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Intermediate monocytes in acute alcoholic hepatitis are functionally activated and induce IL-17 expression in CD4$^+$ T cells

Running title: Intermediate monocytes in acute alcoholic hepatitis

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ABSTRACT

In humans, the three main circulating monocyte subsets are defined by their relative cell surface expression of CD14 and CD16. They are all challenging to study because their characteristics are strongly context specific and this has led to a range of conflicting reports about their function, which is especially so for $CD14^{++}CD16^{+}$ (intermediate) monocytes. *Ex vivo* cultures are also often confounded by the concomitant use of immunosuppressive drugs. We therefore sought to characterize the phenotype and function of intermediate monocytes in the setting of acute inflammation prior to treatment in a cohort of 41 patients with acute alcoholic hepatitis (AH).

Circulating intermediate monocytes were enriched in patients with AH and had an activated phenotype with enhanced expression of CCR2 and CD206 compared to healthy controls. Pro-inflammatory cytokine expression, including IL-1β and IL-23, was also higher than in healthy controls, but both classical ($CD14^{++}CD16^{-}$) and intermediate monocytes in AH were refractory to TLR stimulation. Compared to healthy controls both AH monocyte subsets had greater phagocytic capacity, enhanced ability to drive memory T cell proliferation in co-culture and skewed $CD4^{+}$ T cells to express an increased ratio of IL-17:IFNγ. Furthermore, liver tissue from AH patients demonstrated an enrichment of monocytes including the intermediate subset compared to controls. These data demonstrate that intermediate monocytes are expanded, functionally activated, induce $CD4^{+}$ T cell IL-17 expression and are enriched in the liver of patients with AH.

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25 **KEY POINTS**

26 Circulating intermediate monocytes are enriched in alcoholic hepatitis (AH) patients.

27 AH intermediate monocytes are functionally activated.

28 AH intermediate monocytes induce $CD4^+$ T cell IL-17 and are enriched in the liver.

29

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30 **INTRODUCTION**

31

32 Monocytes consist of a heterogeneous group of myeloid cells with varied function and
33 phenotype. Although they are precursors of tissue resident macrophages and dendritic cells
34 they are also effector cells in their own right (1). Accordingly, they are mobilized from the
35 bone marrow in response to acute inflammatory states such as severe sepsis (2) and play an
36 integral role in responding to pathogen and damage-associated molecular patterns (PAMPs
37 and DAMPs) and shaping $CD4^+$ T cell responses (3, 4). In humans, they are classified on the
38 basis of their cell surface expression of CD14 (lipopolysaccharide [LPS] co-receptor) and
39 CD16 (activatory Fc gamma receptor) into classical ($CD14^{++}CD16^-$), intermediate
40 ($CD14^{++}CD16^+$) and non-classical ($CD14^+CD16^{++}$) subsets (5), each of which is thought to
41 have distinct functions. Currently, the conventional view is that the dominant $CD16^-$ classical
42 subset egress from the bone marrow and are recruited to sites of inflammation where they act
43 as phagocytes and give rise to patrolling pro-inflammatory $CD16^{++}$ cells, with the
44 intermediate monocytes representing a transitional group between these two states (6).

45

46 Consistent with this it is known that the $CD14^{++}CD16^+$ intermediate subset is enriched in the
47 peripheral blood of patients with autoimmune conditions including rheumatoid arthritis (7).
48 The role of these intermediate monocytes is however poorly understood, and we therefore
49 sought to interrogate their function in the context of acute inflammation. To achieve this we
50 recruited patients with acute alcoholic hepatitis (AH) as, at its onset, it is an exemplar disease
51 for rapid-onset, severe systemic inflammation. Unlike chronic autoimmune diseases, on
52 presentation it is typically not confounded by concomitant use of immunosuppressive
53 medications such as glucocorticoids (8).

54

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55 Although it is known that immune activation occurs concurrently with an impaired
56 antimicrobial response in AH (9), and tissue resident hepatic macrophages (Kupffer cells)
57 play an important role in orchestrating the immune mediators of hepatocyte damage (10, 11),
58 little is known about the functional role of monocytes. Nonetheless, alcohol may activate
59 them indirectly by modulating intestinal permeability as a consequence of intestinal dysbiosis
60 (10), resulting in an increased exposure of circulating monocytes to PAMPS such as LPS
61 (11). In this study, we therefore hypothesized that circulating monocytes in the peripheral
62 blood of patients presenting with acute AH would be expanded, phenotypically and
63 functionally pro-inflammatory, in particular with regard to their influence on $CD4^+$ T cells.
64

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MATERIALS AND METHODS

Participants

Regulatory approval for this study was obtained from the UK's National Health Service (NHS) Health Research Authority (07/Q2007/05 and 15/LO/1501) and it was conducted according to the International Council for Harmonization Good Clinical Practice Guidance and the Declaration of Helsinki. Written informed consent was obtained from all study participants.

