Transcriptional, Epigenetic, and Functional Reprogramming of Monocytes From Non-Human Primates Following Chronic Alcohol Drinking

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Chronic heavy drinking (CHD) of alcohol is a known risk factor for increased susceptibility to bacterial and viral infection as well as impaired wound healing. Evidence suggests that these defects are mediated by a dysregulated inflammatory response originating from myeloid cells, notably monocytes and macrophages, but the mechanisms remain poorly understood. Our ability to study CHD is impacted by the complexities of human drinking patterns and behavior as well as comorbidities and confounding risk factors for patients with alcohol use disorders. To overcome these challenges, we utilized a translational rhesus macaque model of voluntary ethanol self-administration that closely recapitulates human drinking patterns and chronicity. In this study, we examined the effects of CHD on blood monocytes in control and CHD female macaques after 12 months of daily ethanol consumption. While monocytes from CHD female macaques generated a hyper-inflamatory response to ex vivo LPS stimulation, their response to E. coli was dampened. In depth scRNA-Seq analysis of purified monocytes revealed significant shifts in classical monocyte subsets with accumulation of cells expressing markers of hypoxia (HIF1A) and inflammation (NFkB signaling pathway) in CHD macaques. The increased presence of monocyte subsets skewed towards inflammatory phenotypes was complemented by epigenetic analysis, which revealed higher accessibility of promoter regions that regulate genes involved in cytokine signaling pathways. Collectively, data presented in this manuscript demonstrate that CHD shifts classical monocyte subset composition and primes the monocytes towards a more hyper-inflammatory response to LPS, but compromised pathogen response.

Keywords: alcohol, inflammation, monocytes, nonhuman primates, scRNA seq, ATAC-Seq
INTRODUCTION

Alcohol consumption is widespread in the United States with 85% of individuals ages 18 and older engaging in this behavior. While most of these individuals are considered moderate drinkers, 7% are classified as heavy alcohol users (National Survey on Drug Use and Health, 2015). Chronic heavy alcohol consumption or chronic heavy drinking (CHD) is associated with multiple adverse health effects including increased incidence of cardiac disease (1, 2), certain types of cancer (3–6), liver cirrhosis (7), and sepsis (8), making it the third leading preventable cause of death in the United States (9). CHD is also associated with higher susceptibility to bacterial and viral infections including pneumonia and tuberculosis (10, 11), hepatitis C virus, and HIV (12, 13). Moreover, CHD compromises tissue repair, resulting in reduced post-operative healing and poor trauma recovery outcomes (14, 15). These observations strongly suggest that CHD dysregulates immunity and host defense.

While CHD can modulate the function of many immune cell populations, data from several laboratories indicate that the most dramatic and consistent changes are evident in the innate immune branch, specifically in myeloid cells (monocytes, macrophages, dendritic cells, and neutrophils) (16–18). Monocytes are relatively short-lived phagocytic cells that circulate in the blood, are constantly repopulated from bone marrow progenitors, and can quickly respond to infection or inflammation by extravasation into tissue and differentiation into tissue-resident macrophages (19). A tightly regulated inflammatory response by monocytes is required for effective infection clearance and tissue repair (20).

Alcohol consumption has been shown to disrupt monocyte and macrophage responses in a dose and time-dependent manner (16). Specifically, acute alcohol treatment of purified primary human monocytes, rodent monocyte-derived macrophages, or human mononuclear cell lines results in decreased production of inflammatory mediators including TNFα following stimulation with lipopolysaccharide (LPS), a TLR4 agonist (21–23). In rodents, acute in vivo (24) exposure to ethanol increases production of the anti-inflammatory cytokine IL-10 through activation of STAT3. Similarly, in healthy human subjects, an acute binge of alcohol increased IL-10 and decreased IL-1β production (25). In contrast, prolonged exposure to alcohol results in increased production of pro-inflammatory mediators, notably TNFα in response to LPS or phorbol myristate acetate (PMA)/ionomycin stimulation (22, 26) potentially due to enhanced activation of NFκB and ERK kinases (27). In line with these observations, monocytes from patients with alcoholic liver disease (ALD) as well as tissue resident macrophage populations including Kupffer cells (28), microglia (29), alveolar macrophages (30), and splenic macrophages (17, 31) produce higher levels of TNF-α at resting state as well as in response to LPS (32). The enhanced inflammatory response by tissue-resident myeloid cells in the context of CHD is linked to organ damage, most notably in the liver (33) but also the intestine (34), brain (35, 36), and lungs (37).

Despite the studies described above, our understanding of the mechanisms by which chronic alcohol consumption reprograms circulating monocytes and tissue-resident macrophages remains incomplete. Some studies have suggested that CHD-induced gut “leakiness” and translocation of bacterial products including LPS across the gut barrier into the circulation could lead to chronic activation and subsequently organ damage (34). Whether monocytes in circulation are activated prior to organ damage by circulating bacterial products, ethanol, ethanol metabolites, or a combination of these factors remains unanswered.

Data from clinical studies are confounded by self-reported alcohol intake, the use of nicotine, recreational or illicit drugs, nutritional deficiencies, and presence of organ damage (38). Addressing these questions requires access to a reliable animal model that recapitulates critical aspects of human CHD. Therefore, to better model human CHD and relate immune response of peripheral monocytes to quantified alcohol intakes in the absence of confounders listed above, we leveraged a rhesus macaque model of chronic voluntary ethanol self-administration (39, 40). Using this model, we reported that CHD disrupts the resting transcriptome and results in heightened inflammatory responses by peripheral blood mononuclear cells (PBMC) from male and female macaques (18, 41). Additionally, splenic macrophages from CHD monkeys generate a hyper-inflammatory response following LPS stimulation that is accompanied by increased chromatin accessibility at promoters and intergenic regions that regulate genes important for inflammatory responses (17).

