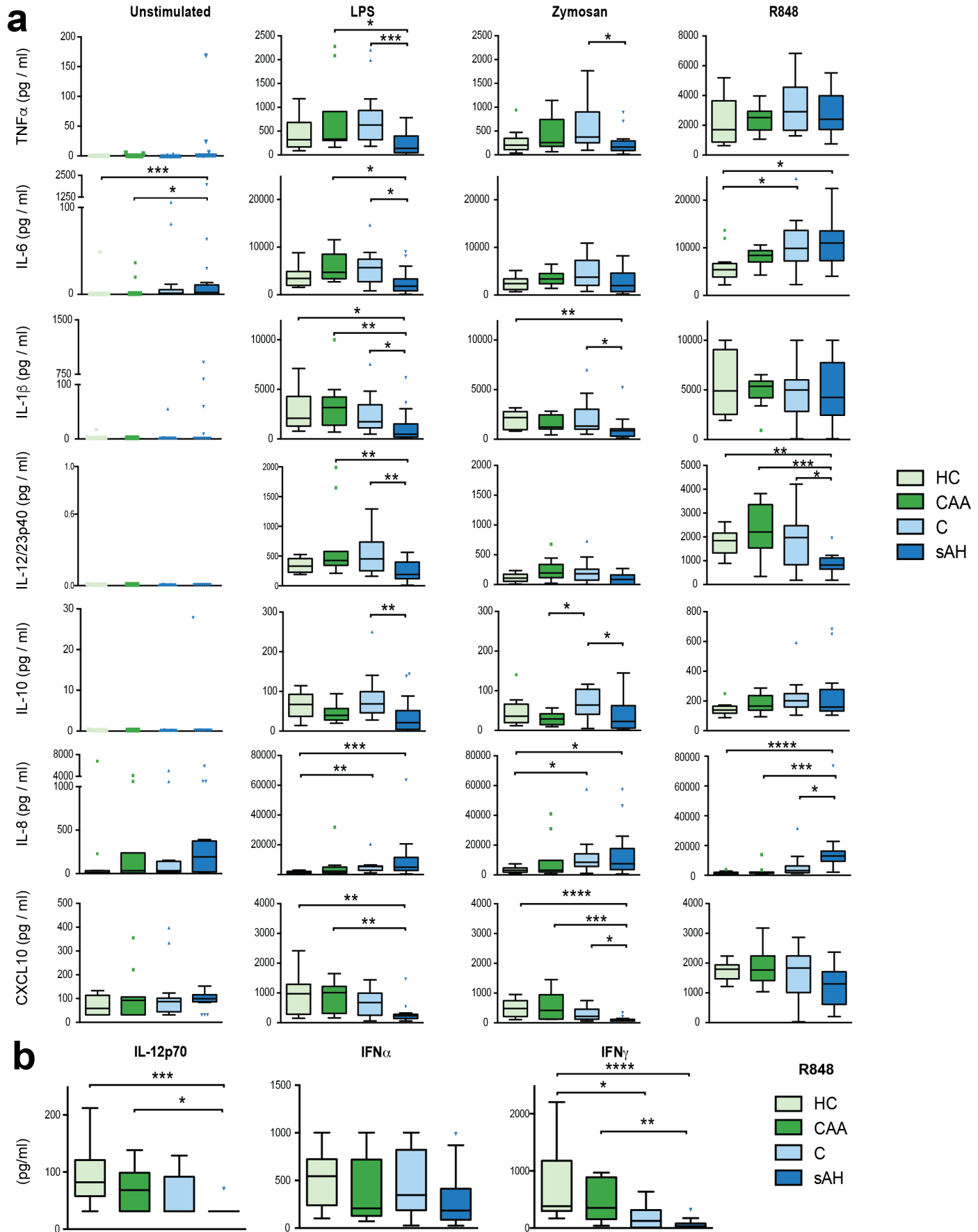


Figure 2

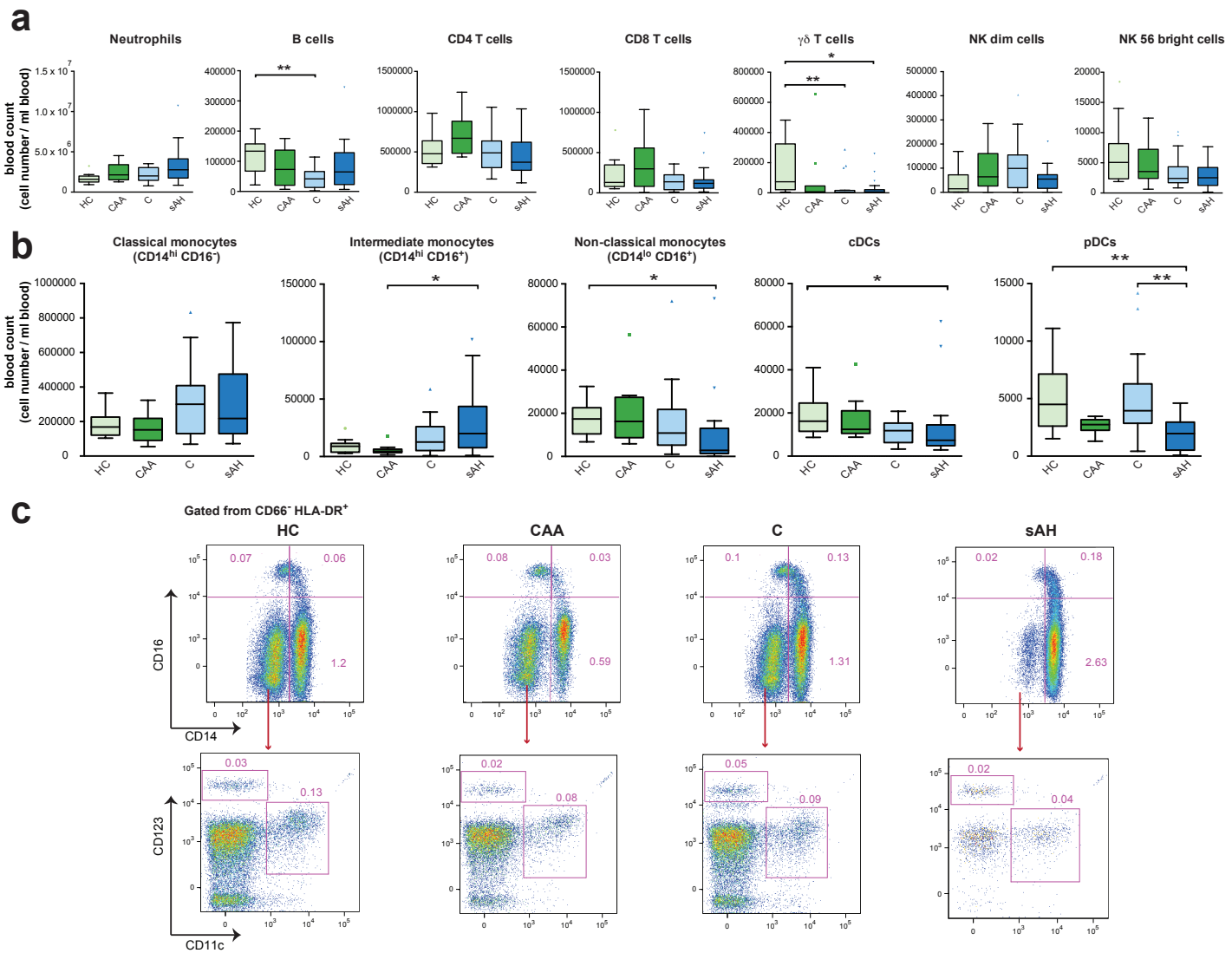