Patients admitted to University Hospitals Bristol NHS Foundation Trust (UHBristol) and University Hospitals Plymouth NHS Trust with a diagnosis of severe AH were prospectively recruited. Severe AH was defined in accordance with recently published trial standards (12) as recent onset jaundice (within previous 3 months) with a serum bilirubin level > 80 µmol/L in a heavy alcohol drinker (more than 60 (males) or 40 (females) g alcohol per day for more than 6 months) with aspartate aminotransferase / alanine aminotransferase > 1.5 and discriminant function > 32 (13). Peripheral blood was drawn *prior* to commencement of any medical therapy. Clinical and biochemical data were recorded, and survival was determined at day 90.

Control blood samples were obtained both from healthy volunteers (HVs) and age and sex-matched patients with alcohol related liver disease (ALD) in the absence of systemic liver inflammation. Patients with ALD were defined as heavy alcohol consumers (> 60 (males) or > 40 (females) g/alcohol per day for more than 6 months) with a diagnosis of cirrhosis (confirmed on imaging or histology) without severe AH (bilirubin < 80 µmol/L and discriminant function < 32) who had an unplanned hospital admission. Patients with ALD

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90 received usual clinical care. The ALD control group was selected as these patients had many
91 similar characteristics to the AH group (demographics, alcohol consumption, underlying liver
92 disease and unplanned hospital admission). The key difference was the absence of the acute
93 inflammatory state of AH. HVs were recruited from local laboratory and hospital workers
94 who provided a self-declaration that they did not have any chronic health problems. Both
95 ALD and HV donors gave a single sample of peripheral blood.

96

97 An historic cohort of AH patients recruited at UHBristol between 2007 and 2010, underwent
98 transjugular liver biopsy and tissue surplus to diagnostic requirement was used for this study.
99 The median time of the biopsy after clinical diagnosis of AH was 9 days (range -1 to 143
100 days). Only samples taken within 10 days of diagnosis were included in this study. In ALD
101 patients who also underwent liver biopsy for clinical reasons (n=4) surplus tissue was used
102 for this study.

103

104 **Peripheral blood mononuclear cell (PBMC) isolation and magnetic activated cell sorting**
105 PBMCs were isolated from whole blood by density gradient centrifugation using Ficoll-
106 Paque PLUS (GE Healthcare, Cardiff, UK). Autologous $CD4^{+}CD45RO^{+}$ memory T cells and
107 monocytes were isolated from PBMCs using a memory $CD4^{+}$ T cell isolation kit (Miltenyi
108 Biotec Ltd, Surrey, UK) and a pan-monocyte negative selection kit (Miltenyi), respectively,
109 according to the manufacturer's instructions. Classical ($CD16^{-}$) and intermediate ($CD16^{+}$)
110 monocyte subsets were subsequently separated using CD16 microbeads (Miltenyi).

111

112

113

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114 **Cell sorting by flow cytometry**

115 Flow cytometry was performed using the BD Influx flow cytometer (BD Biosciences,
116 Oxford, UK) based on cell surface expression of CD3, CD4, CD45RO (memory $CD4^{+}$ T
117 cells) and HLA-DR, CD14 and CD16 (monocyte subsets, $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and
118 $CD14^{+}CD16^{++}$). Dead cells were excluded using 7-aminoactinomycin D (7AAD; Thermo
119 Fisher Scientific, Loughborough, UK). Monocytes were classified into three subsets
120 according to the recommendations of an expert consensus panel based on CD14 and CD16
121 expression (5). The gating strategy was performed according to a previously published report,
122 which demonstrated accurate classification of monocytes in patients with acute liver disease
123 (14) and is shown in Supplementary Figure 1A. Purity of sorted cell populations was
124 assessed by flow cytometry and was > 95% (data not shown). Details of antibodies are found
125 in Supplementary Table I.

126

127 **Cell characterization by flow cytometry**

128 PBMCs were incubated with antibodies to CD3, CD4, CD14, CD16, CCR2, CCR5, CX_3CR1,
129 HLA-DR, CD206 and CD80 then washed and analyzed on a BD LSR II flow cytometer (BD
130 Biosciences). Gating was performed using 'fluorescence minus one' controls for each
131 fluorochrome. Details of antibodies are found in Supplementary Table I. Monocyte subsets
132 were gated as per the sort strategy but without CD45RO (Supplementary Figure 1A).