In this study, we examine how CHD alters the ability of blood monocytes to respond properly to stimulation. We show that CHD is associated with increased numbers of circulating monocytes that exhibit a heightened transcriptional and immune mediator response to LPS stimulation, but lower response to bacterial pathogens. To determine the molecular mechanisms of this dysregulated response, we profiled the transcriptome of the monocytes by single cell RNA-Seq and their epigenetic landscape by ATAC-Seq. Our results indicate that CHD significantly alters the epigenetic and transcriptional profiles of circulating monocytes, priming them towards an inflammatory state but altering their effector function to towards pathogens.

METHODS AND MATERIALS

Animal Studies and Sample Collection

These studies used samples from a non-human primate model of voluntary ethanol self-administration established through schedule-induced polydipsia (39, 40, 42). Briefly, in this model, rhesus macaques are introduced to a 4% w/v ethanol solution during a 90-day induction period followed by concurrent access to the 4% w/v solution and water for 22 hours/day for one year. During this time, the macaques adopt a stable drinking phenotype defined by the amount of ethanol consumed per day and the pattern of ethanol consumption (g/kg/day) (39). Blood samples were taken from the saphenous vein every 5–7 days at 7 hrs after the onset of the 22 hr/day access to ethanol and
assayed by headspace gas chromatography for blood ethanol concentrations (BECs).

For these studies, blood samples were collected from 9 female and 3 male rhesus macaques (average age 5.68 yrs), with 5 animals serving as controls and 8 classified as chronic heavy drinkers (CHD) based on 12 months of ethanol self-administration (tissue and drinking data obtained from the Monkey Alcohol Tissue Research Resource: www.mattr.com). These cohorts of animals (Cohorts 6 and 7a on matrr.com) were described in two previous studies of innate immune system response to alcohol (18, 41). Peripheral Blood Mononuclear Cells (PBMC) were isolated by centrifugation over histopaque (Sigma, St Louis, MO) as per manufacturer’s protocol and cryopreserved until they could be analyzed as a batch. The average daily ethanol intake for each animal is outlined in Supplementary Table 1. CHD animals did not show signs of overt liver damage as assessed by transaminase (AST) enzyme levels [Supplementary Figure 2G and (43)].

LAL and IgM Assays
Endotoxin-core antibodies in plasma samples were measured using an enzyme-immunoassay technique (ELISA) after 12 months of alcohol consumption using EndoCab IgM ELISA kit (Hycult Biotech, Catalog# HK504-IgM). Plasma samples were diluted 50x.

Circulating endotoxin was measured from plasma using a Limulus amebocyte lysate (LAL) assay (Hycult Biotech) following the manufacturers protocol.

Flow Cytometry Analysis
1×10⁶ freshly thawed PBMC or 1×10⁵ purified CD14+ monocytes were cultured in RPMI supplemented with 10% FBS with or without 100 ng/mL LPS (TLR4 ligand, E. coli 055:B5; Invivogen, San Diego CA) for 16 or 6 hours, respectively, in 96-well tissue culture plates at 37°C in a 5% CO₂ environment. 3.5×10⁴ purified CD14+ monocytes were cultured in RPMI supplemented with 10% FBS with or without 6×10⁵ cfu/mL heat-killed E. coli (Escherichia coli (Migula) Castellani and Chalmers ATCC 11775) for 16 hours in 96-well tissue culture plates at 37°C in a 5% CO₂ environment. Plates were spun down: supernatants were used to measure production of immune mediators and cell pellets were resuspended in Qiазol (Qiagen, Valencia CA) for RNA extraction. Both cells and supernatants were stored at -80°C until they could be processed as a batch.

Luminex Assay
Immune mediators in the supernatants from PBMC or purified monocytes stimulated with LPS were measured using a 30-plex panel measuring levels of cytokines (IFNγ, IL-1b, IL-2, IL4, IL-6, IL-7, IL-12, IL-15, IL-17, IL-18, IL-23, TNFα, IL-1RA, IFN-b, and IL-10), chemokines (MCP-1, MIP-1a, MIP-1b, Eotaxin, IL-8, MIG, I-TAC, BCA-1, IP-10, and SDF-1a), and other factors (PD-L1, PDGF-BB, S100B, GM-CSF, and VEGF-A) validated for NHP (R&D Systems, Minneapolis, MN, USA). Standard curves were generated using 5-parameter logistic regression using the xPONENT™ software provided with the MAGPIX instrument (Luminex, Austin TX).

Immune mediators in the supernatants from monocytes stimulated with E. coli were measured using a more sensitive ProcartaPlex 31-plex panel measuring levels of cytokines (IFNγ, IFNβ, IL-10, IL-12p70, IL-15, IL-17A, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, MIF, and TNFα), chemokines (BLC(CXCL13), Eotaxin (CCL11), I-TAC(CXCL11), IL-8(CXCL8), IP-10(CXCL10), MCP-1(CCL2), MIG(CXCL9), MIP-1a(CCL3), MIP-1b(CCL4)), growth factors (BDNF, G-CSF, GM-CSF, PDGF-BB, VEGF-A) and other factors (CD40L, Granzyme B) (Invitrogen, Carlsbad, CA). Differences in induction of proteins post stimulation were tested using unpaired t-tests with Welch’s correction. Dose-dependent responses were modeled based on gg/day ethanol consumed and tested for linear fit regression analysis in Prism (GraphPad, San Diego CA). Raw data included in Supplementary Table 2.