Figure 3

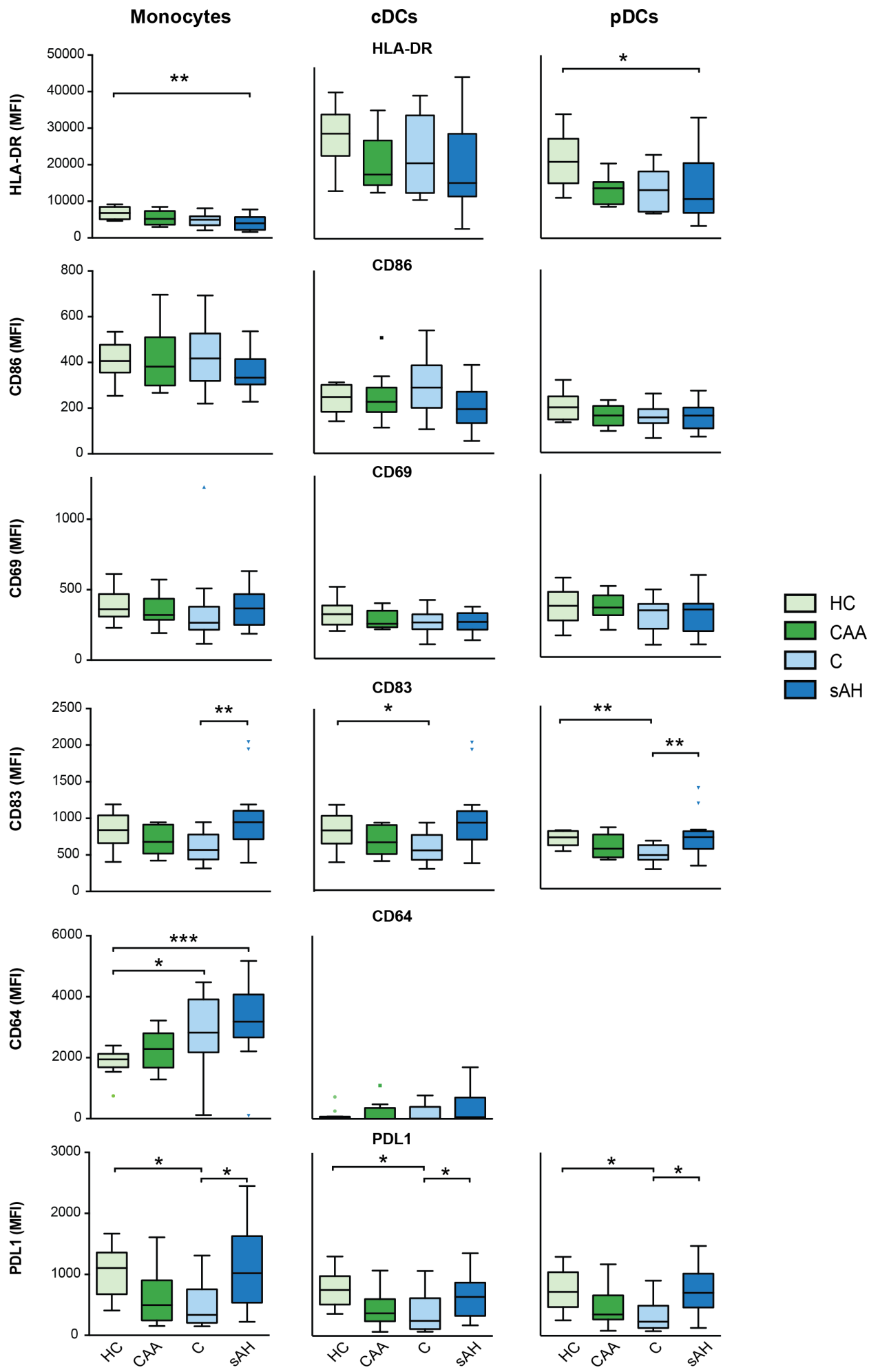


Figure 4