133

134 Monocyte subsets sorted by flow cytometry were cultured at 0.5 x 10^{6} / ml in complete media
135 (RPMI supplemented with 10% fetal bovine serum [GE Healthcare], L-glutamine [Sigma-
136 Aldrich, Poole, UK] and penicillin/streptomycin [Sigma-Aldrich]) in the presence or absence
137 of 100 pg/ml LPS (Sigma-Aldrich) for 18-24h with the addition of 1 µg/ml BD GolgiPlug
138 (BD Biosciences) in a 37°C, 5% CO_2 humidified incubator. Cells were permeabilized and

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139 intracellular staining was performed with antibodies to IL-1β, IL-6, IL-8, IL-12p40, IL-23p19

140 and TNFα. Gating was performed using 'fluorescence minus one' controls for each

141 fluorochrome. Similar replicates were performed for other TLR ligands: peptidoglycan

142 (TLR2/1) at 1 μg/ml, polyinosinic-polycytidylic (PI:C; TLR3) at 5 μg/ml) and Resiquimod

143 (R848; TLR7/8) at 2.5 μg/ml (all TLR ligands from Invivogen, San Diego, USA). To assess

144 whether endogenous stimuli affected cytokine expression, further replicates of monocyte

145 subsets were stimulated with 200 ng/ml interferon alpha (IFNα; Biolegend, San Diego, USA)

146 using an identical protocol.

147

148 **Phagocytosis assay**

149 Monocytes isolated by MACS were cultured at 0.5 x 10^6 / ml with 1 ng/ml LPS (Sigma-

150 Aldrich) and 1 x 10^8 fluorescently labelled microparticles (Park Scientific, Northampton,

151 UK) for 10 minutes under tissue culture conditions. Cells were washed and stained with

152 antibodies to CD14 and CD16 before fixation. Phagocytosis of fluorescent microparticles was

153 quantified in each monocyte subset by flow cytometry (Figure 3A) (15).

154

155 **Monocyte and T cell co-culture assay**

156 Carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific) labelled T cells and

157 unlabeled monocyte subsets were co-cultured in complete media at a ratio of 5:1 (1x10^5 T

158 cells: 2x10^4 monocytes) for 5 days under tissue culture conditions, in wells pre-coated for 4hr

159 at 37^{0}C with 1 μg/ml anti-CD3 (Thermo Fisher Scientific). A control well without

160 monocytes but containing both anti-CD3 and anti-CD28 (both 1μg/ml; Thermo Fisher

161 Scientific) was also included. For the final 4 hours of culture, cells were pulsed with 20ng/ml

162 phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1μM ionomycin (Sigma-Aldrich)

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163 and 1 μg/ml BD GolgiStop (BD Biosciences). Cells were assessed for intracellular cytokine
164 expression (IL-17A and IFNγ) using flow cytometry (BD LSR II).
165
166 **Genetic analysis**
167 RNA was prepared from RNA stabilized samples (RNA protect, Qiagen, Manchester, UK)
168 using the RNeasy plus micro kit (Qiagen). RNA was extracted from classical and
169 intermediate monocytes isolated by cell sorting from 3 AH and HV subjects. RNA yields
170 were determined by Nanodrop measurement ($A_{260,}$ Thermo Fisher Scientifc) and RNA
171 integrity was assessed by RNA electrophoresis (Bioanalyser 2100, Agilent, Stockport, UK).
172 Gene expression analysis was carried out using nCounter Human Immunology V2 code set
173 (Nanostring technologies, Seattle, WA, USA). Quality control assessment and normalisation
174 of Nanostring® reporter code counts (RCC) were performed following manufacturer
175 recommended methods using nSolver™ Analysis Software version 4 (Nanostring
176 technologies). Heatmaps were generated for the most differentially expressed genes for each
177 monocyte subset for HV vs AH.
178
179 **Sample workflow**
180 In all participants, PBMCs were isolated and characterized by CD3, CD4, CD14 and CD16
181 expression. In a pre-specified analysis, the first 14 HV and AH samples obtained underwent
182 the full surface marker phenotype panel. The subsequent 10 AH patients were analyzed for
183 phagocytosis. Cell sorting experiments required 150 mL fresh blood from each participant.
184 Due to the volume required, we pre-specified an analysis of 10 unselected AH patients.
185
186
187