RNA Isolation and Library Preparation
Total RNA was isolated from PBMC or purified CD14+ monocytes from female macaques using the mRNeasy kit (Qiagen, Valencia CA) following manufacturer instructions and quality assessed using Agilent 2100 Bioanalyzer. Libraries from PBMC RNA were generated using the TruSeq Stranded RNA LT kit (Illumina, San Diego, CA, USA). Libraries from purified CD14+ monocytes RNA were generated using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, Ipswitch, MA, USA), which allows for lower input concentrations of RNA (10ng). For both library prep kits, rRNA depleated RNA was fragmented, converted to double-stranded cDNA and ligated to adapters. The roughly 300bp-
long fragments were then amplified by PCR and selected by size exclusion. Libraries were multiplexed and following quality control for size, quality, and concentrations, were sequenced to an average depth of 20 million 100bp reads on the HiSeq 4000 platform.

**Bulk RNA-Seq Data Analysis**

RNA-Seq reads were quality checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), adapter and quality trimmed using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), retaining reads at least 35bp long. Reads were aligned to *Macaca mulatta* genome (Mmul_8.0.1) based on annotations available on ENSEMBL (Mmul_8.0.1.92) using TopHat (44) internally running Bowtie2 (45). Aligned reads were counted gene-wise using GenomicRanges (46), counting reads in a strand-specific manner. Genes with low read counts (average <5) and non-protein coding genes were filtered out before differential gene expression analyses. Read counts were normalized using RPKM method for generation of PCA and heatmaps. Raw counts were used to test for differentially expressed genes (DEG) using edgeR (47), defining DEG as ones with at least two-fold up or down regulation and an FDR controlled at 5%. Functional enrichment of gene expression changes in resting and LPS-stimulated cells was performed using Metascape (48) and DAVID (49). Networks of functional enrichment terms were generated using Metascape and visualized in Cytoscape (50). Transcription factors that regulate expression of DEG were predicted using the ChEA3 (51) tool using ENSEMBL ChIP database.

**10X scRNA-Seq Data Analysis**

PBMC from control (n=3) and CHD (n=3) female macaques were thawed and stained with anti-CD14, HLA-DR antibodies and sorted for live CD14+/HLA-DR+ cells on a BD FACSaria Fusion on the same day. Sorted monocytes were pooled and resuspended at a concentration of 1200 cells/ul and loaded into the 10X Chromium gem aiming for an estimated 10,000 cells per sample. cDNA amplification and library preparation (10X v3 chemistry) were performed according to manufacturer protocol and sequenced on a NovaSeq S4 (Illumina) to a depth of >50,000 reads/cell.

Sequencing reads were aligned to the Mmul_8.0.1 reference genome using cellranger v3.1 (52) (10X Genomics). Quality control steps were performed prior to downstream analysis with Seurat, filtering out cells with fewer than 200 unique features and cells with greater than 20% mitochondrial content. Control and CHD datasets were down sampled to 4680 cells each and integrated in Seurat (53) using the *IntegrateData* function. Data normalization and variance stabilization were performed, correcting for differential effects of mitochondrial and cell cycle gene expression levels. Clustering was performed using the first 20 principal components. Small clusters with an over-representation of B and T cell gene expression were removed for downstream analysis. Clusters were visualized using uniform manifold approximation and projection (UMAP) and further characterized into distinct monocyte subsets using the *FindMarkers* function (Supplementary Table 3).

**Pseudo-Temporal Analysis**

Pseudo-time trajectory monocytes was reconstructed using Slingshot (54). The UMAP dimensional reduction performed in Seurat was used as the input for Slingshot. For calculation of the lineages and pseudotime, the most abundant classical monocyte cluster, MS1, was set as the root state.

**Differential Expression Analyses**

Differential expression analysis (CHD to Control) was performed using DESeq under default settings in Seurat. Only statistically significant genes (Fold change cutoff ≥ 1.2; adjusted p-value ≤ 0.05) were included in downstream analysis.

**Module Scoring and Functional Enrichment**

For gene scoring analysis, we compared gene signatures and pathways from KEGG (https://www.genome.jp/kegg/pathway.html) (Supplementary Table 4) in the monocytes using Seurat’s *AddModuleScore* function. Over representative gene ontologies were identified using 1-way, 2-way or 4-way enrichment of differential signatures using Metascape (48). All plots were generated using ggplot2 and Seurat.

**ATAC-Seq Library Preparation**

Following the Omni-ATAC protocol, 2x10⁴ purified monocytes from female macaques were lysed in lysis buffer (10mM Tris-HCl (pH 7.4), 10mM NaCl, 3mM MgCl₂, 10% NP-40, 10% Tween, and 1% Digitonin) on ice for 3 minutes (55). Immediately after lysis, nuclei were spun at 500 g for 10 minutes at 4°C to remove supernatant. Nuclei were then incubated with Tn5 transposase for 30 minutes at 37°C. Tagmented DNA was purified using AMPure XP beads (Beckman Coulter, Brea, CA) and PCR was performed to amplify the library under the following conditions: 72°C for 5 min; 98 for 30s; 5 cycles of 98°C for 10s, 63C for 30s, and 72°C for 1min; hold at 4C. Libraries were then purified with warm AMPure XP beads and quantified on a Bioanalyzer (Agilent Technologies, Santa Clara CA). Libraries were multiplexed and sequenced to a depth of 50 million 100bp paired reads on a NextSeq (Illumina).