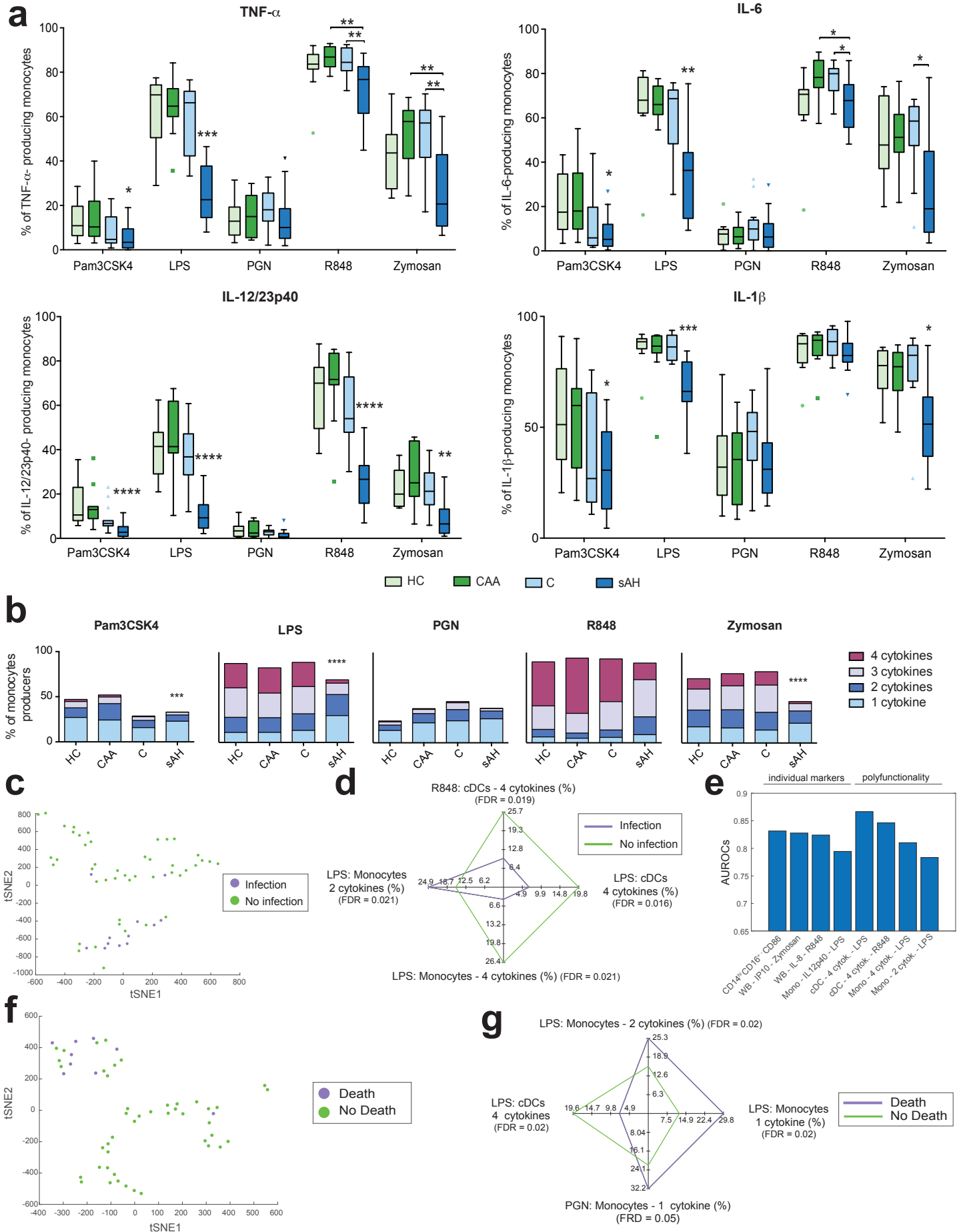


Figure 5

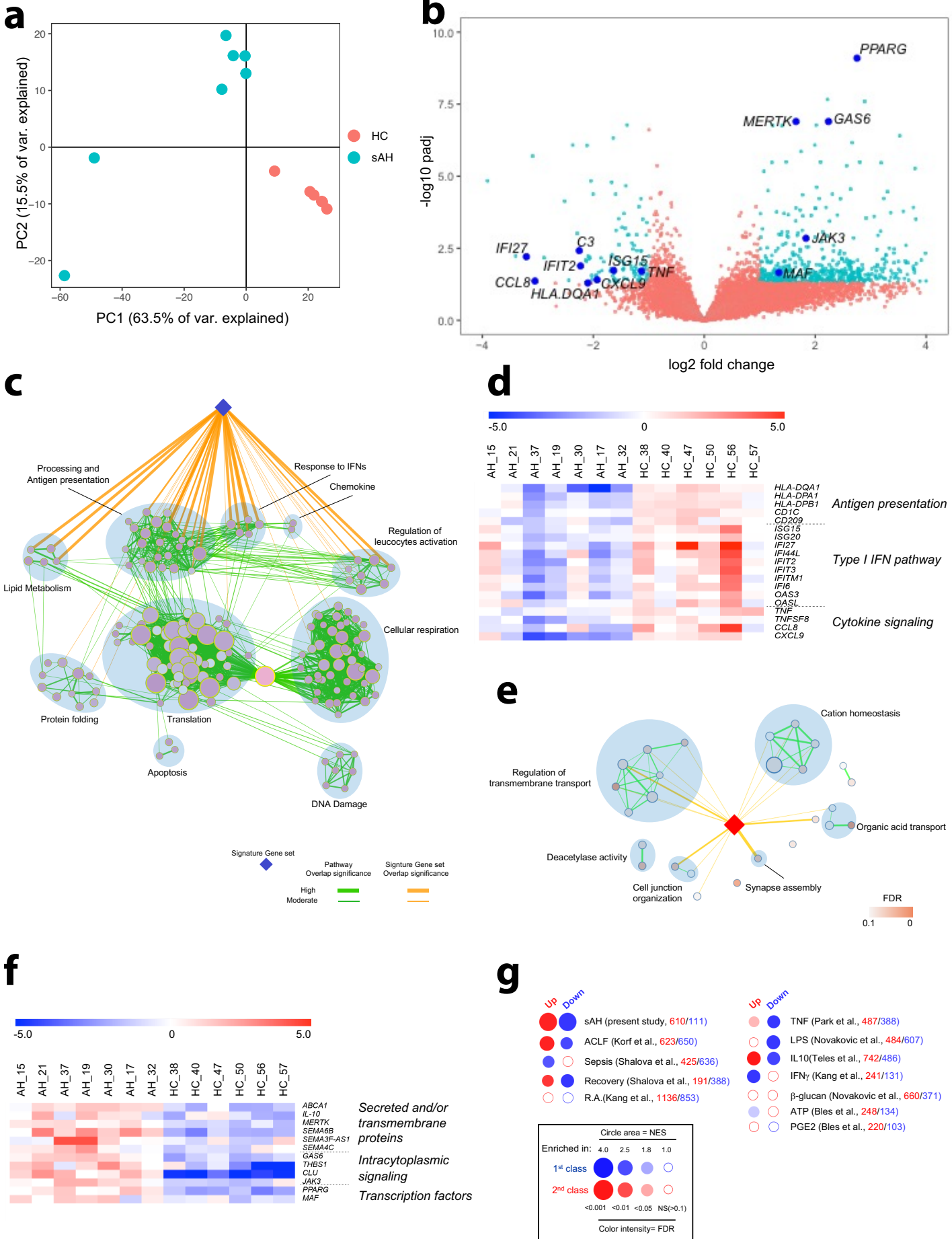