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188 **Liver tissue immunostaining**

189 Pathology specimens were acquired from 7 AH and 4 ALD patients who had undergone
190 transjugular liver biopsy performed by an interventional radiologist at UHBristol. 5 µm
191 sections of the formalin fixed and paraffin embedded tissues samples were obtained using a
192 microtome and mounted onto slides. Samples were deparaffinised in Xylene (Sigma-Aldrich)
193 and rehydrated in ethanol before antigen retrieval in 10 mM citrate buffer and incubating with
194 primary antibodies to CD14 and CD16 (both Abcam, Cambridge, UK) overnight at 4°C.
195 Bound antibodies were detected using fluorescently-labelled secondary antibodies (Abcam),
196 mounted with DAPI (Vector Labs, Peterborough, UK) and fluorescence was visualized under
197 confocal microscopy. Semi-quantitative analysis was performed by manual counting of
198 fluorescent cells per x20 power field in 3 separate portal and lobule regions each. Mean cell
199 counts per field were compared.

200

201 **Statistical analysis**

202 Continuous variables were compared using Mann-Whitney U tests for independent samples
203 or Wilcoxon signed rank tests for paired samples. Categorical variables were compared using
204 Fisher exact test. All analysis was performed using SPSS version 21 (IBM, New York, USA).
205 Multiparametric cytokine expression was analyzed using SPICE v6.0 (freely available from
206 http://exon.niaid.nih.gov/spice/).

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RESULTS

Peripheral blood analyses were conducted on 41 patients with severe AH (mean age 50.2 years, 52% female). Mean disease severity scores of discriminant function (DF) and model for end-stage liver disease (MELD) were 60.8 (standard deviation [sd] 23.9) and 18.1 (sd 6.6) respectively. Overall, there was 14% and 24% 28- and 90-day mortality respectively. Age, gender, disease severity and outcome were similar in 10 patients with ALD who had mean age of 51.8 years, 50% female and MELD 15.1 (sd 5.4). There was 10% and 30% 28- and 90-day mortality respectively (Table 1). Peripheral blood analyses were also performed on 47 HVs.

Intermediate monocytes are enriched in patients with AH

Baseline total monocyte count was significantly greater than the healthy population median (0.71 v 0.60; $p<0.01$; Figure 1A). The proportion of peripheral blood intermediate monocytes, as measured by flow cytometry (Figure 1B) was significantly higher in patients with AH compared to HVs (16.7% v 7.4%; $p<0.001$; Figure 1C). Compared to HVs there is a commensurate reduction in the proportion of classical monocytes (78.4% v 83.0%; $p<0.01$; Figure 1C) and almost an absence in non-classical monocytes in patients with AH compared with HVs (0.7% v 3.9%; $p<0.001$; Figure 1C). The proportions of circulating monocyte subsets in patients with AH were also significantly different to those in patients with ALD (Figure 1C). Classical and intermediate proportions in ALD patients did not differ from HVs (Figure 1C).

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232 **Monocyte surface marker phenotype is altered in AH**

233 Flow cytometry was used to quantify surface marker phenotype in AH patients compared to
234 HVs (Figure 1D). Intermediate monocytes from AH patients were phenotypically altered
235 compared to HVs with higher expression of CCR2 (0.6 v 0.3 [expression normalized to HV
236 classical monocytes]; $p<0.01$) and CD206 (3.4 v 1.2; $p<0.01$) and lower expression of HLA-
237 DR (1.8 v 4.9; $p<0.001$). In AH patients, intermediate monocytes expressed lower CCR2 (0.6
238 v 1.0; $p<0.05$) but higher HLA-DR (1.8 v 0.5; $p<0.001$), CD206 (2.8 v 1.8; $p<0.05$) and
239 CD80 (1.5 v 1.1; $p<0.05$) compared to classical monocytes. Although a small population,
240 non-classical monocytes in AH patients were phenotypically distinct to intermediate
241 monocytes with lower expression of CCR2 (0.2 v 0.8; $p<0.001$) and CCR5 (0.8 v 1.2;
242 $p<0.05$). The percentage of circulating intermediate monocytes was higher and their HLA-
243 DR expression was lower in AH patients who died within 90 days compared to those that
244 survived (23% v 16% and 18,000 v 37,000 mean fluorescence intensity, respectively; both
245 $p<0.05$; Supplementary figure 1B and C).