**ATAC-Seq Data Analysis**

Paired ended reads from sequencing were quality checked using FastQC and trimmed to a quality threshold of 20 and minimum read length 50. Trimmed reads were aligned to the Macaca Mulatta genome (Mmul_8.0.1) using Bowtie2 (-X 2000 -k 1 –very-sensitive –no-discordant –no-mixed). Reads aligning to mitochondrial genome were removed using Samtools and PCR duplicate artifacts were removed using Picard. Samples from each group were concatenated to achieve greater than 5x10⁶ non-duplicate, non-mitochondrial reads per group.

Accessible chromatin peaks were called using Homer’s *findPeaks* function (56) (FDR<0.05) and differential peak analysis was performed using Homer’s *getDifferentialPeaks* function (P < 0.05). Genomic annotation of open chromatin regions in monocytes and differentially accessible regions (DAR) with CHD was assigned using ChIPseeker (57). Promoters were defined as -1000bp to +100bp around the transcriptional start
site (TSS). Functional enrichment of open promoter regions was performed using Metascape (http://metascape.org).

Due to the lack of available macaque annotation databases, distal intergenic regions from the macaque assembly were converted to the human genome (hg19) coordinates using the UCSC liftOver tool. Cis-Regulatory roles of these putative enhancer regions were identified using GREAT (http://great.stanford.edu/public/html/). Transcription factor motif analysis was performed using Homer’s findMotifs function with default parameters. Promoter regions for every annotated macaque gene were defined in ChIPSeeker as -1000bp to +100bp relative to the TSS. A counts matrix was generated for these regions using featureCounts (58), where pooled bam files for each group were normalized to total numbers of mapped reads.

Statistical Analysis
All statistical analyses were conducted in Prism 7(GraphPad). Data sets were first tested for normality. Two group comparisons were carried out an unpaired t-test with Welch’s correction. Differences between 4 groups were tested using one-way ANOVA (α=0.05) followed by Holm Sidak’s multiple comparisons tests. Error bars for all graphs are defined as ±SEM. Linear regression analysis compared significant shifts in curve over horizontal line, with spearman correlation coefficient reported. Statistical significance of functional enrichment was defined using hypergeometric tests. P-values less than or equal to 0.05 were considered statistically significant. Values between 0.05 and 0.1 are reported as trending patterns.

RESULTS
Chronic Heavy Drinking Results in Enhanced Responses to LPS by PBMC
Recent bulk RNA seq analysis of resting PBMC obtained from female rhesus macaques after 12 months of chronic heavy drinking (CHD) indicated that most of the differential gene expression originated from innate immune cells [monocytes and dendritic cells (DCs)] (18). Moreover, studies using PBMC from male macaques showed increased responses to ex vivo LPS stimulation after 12 months of chronic heavy ethanol consumption (41). To assess if CHD also led to changes in inflammatory responses by circulating innate immune cells in female macaques, PBMC obtained from CHD (n=6) and control (n=3) females were stimulated ex vivo with LPS for 16 hours followed by measurement of immune mediator production by Luminex assay and transcriptional changes by bulk RNA Seq (Supplementary Figure 1A). LPS stimulation resulted in a robust inflammatory response (TNFα, IL-6, IL-18, IL-4, GMCSF and S100B) by PBMC from both CHD and control animals (Figure 1A). However, PBMC from CHD animals produced significantly elevated levels of additional inflammatory mediators notably IL-1β, IL-12, IFNγ, CCL3, and CCL4 compared to their unstimulated condition (Figure 1A). Moreover, production of inflammatory cytokines IL-6, IL-23 and to lesser extent soluble PD-L1 was higher by CHD PBMC following LPS stimulation compared to controls (Figure 1A). In addition, levels of TNFα, CCL4, IL-6, IL-15, IL-23, and sPD-L1 were positively correlated with ethanol dose (Figure 1B and Supplementary Figure 1B).

We next assessed transcriptional differences in response to LPS between the two groups. Differential analysis indicated a heightened response to LPS by CHD PBMC relative to control PBMC (Figure 1C). While signatures of immune activation were observed in both groups following LPS stimulation, DEGs upregulated in PBMC from controls enriched more significantly to pathways involved in antiviral immunity (“response to IFNγ” and “response to virus”), whereas DEGs upregulated in PBMC from CHD animals enriched more significantly to leukocyte activation and inflammation pathways (Figure 1D and Supplementary Figure 1C). Enrichment of the downregulated genes shows significant enrichment to GO terms associated with chemotaxis, leukocyte differentiation and metabolism in the PBMC from control animals (Figure 1E and Supplementary Figure 1D). These findings indicate disruption of innate immune responses with CHD and further highlight that the heightened inflammatory response to LPS by PBMC is not restricted to male macaques.