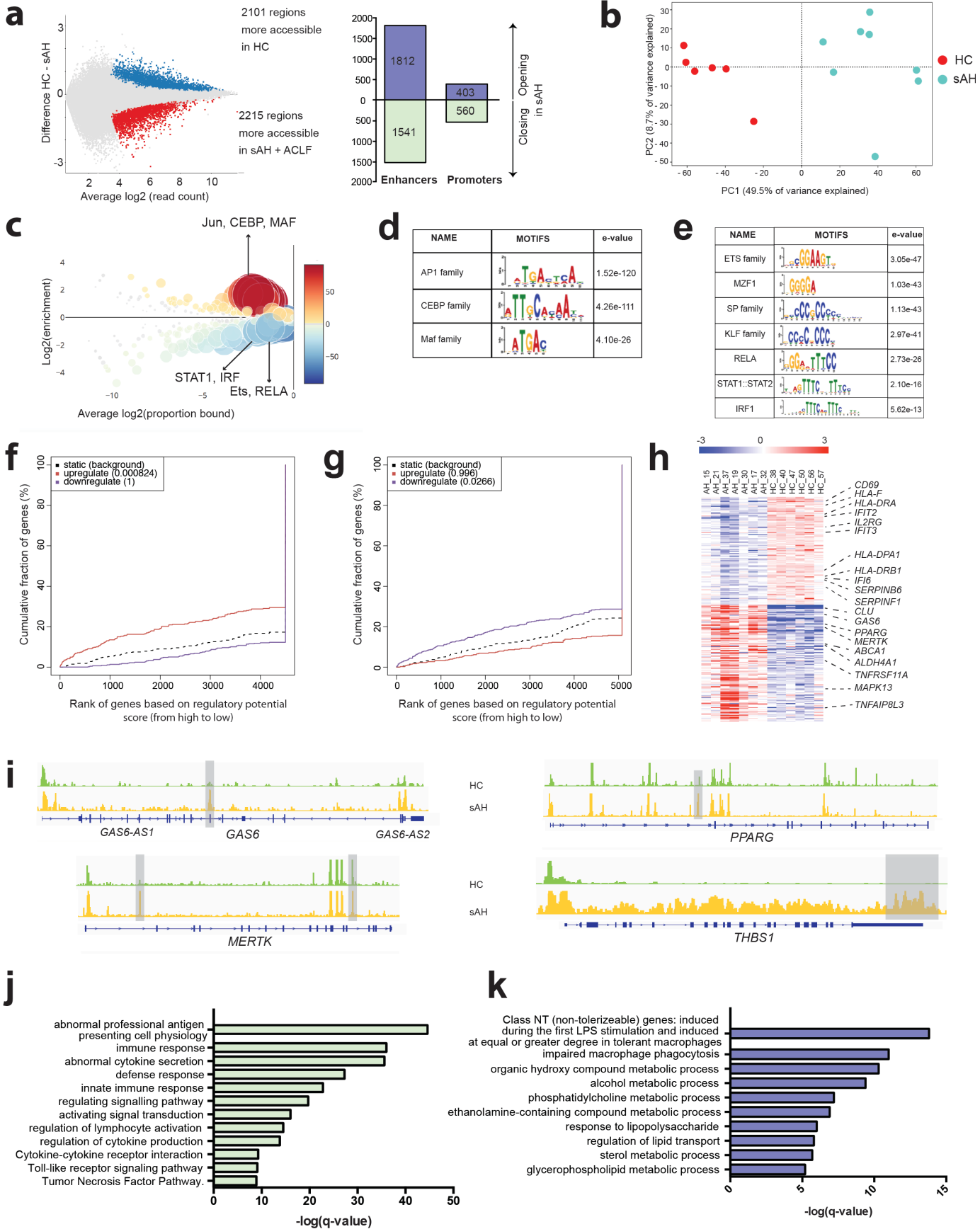