246

247 **Monocyte subsets from AH patients are primed to produce pro-inflammatory cytokines**
248 **but refractory to stimulation**

249 High proportions of unstimulated AH monocytes produce pro-inflammatory cytokines and
250 have greater co-expression of multiple cytokines compared to HV monocytes (Figure 2A).
251 SPICE analysis showed similar cytokine profiles between unstimulated AH classical and
252 intermediate monocytes but increased cytokine expression in HV intermediate versus
253 classical subsets (Figure 2A). Global cytokine expression in AH v HV classical and
254 intermediate subsets was 85% v 41% ($p<0.05$) and 89% v 59% ($p<0.05$) respectively (Figure
255 2D).

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257 Specific cytokine analysis revealed that classical monocytes in AH patients produce higher
258 levels of IL-6 (37% v 3%; $p<0.05$), IL-8 (64% v 17%; $p<0.05$) and IL-23 (11% v 1%;
259 $p<0.05$) compared with HVs (Figure 2B). Intermediate monocytes from AH patients produce
260 significantly higher levels of IL-1β (72% v 24%; $p<0.05$), IL-8 (64% v 10%; $p<0.05$) and IL-
261 23 (11% v 2%; $p<0.05$) compared with HVs (Figure 2C). HV monocyte subsets have
262 minimal expression of IL-6, IL-12 and IL-23, but do produce low levels of other pro-
263 inflammatory cytokines (Figure 2B and C).
264
265 Gene expression analysis comparing unstimulated *ex vivo* AH and HV monocyte subsets
266 revealed a greater number of differentially expressed genes in intermediate than classical
267 monocytes (Supplementary figure 2A-C). Increased expression was noted in a range of
268 chemokines, cytokines and their receptors (Supplementary figure 2B). *CCR2* was the second
269 most differentially expressed gene in AH compared to HV intermediate monocytes after
270 *CLEC4E*, a calcium-dependent lectin involved in innate pattern recognition receptor
271 (Supplementary figure 2B). Consistent with protein analysis, AH intermediate monocytes
272 expressed higher levels of *IL1B* and *IL8* RNA than HVs. Compared to AH classical
273 monocytes, AH intermediate monocytes generally expressed greater levels of genes involved
274 in inflammatory pathways and cell-cell interaction (Supplementary figure 2D-E).
275
276 With regard to monocyte capacity to produce pro-inflammatory cytokines, TLR4 stimulation
277 with LPS of classical monocytes increased global cytokine expression by 1.4 fold in HVs
278 compared to no change in AH patients ($p<0.05$; Figure 2E). This was particularly apparent in
279 increased expression of both IL-1β and IL-6 compared to AH classical monocytes
280 (Supplementary Figure 1D). No changes in global cytokine expression were noted in TLR4
281 stimulated HV or AH intermediate monocytes (Figure 2E). Similar findings were true of

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282 TLR2/1 (peptidoglygan), TLR3 (PI:C) and TLR 7/8 (R848) stimulation (Supplementary
283 Table II). AH classical and intermediate monocytes were also unresponsive to endogenous
284 stimulation with IFNα (Supplementary Table II).
285
286 **Monocytes from patients with AH are functionally activated**
287 LPS stimulated phagocytosis of fluorescently labelled microparticles was significantly higher
288 in both classical and intermediate monocytes from AH patients compared to HV monocytes
289 (65% v 46% in classical monocytes; $p<0.05$ and 52% v 19%; $p<0.001$ in intermediate
290 monocytes; Figure 3B). In HVs, phagocytic capacity was lower in intermediate compared to
291 classical monocytes (19% v 46%; $p<0.01$; Figure 3B). Of monocytes actively phagocytosing
292 microparticles, intermediate monocytes from AH patients had enhanced ability to
293 phagocytose more than one particle per monocyte compared to HVs (62% v 36%; $p<0.01$;
294 Figure 3C) which was similar to AH classical monocytes (62% v 61%; $p>0.05$).
295
296 **Classical and intermediate monocytes from AH patients drive similar T cell responses**
297 In patients with AH, classical and intermediate monocytes have similar effects on memory
298 $CD4^+$ T cell proliferation and polarization with equal proliferation (65% v 64%; Figure 4A),
299 IL-17 expression (15.3% v 12.6%; Figure 4B) and IFNγ expression (13.7% v 14.7%; Figure
300 4C). However, in HVs, intermediate monocytes drove significantly less memory $CD4^+$ T cell
301 proliferation (36% v 46%; $p<0.01$; Figure 4A) and IL-17 expression (6.5% v 8.6%; $p<0.05$;
302 Figure 4B) than classical monocytes and had a higher IFNγ/IL-17 ratio (3.2 v 2.1; $p<0.01$;
303 Figure 4E). Compared to HV intermediate monocytes, those from AH patients drove greater
304 T cell proliferation (64% v 36%; $p<0.05$; Figure 4A) and IL-17 expression (12.6% v 6.5%;
305 $p<0.05$; Figure 4B) with a trend to lower IFNγ expression (4.2% v 18.0%; $p=0.09$; Figure
306 4C).