Chronic Heavy Drinking Impacts Monocyte Frequencies and Functional Responses to LPS Stimulation
To measure changes in frequencies and phenotypes of monocyte subsets with CHD in female macaques, we profiled PBMC by flow cytometry. Interestingly, while the total white blood cell numbers were unchanged with CHD (18), the proportions of monocytes within PBMC from CHD macaques were significantly elevated (Figure 2A). However, the relative distribution of the monocyte sub-populations were comparable between the groups (Supplementary Figures 2A, B). We profiled several TLRs, chemokine, and activation receptors on monocytes by flow cytometry, but found no significant differences (Supplementary Figure 2C). To assess whether the heightened inflammatory response detected in PBMC was due to increased numbers of monocytes or cell-intrinsic changes caused by CHD, monocytes were purified from each group and subjected to bulk RNA-Seq at resting state and after LPS stimulation (Supplementary Figure 1A). A modest number of DEG were detected at resting state (58 upregulated and 112 downregulated) between CHD and control monocytes (Figure 2B). The DEG upregulated with CHD enriched to pathways associated with inflammatory response such as “pattern recognition receptor activity” (e.g. NLRP3) and “cytokine production” (e.g. FCN1) (Figure 2C, and Supplementary Figure 2D). DEG downregulated with CHD mapped to gene ontology (GO) terms “activation of immune response” (e.g. IFNG) “lymphocyte proliferation” (e.g. HSPD1) and “chemotaxis” (e.g. CCR7) (Figure 2C). One potential mechanism for this enhanced monocyte transcriptional activation at resting state could be increased microbial products in circulation due to altered barriers (31). Therefore, we measured circulating levels of bacterial endotoxin (LAL) and...
IgM bound endotoxin. We found slightly increased levels of circulating LAL with CHD, but no changes in IgM-bound endotoxin (Supplementary Figures 2E, F). Additionally, levels of alanine transaminase (ALT) and aspartate transaminase (AST) levels, both of which are indicators of liver damage, were also comparable between the two groups (Supplementary Figure 2G).

Secreted levels of cytokines, chemokines, and growth factors were measured after 6-hour incubation in the presence or absence of LPS by Luminex assay. No significant differences in immune mediators’ secretion were noted in the non-stimulated conditions. As observed for PBMC, significantly enhanced production of pro-inflammatory cytokines TNFα, IL-6, and IL-15, chemokines CCL4, and CXCL11, and to a lesser extent IL-4 and IL-7 by CHD monocytes were noted (Figure 2D and Supplementary Figure 3A). The LPS response was also assessed at the transcriptional level using RNA-Seq. Principal component analysis (PCA) showed that stimulation accounts for the bulk of the transcriptional changes (PC1, 75% of the differences) (Supplementary Figure 3B) with 357 and 262 DEG between CHD and controls in the NS, and LPS conditions, respectively (Supplementary Figures 3C, D). The DEG from these comparisons showed that genes associated with myeloid inflammatory pathways were upregulated (TNFSF21, MMP2, TLR2, MMP1) while genes associated with adaptive immune activation were downregulated (IL21R, CD40, MAMU-DOA, CCR6) in CHD compared to control monocytes (Supplementary Figures 3C, D).

We then identified DEG in the LPS relative to NS condition for each group. A greater number of DEG was detected in the CHD group, with a large overlap between the two groups, and few DEG detected solely in the control group (Figure 2E). As expected, DEG common between the two groups enriched to GO terms associated with monocyte activation including “regulation cytokine production”, “leukocyte migration”, and “JAK-STAT cascade” (Figure 2F). The DEG detected only in control animals mapped to “Carbohydrate catabolic process”. The DEG unique to monocytes from CHD animals mapped to cytokine
FIGURE 2 | Impact of CHD on the monocyte functional and transcriptomic response to LPS. (A) Abundance of live CD14+ cells in PBMC from control (n = 3) and CHD (n = 5) female macaques measured by flow cytometry. Significance calculated by t-test with Welch’s correction. (B) Volcano plot representing up- and downregulated differentially expressed genes (DEG) with CHD in resting monocytes. Red = significant with an FDR ≤ 0.05 and fold change ≥ 2. (C) Heatmap of significant GO terms (identified using Metascape) to which DEGs downregulated and upregulated with CHD in monocytes enriched. The scales of the heatmaps are -log(q-values) associated with the enrichment to selected pathways. (D) Purified monocytes were stimulated with LPS for 6 hours and supernatants were analyzed by Luminex assay. Bar plots showing concentration (pg/ml) of selected immune mediators (TNFα, IL-6, IL-15, CCL4) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 between LPS and unstimulated condition, #p < 0.05, ##p < 0.01, ###p < 0.001 between CHD and control for each stimulation condition calculated by One-way ANOVA). (E) Venn diagram showing overlap between DEG identified in control and CHD monocytes after LPS stimulation. (F) Network visualization of functional enrichment analysis of DEG upregulated with LPS in monocytes from controls only, CHD only, and both using Metascape. The size of each node represents the number of DEGs associated with a given gene ontology (GO) term and the pie chart filling represents relative proportion of DEGs from each group that enriched to that GO term. Similar GO terms are clustered together and are titled with the name of the most statistically significant GO term within that cluster. The gray lines denote shared interactions between GO terms. Density and number of gray lines indicates the strength of connections between closely related GO terms. (G) Heatmap of average normalized expression of genes associated with inflammatory response pathways.
production and signaling pathways (Figure 2F). The expression of inflammatory genes was broadly upregulated in the CHD monocytes following LPS stimulation including CEBPB, CCL20, CCL4, STAT3, FOS, S100A8, HIF1A, and TLR4 (Figure 2G). Additional predictive analysis revealed that LPS-responsive DEGs detected in the CHD monocytes are regulated by canonical transcription factors NFkB2, RELB, and JUNB with over 250 genes mapping to each (Supplementary Figure 3E).