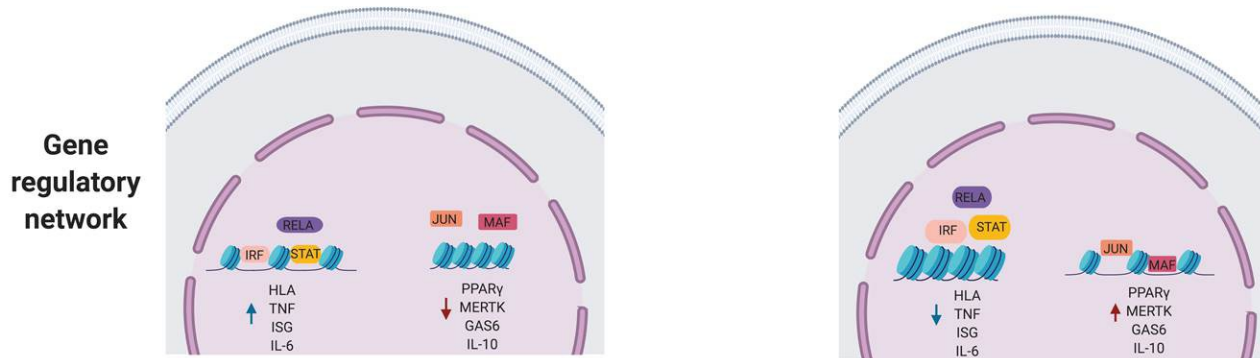
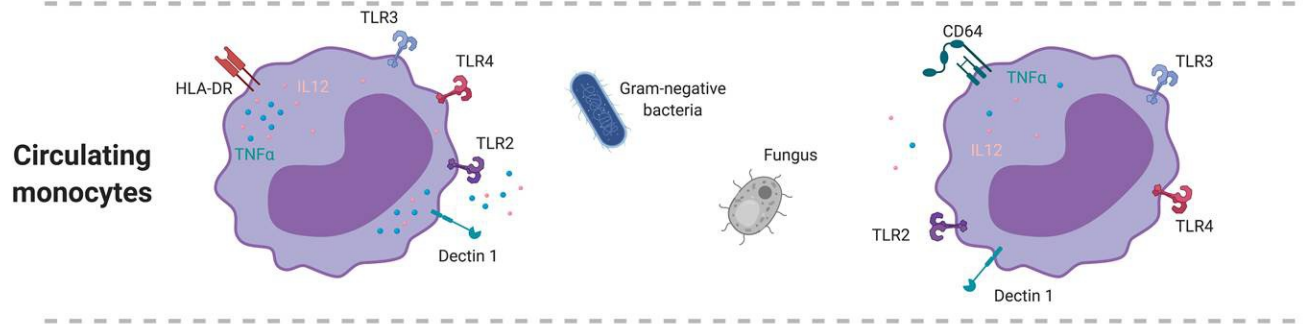