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307

308 **Intrahepatic monocytes**

309 Liver tissue from AH patients, taken a median of 7.5 days after clinical diagnosis was made,

310 demonstrated a significant increase in $CD14^+$ monocytes compared to ALD controls (25.7 v

311 10.5 cells per field; $p<0.01$; Figure 5B). This difference was particularly evident in portal

312 regions in both dual positive $CD14^+CD16^+$ (Figure 5C) and single positive $CD14^+CD16^-$

313 (Figure 5D) cells versus ALD patients (6.3 v 1.3 cells per field; $p<0.05$ and 15.7 v 9.0 cells

314 per field; $p<0.05$ respectively).

315

316

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318 **DISCUSSION**

319

320 In this study, we have demonstrated an enrichment of intermediate monocytes in the
321 peripheral blood of patients with acute severe AH. These are characterized by the
322 upregulation of CCR2 and expression of pro-inflammatory cytokines. They are also
323 refractory to TLR stimulation. Furthermore, intermediate monocytes are functionally similar
324 to classical monocytes in patients with AH. Compared to HVs, both subsets have an activated
325 phenotype with greater phagocytic capacity, enhanced ability to drive memory T cell
326 proliferation in co-culture and favor a Th17 phenotype. Liver tissue from AH patients
327 demonstrates an enrichment of monocytes including the intermediate subset compared to
328 ALD controls. This leads us to hypothesize that activated circulating monocytes home to the
329 liver and contribute to disease pathogenesis.

330

331 Severe AH is associated with an enrichment of circulating intermediate monocytes, as
332 described in other inflammatory diseases (7, 8, 14, 16), which is not a result of either alcohol
333 related cirrhosis or active heavy alcohol use itself (Figure 1C). Both the percentage of
334 circulating intermediate monocytes and their activation status (HLA-DR expression) are
335 associated with clinical outcome (Supplementary figure 1B and C). Protein and gene
336 expression and functional analyses have enabled characterization of these monocytes in
337 comparison to HVs. Based on the canonical cell surface markers for classical (CCR2),
338 intermediate (CCR5) and non-classical monocytes (CX_3CR1) (14, 17-19), the elevated
339 expression of CCR2 in AH intermediate monocytes suggest that they have the ability to
340 migrate to the inflamed liver, consistent with our liver immunostaining findings. CCL2, the
341 ligand for CCR2, is highly expressed in AH liver tissue (20). Furthermore, in comparison to
342 HV intermediate monocytes, CCR5 expression is downregulated in AH intermediate

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343 monocytes. Reduced CCR5 and elevated CCR2 expression are indicative of an immature
344 monocyte phenotype (21, 22), suggesting that these monocytes have recently egressed from
345 the bone marrow as part of the inflammatory response. Other surface proteins involved in
346 cell-cell interactions were also upregulated including CD80 and CD206, consistent with their
347 enhanced phagocytosis and proliferative T cell drive (Figures 3 and 4). Furthermore, AH
348 intermediate monocytes express greater levels of genes involved in inflammatory pathways
349 and cell-cell interactions compared to classical monocytes suggesting an activated phenotype
350 (Supplementary figure 2).
351
352 AH monocytes are functionally activated. Intermediate monocytes from AH patients
353 demonstrated high phagocytic capacity (in both the proportion able to phagocytose and the
354 number of particles phagocytosed per monocyte; Figure 3). They secreted high levels of pro-
355 inflammatory cytokines but were refractory to further modulation by a range of bacterial,
356 fungal and viral TLR ligands. Hence, they may already be maximally stimulated *in vivo* and
357 cannot further upregulate their intracellular cytokine expression. Importantly, expression of
358 IL-1β, and IL-23, were higher in classical and intermediate monocytes from patients with AH
359 compared to HVs (Figure 2). As these cytokines are essential for the differentiation of Th17
360 cells from naïve $CD4^+$ T cells (3, 23), this suggests that these monocytes are skewed to
361 polarize T cells to a Th17 phenotype in the context of acute inflammation.
362
363 We tested this *in vitro* and demonstrated that classical rather than intermediate monocytes
364 from healthy volunteers induce greater memory T cell proliferation and more IL-17
365 expression with a higher IFNγ:IL-17 ratio. This replicated previous findings (8). However, in
366 AH patients, in the context of increased expression of IL-1β and IL-23 from their classical
367 and intermediate monocytes, this was reversed and co-cultured memory T cells had a reduced