**Chronic Heavy Drinking Alters the Monocyte Transcriptome and Cell Subset Distribution at the Single Cell Level**

To gain a deeper understanding of the heightened activation state of monocytes with CHD, we performed single cell RNA sequencing (scRNA-Seq) on sorted CD14+HLA-DR+ monocytes from CHD and control female macaques (n=3 pooled samples/group). UMAP clustering of all 9,360 monocytes revealed 9 distinct monocyte subsets (MS) (Figure 3A). These clusters could be categorized into the three major monocyte subtypes typically identified by flow cytometry: non-classical, intermediate, and classical based on expression of CD14, MAMU-DRA, and FCGR3 (CD16) (Supplementary Figure 4A). As shown by flow cytometry, the frequency of these three major subsets was comparable between controls and CHD animals by scRNA-Seq analysis (Supplementary Figure 4B). The non-classical monocytes formed one cluster (MS6) exhibiting high expression of CX3CR1, MS4A7, and FCGR3 and lower expression of CD14 and MHC II molecule MAMU-DRA (Figure 3A, B, and Supplementary Figure 4C). The intermediate monocytes also fell into one cluster (MS4), expressing lower levels of non-classical (MS4A7, FCGR3A) and classical (CD14, S100A8) markers with higher expression of MAMU-DRA (Figures 3A, B, and Supplementary Figure 4C). Finally, 7 clusters of classical monocytes were identified (MS1, MS2, MS3, MS5, MS7, MS8, and MS9), based on expression of CST3, GOS2, S100A8, S100A9, HIF1A, SOD2, EGR1, and HERC5 (Figures 3A, B, and Supplementary Figure 4C). These data reveal considerable, previously unappreciated transcriptional heterogeneity within the classical monocytes. Subsets MS5, MS7 were primarily detected in monocytes from CHD animals while MS8 was primarily detected in monocytes from control animals (Figure 3 and Supplementary Figure 4D). Module scoring revealed that MS5 had the lowest expression of genes that play a role in antigen presentation as well as hypoxia, while MS5 and MS7 had higher expression of genes within the TLR- and IL-6 signaling pathways (Supplementary Figure 4E).

To identify the biological implications of these subsets, we extracted the genes that define each cluster and performed functional enrichment (Figure 3C). Although genes that define MS5 (classical – CHD) and MS8 (classical – control) subsets enriched to similar GO terms ("Regulated exocytosis", "granulocyte activation/migration"), only genes highly expressed in MS5 (CHD animals) enriched to "Response to alcohol" and "Wound healing", notably FOSB, HIF1A, and FN1. Interestingly, genes that define MS7 subset (classical – CHD) enriched to "Cytokine-mediated signaling pathway" including CSF3R, IRF1, IRF7, NFKBIA, STAT1, STAT3, and VEGFA. Classical subsets MS1 and MS2 were slightly more abundant in monocytes from control animals and expressed high levels of CST3 and GOS2 as well as S100A8 and S100A9, respectively.

To better understand the relationships between the classical monocyte clusters and their differentiation/activation states, we performed a trajectory analysis. The monocyte clusters were ordered by pseudotime, starting with the most abundant MS1 cluster which resulted in four unique trajectory lineages (Figure 3D). Lineages 1, 2 and 4 indicate transitions culminating in MS8, MS3, and MS5, respectively. Lineage 3 is the classical to non-classical differentiation trajectory. Density plots revealed enrichment of control monocytes at the start of each lineage (less differentiated) and enrichment of the CHD monocytes at the end of the lineages (more differentiated), most notably in Lineage 4 (Figure 3E).

We next assessed differential gene expression with CHD in the 3 major subsets (Supplementary Figure 4F). This analysis showed that IFN-inducible genes WARS and IDO were upregulated in the classical CHD monocytes, whereas MHC-II gene MAMU-DRB1 was downregulated (Supplementary Figure 4F). In the intermediate monocytes, CHD induced upregulation of inflammatory signaling genes MAP3K and S100P but led to decreased expression of FC-receptor gene FCGR3 (Supplementary Figure 4F). The 33 DEG downregulated in the non-classical monocytes from the CHD group relative to controls enriched to antigen processing and IFNγ signaling pathways (Supplementary Figure 4G). Finally, we detected a significant reduction in lysosome and Fcγ-receptor mediated phagocytosis modules in the CHD monocytes (Figure 3F) while gene modules for NFKB signaling and HIF1A signaling were increased (Figure 3G).