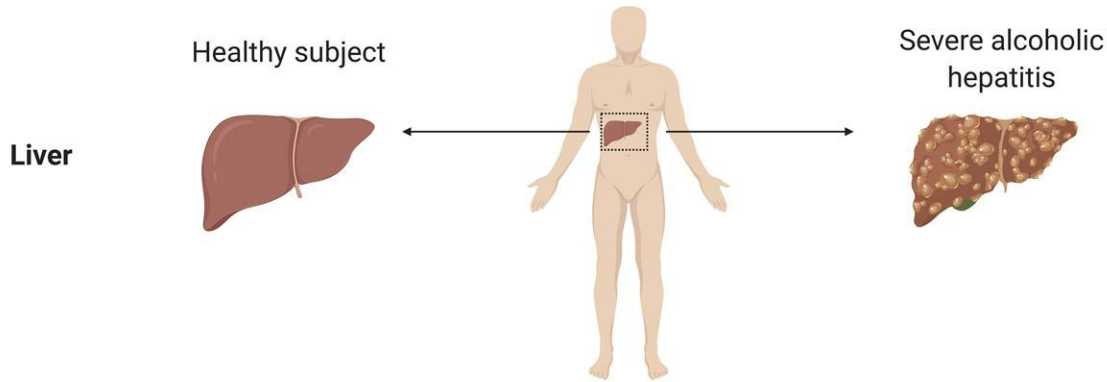


Figure 6





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Increased susceptibility to infections & increased mortality