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368 ratio of IFNγ:IL-17 and favored IL-17 expression (Figure 4). This is consistent with previous
369 reports in patients with alcohol related liver disease (24, 25), but the consequences of this
370 switch are unclear, as a skew to a Th17 phenotype may either be pro-inflammatory and
371 pathogenic (26), or control disease through the recruitment of neutrophils which enhance
372 hepatic regeneration and are associated with an improved prognosis (27, 28).
373
374 Additionally, our data demonstrate an enrichment in intrahepatic monocytes in AH patients
375 compared to ALD controls (Figure 5) suggesting the acute inflammatory state in AH and not
376 just heavy alcohol consumption or cirrhosis itself is associated with intrahepatic monocyte
377 trafficking. Given the increased expression of liver homing chemokine receptors and the
378 activated monocyte phenotype of circulating monocytes it is possible that these are the same
379 cells we have documented within the liver, where they may be contributing to disease
380 pathogenesis. Further studies are required to examine the dynamics of monocyte liver
381 infiltration.
382
383 We have compared our findings in patients with AH to healthy controls as well as ALD
384 patients. The latter group were selected as appropriate disease controls as they shared many
385 clinical characteristics with AH patients (ongoing alcohol consumption, demographics,
386 underlying liver disease and were hospitalizsed). This has allowed us to conclude that the
387 differences seen in AH patients were not simply due to alcohol consumption or underlying
388 liver disease but related to the inflammatory state of AH.
389
390 There remains debate over the functional phenotype of the intermediate monocyte (29) with
391 observations of similarities to both the classical (30) and non-classical (18) subsets. Some
392 studies have reported that intermediate monocytes are the main producers of pro-

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393 inflammatory cytokines (30) while others that they are anti-inflammatory with high
394 expression of IL-10 in response to TLR4 ligand (31). What is clear is that there is significant
395 heterogeneity of monocytes, which has been well defined in the healthy state (18, 19, 32, 33).
396 Recent single-cell RNA sequencing has further categorized $CD16^{+}$ monocytes into three
397 distinct subsets with some functional overlap with classical and non-classical monocytes (34).
398 Although the expansion of intermediate monocytes has been reported in several inflammatory
399 conditions and proposed as a biomarker of outcome (35), there are few reports of their
400 function in inflammatory states. Consistent with our findings, the best data are from patients
401 with rheumatoid arthritis in which intermediate monocytes induced IL-17 expression from
402 $CD4^{+}$ T cells (36). However, further functional phenotyping has not been conducted.
403
404 Here were present the first report of the functional phenotype of intermediate monocytes in
405 AH. We conclude that, in AH, intermediate monocytes are functionally activated. The
406 intermediate monocyte subset is expanded in the peripheral circulation, tracks to the site of
407 inflammation and has increased phagocytic capacity and ability to drive both T cell
408 proliferation and IL-17 expression than in normal homeostatic conditions. In this acute
409 inflammatory condition, intermediate monocytes may play a role in disease pathogenesis and,
410 therefore, are candidates for further study in the development of disease-specific biomarkers
411 and targeted immune therapies.

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419

420

421 **Conflict of interest disclosure**

422 None of the authors have any conflict of interest to declare

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549 **FIGURE LEGENDS**

550

551 **Figure 1. Intermediate monocytes are enriched in patients with AH and have an altered**
552 **phenotype compared to HVs.** (A) Absolute monocyte counts at baseline of treatment with
553 glucocorticoids in 41 patients with AH (mean and SD). Normal range for the local population
554 is shown (dashed lines). (B) Representative FACS dotplot of CD14 and CD16 expression on
555 monocytes in a HV and a patient with AH. (C) Monocyte subsets as a percentage of total
556 monocyte numbers in HVs (n=47), patients with AH at initial presentation (n=41) and ALD
557 patients (n=10) for classical, intermediate and non-classical subsets. Scatter dot plots show
558 mean and SEM. (D) CCR2, CCR5, CX_3CR1, HLA-DR, CD206 and CD80 surface protein
559 expression in monocyte subsets in HVs (n=14) and patients with AH (n=14) was quantified
560 by flow cytometry. Mean fluorescence intensity was normalized to HV classical monocyte
561 expression of each marker and expressed as a fold difference. Bars charts show mean with
562 SEM. *p<0.05; **p<0.01; ***p<0.001.