**Chronic Heavy Drinking Impacts the Epigenome of Circulating Monocytes**

To uncover epigenetic basis for the altered transcriptional profile in resting monocytes and altered functional responses with CHD, we profiled the chromatin accessibility in purified resting monocytes from female macaques (n=3/group) using the assay for transposase-accessible chromatin sequencing (ATAC-Seq) (55). We identified 11,717 differentially accessible regions (DAR) that were open in the CHD monocytes compared to 9,173 DAR that were accessible in the controls (Figure 4A). More than 25% of the accessible regions in the CHD monocytes mapped to promoter regions and were closer to the transcription start site compared to accessible regions in monocytes from control animals (Figure 4A and Supplementary Figure 5A). Genes regulated by promoter regions open in the CHD monocytes enriched to GO terms associated with cytokine production and myeloid cell activation (Figure 4B). Additional analysis using GREAT showed that cis-regulatory elements in the distal intergenic regions open in CHD monocytes enriched to processes involved with apoptotic signaling, MAPK signaling cascade, and myeloid cell differentiation (Supplementary Figure 5B). Motif enrichment analysis of open chromatin
FIGURE 3 | Single cell RNA-Seq analysis of monocytes with CHD. Monocytes (n = 3 control/3 CHD) were purified from total PBMC from female macaques and subjected to 10X scRNA-Seq analysis. (A) Visualization of 9,360 monocytes by uniform manifold approximation and projection (UMAP) colored by group (control and CHD) as well as by identified clusters. (B) Stacked violin plots representing expression of key genes used for cluster identification, grouped by monocyte subset cluster. (C) GO Biological Process enrichment from Metascape of genes highly expressed in MS5, MS7, and MS8, defined by q-value. (D) Trajectory analysis of monocytes determined using Slingshot. (E) Cell density plots for Control and CHD groups across each of four trajectory lineages determined by Slingshot. (F, G) Violin plots comparing (F) Lysosome and Fcγ receptor-mediated phagocytosis and (G) NFκB signaling and HIF1A signaling pathway module scores in all monocytes of both groups. Statistical analysis of module scores was performed using a t-test with Welch's correction where ****p < 0.0001.
regions in the CHD and control monocytes demonstrated increased putative binding sites for transcription factors important for monocyte differentiation and activation SP1, FRA1, FOS, JUNB, BATF, and PU.1 with CHD (59–63) (Figure 4C). To link the resting epigenome of the CHD monocytes to the enhanced transcriptional response after LPS stimulation, we compared the genes associated with the open promoter regions (<1kb) and the DEG identified after LPS stimulation and identified 281 common genes (Figure 4D). Functional enrichment of these genes showed significant mapping to Biological Process “Cytokine-mediated signaling” including transcription factors FOS, HIF1A, STAT3 and JUNB (Figure 4E). These genes as well as inflammatory CCL20, TLR2, CD81 and TNFSF14 had both open promoters in the resting CHD monocytes as well as upregulated gene expression in the LPS-stimulated CHD monocytes, linking open chromatin under resting conditions with CHD to transcriptional LPS responses (Figure 4F).

CHD Impairs Monocyte Response to E. coli

We next asked whether CHD would affect the monocyte response to pathogens. Monocytes from male and female macaques (n=3...
controls; n=6 CHD) were co-cultured with heat-killed E. coli for 16 hours and immune mediator production was measured using Luminex (Supplementary Figure 1A). In contrast to the response to purified LPS, monocytes from CHD animals generated a dampened response to E. coli characterized by reduced levels of IL-1β, IL-6, MIP-1β, and IL-15 and to a lesser extent I-TAC and IL-5 compared to their control counterparts (Figures 5A, B). No sex differences were noted in these assays, indicating that defects in monocyte responses to bacterial pathogens are comparable in male and female macaques. Moreover, these data suggests that CHD differentially impacts pathogen recognition and activation pathways within monocytes.

DISCUSSION

It is well-appreciated that chronic alcohol drinking exerts a profound impact on peripheral innate immune cells. However, the limitations provided by the complexity of studying alcohol consumption in humans has left major gaps in understanding mechanisms that underlie the immune response under conditions of heavy alcohol drinking. In this study, we utilized a macaque model of voluntary ethanol self-administration to profile peripheral monocytes in animals after 12 months of daily alcohol drinking. We first demonstrated that circulating monocytes from CHD animals generate a hyper-inflammatory response to ex vivo LPS stimulation at the transcript and protein level. A comprehensive profiling of the monocytes by scRNA-Seq and ATAC-Seq revealed alterations in differentiation state as well as epigenetic landscape with CHD. In contrast to what we observed following ex vivo stimulation with LPS, monocytes from CHD animals generated a dampened production of immune mediators following ex vivo E. coli stimulation. This highlights critical aspects of the complexity of immune cell responses and the necessity of utilizing both purified stimulants as well as real pathogens to uncover the underlying mechanisms of response.

The enhanced inflammatory responses in response to ex vivo stimulation with LPS by PBMC from CHD female macaques are in line with our previous data for PBMC from CHD male macaques (41), indicating that this consequence of CHD is sex independent. Our follow up phenotyping revealed that there were in fact more CD14+ monocytes in the PBMC of CHD animals, but no changes were observed in the distribution of classical, non-classical, and intermediate monocyte populations. Further analysis of purified monocytes indicated that this hyper-inflammatory response is mediated largely by intrinsic changes within monocytes and not just increased cell numbers. Indeed, CHD induced significant transcriptional differences consistent with activation of inflammatory pathways in monocytes at resting state. Moreover, LPS stimulation resulted in a drastic increase in numbers of DEG from the CHD monocytes with increased expression of genes associated with inflammatory response. In contrast, genes involved in carbohydrate catabolic processes were not upregulated in monocytes from CHD animals suggesting metabolic rewiring associated with a heightened inflammatory state (64). The increased non-specific inflammatory

![Figure 5](image_url)
mediator production in response to ex vivo LPS stimulation are in line with previous in vitro studies that have reported hyper-activation of myeloid cells with prolonged alcohol exposure. This also fits with hyper-inflammatory phenotypes associated with patients with alcohol use disorders, especially those with alcoholic liver disease (16, 27, 65).

To elucidate the molecular basis for this dysregulated inflammatory response, we profiled monocytes from CHD and control animals by scRNA-Seq. This analysis revealed tremendous diversity amongst classical monocytes that has not been previously appreciated. We identified two unique classical clusters within monocytes from CHD animals expressing high levels of hypoxia factor HIF1A (MS5), and antioxidative defense molecule SOD2 (MS7). Alcohol and the products of its metabolism can induce oxidative stress which can alter cellular transcriptional profiles, potentially explaining the profile of the MS5 cluster (66). CHD animals also had a higher number of cells within the anti-viral cluster MS9 that expressed high levels of interferon stimulated (ISG) genes such as HERC5. This observation is in line with our earlier study that reported higher levels of ISG within PBMC from these same animals (18). Functional module scoring of all the monocytes revealed downregulation of genes involved in lysosome function and Fcγ receptor mediated phagocytosis, but upregulation of NFκB signaling and HIF1A signaling. Moreover, trajectory within the monocyte subsets revealed that CHD accelerates differentiation/activation of classical monocyte subsets making them potentially poised towards a hyper-inflammatory response.

Although the non-classical and intermediate monocytes fell into single clusters with equal numbers of cells from controls and CHD animals, differential gene expression analysis revealed significant downregulation of genes associated with MHC class II, antigen processing and presentation, and IFNγ signaling pathways in the CHD monocytes. This observation provides a potential explanation for the reduced response to vaccination observed in CHD animals (67). Further, monocytes from CHD male and female macaques generated a dampened cytokine and chemokine response to heat killed E. coli. This was an unexpected outcome since stimulation with LPS resulted in a heightened inflammatory response and suggests a rewiring of monocytes with CHD towards non-specific inflammatory responses and away from anti-microbial functions. This discrepancy could be explained by the fact that LPS signals exclusively through TLR4, whereas heat killed E. coli could trigger a number of TLRs including TLR2/6 (68). Additionally, CHD interferes with phagocytosis and ROS production in macrophages (69), which could interfere with pathogen sensing. While the mechanisms underlying the differences between the heightened response to LPS and dampened response to whole organisms is complex and require further study, our observations provide potential explanation for the reported increased susceptibility of patients with alcohol use disorders to bacterial infections (10, 11).

Ethanol metabolites notably acetaldehyde and acetate in addition to minor byproducts such as reactive oxygen species (ROS) and lipid metabolism products can modulate gene expression by binding transcription factors and/or modifying chromatin accessibility (66). Therefore, we profiled chromatin accessibility of monocytes from control and CHD animals by ATAC-Seq. We discovered that promoter regions that regulate genes important for cytokine production and myeloid cell activation were more accessible in monocytes from CHD animals. These open regions contained putative binding sites for transcription factors important for monocyte activation and differentiation. Integrative analyses showed correlation between chromatin accessibility and expression levels of key inflammatory genes in CHD monocytes, including FOS, HIF1A, and JUNB. This suggests that the monocytes are poised epigenetically towards heightened inflammatory responses.

One proposed mechanism of this hyper-inflammatory state in monocytes (33, 35–37) and macrophages with CHD is the translocation of bacterial products into circulation through impaired gut barrier caused by ethanol consumption (34). We detected modestly elevated levels of LAL in circulation but no changes in IgM bound endotoxin. A small increase in these circulating bacterial products could significantly impact the activation state of monocytes; however, it is difficult to tease these effects out from circulating ethanol and its metabolic products (acetaldehyde, acetate). Additionally, liver enzyme levels remained within normal ranges for the duration of the study. Long term exposure to activating agents, like LPS, is believed to lead to tolerance and a decreased response to secondary stimulation in monocytes and macrophages defined by specific changes in chromatin structure (70–73). Interestingly, we see enhanced inflammatory responses of monocytes and macrophages from CHD animals with LPS, akin to innate immune training (74, 75). Indeed, ethanol and its metabolites have been found to directly act on histone modifications, creating changes in accessible chromatin (66, 76, 77). This could be occurring in circulating monocytes, tissue macrophages, or the progenitors of these cells in the bone marrow; perhaps on all three levels, altering the epigenetic structure and therefore functional capacities of these cells (78). In fact, observations in patients with alcohol use disorders suggest an effect of alcohol on hematopoiesis (79). Because blood monocytes are under constant repopulation from bone marrow progenitors, it is conceivable that the progenitors themselves or the process of myelopoiesis are disrupted with CHD.

In summary, this study provides a novel, in-depth, and integrative analysis of the effects of long-term in vivo alcohol drinking on monocytes in non-human primates. Our findings indicate that alcohol drinking alters the baseline epigenetic and transcriptional states of monocytes, which is associated with heightened inflammatory responses but reduced anti-microbial activity. Future studies will focus on linking epigenetic modifications to functional outcomes and investigating the impact of alcohol drinking on bone marrow progenitors.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and
accession number(s) can be found below: (https://www.ncbi.nlm.nih.gov/, PRJNA723053).

ETHICS STATEMENT

The study was approved by the Institutional Animal Care and Use Committee at the Oregon National Primate Research Center.

AUTHOR CONTRIBUTIONS

SL, KG, and IM conceived and designed the experiments. SL, SS, and BD performed the experiments. SL and BD analyzed the data. SL and IM wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724015/full#supplementary-material

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