563

564 **Figure 2. Monocytes from patients with AH express higher levels of pro-inflammatory**
565 **cytokines than healthy volunteers.** Classical and intermediate monocyte subsets from HVs
566 (n=3) and patients with AH (n=5) were sorted by flow cytometry and cultured for 18-24h
567 with or without LPS stimulation before intracellular cytokine expression was measured by
568 flow cytometry. (A) Simplified Presentation of Incredibly Complex Evaluations (SPICE)
569 analysis of intracellular cytokines demonstrates greater cytokine expression and co-
570 expression of multiple cytokines in both classical and intermediate monocytes from AH
571 patients versus HVs. (B) and (C) Unstimulated individual cytokine expression in classical (B)
572 and intermediate (C) monocytes in HVs and patients with AH. (D) Global cytokine
573 expression (total percentage positive for any cytokine) in classical and intermediate

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574 monocytes in HVs and patients with AH. Unstimulated classical and intermediate monocytes
575 secrete more cytokines from AH patients than HVs (85% v 41%; $p<0.05$ and 89% v 59%;
576 $p<0.05$ respectively). (E) Fold change in global cytokine expression in the presence of LPS.
577 Addition of LPS does not yield any difference in cytokine expression AH monocyte subsets
578 or in HV intermediate monocytes. Bars charts show mean with SEM.
579

580 **Figure 3. AH monocytes have enhanced phagocytic capacity compared to HV**
581 **monocytes.** Monocytes isolated by magnetic bead separation from HVs (n=5) and patients
582 with AH (n=10) were cultured with fluorescent coated microspheres in the presence of LPS.
583 (A) Example flow cytometry dotplot of monocyte subsets cultured with fluorescent
584 microparticles. Histograms demonstrate monocytes positive and negative for fluorescently
585 labelled microparticles from classical and intermediate monocyte subsets. Intensity of
586 fluorescence represents the number of microparticles phagocytosed by each monocyte. (B)
587 The proportion of monocytes with intracellular microparticles was quantified in each
588 monocyte subset by flow cytometry. Monocytes from AH patients were more efficient at
589 phagocytosis compared to those from HVs. (C) The proportion of monocytes from each
590 subset which had phagocytosed 2 or more microparticles each was quantified in monocytes
591 from HVs and patients with AH. Intermediate monocytes from AH patients had greater
592 capacity to phagocytose more than 1 microparticle each compared to those from HVs. Scatter
593 dot plots show mean and SEM. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.
594

595 **Figure 4. Monocytes from AH patients drive greater T cell proliferation and IL-17A**
596 **expression than HV monocytes.** Memory T cells and monocyte subsets were isolated from
597 HVs (n=16) and patients with AH (n=5) and co-cultured. T cells were labelled with CFSE
598 and proliferation was determined by CFSE dilution and intracellular cytokine expression

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599 determined by flow cytometry. (A) Percentage of $CFSE^{lo}$ memory T cells. (B) and (C).

600 Percentage of single positive IL-17 (B) and IFNγ (C) expressing cells. (D) Percentage of cells

601 co-expressing IL-17 and IFNγ. (E) Ratio of IFNγ/IL-17. Bars charts show mean with SEM.

602 *$p<0.05$; **$p<0.01$

603

604 **Figure 5. Intrahepatic monocytes are enriched in patients with AH.** (A) Representative

605 confocal immunostaining images from the portal tract of a liver biopsy from a patient with

606 AH (left) and ALD control (right) with multiple dual staining cells (yellow) seen in the AH

607 sample only. ALD controls are actively drinking patients with cirrhosis but no acute

608 inflammatory component. (B) The mean number of $CD14^+$ monocytes in liver tissue from

609 patients with AH (n=7) and patients with ALD (n=4) taken a median of 7.5 days after

610 commencement of glucocorticoid treatment averaged from 3 portal and 3 lobular fields. (C)

611 There are more $CD14^+CD16^+$ monocytes in portal areas in AH compared to ALD patients

612 (6.3 v 1.3; $p<0.05$). (D) There are more $CD14^+CD16^-$ monocytes in portal regions in AH

613 compared to ALD patients (15.7 v 9.0; $p<0.05$). Scatter dot plots show mean with SEM.

614 *$p<0.05$.

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TABLES

Table 1. Participant characteristics for AH and ALD patients at baseline and mortality at days 28 and 90.

	Disease category	
	AH (n=41)	**ALD (n=10)**
Age	50.2 (13.4)	51.8 (9.8)
Gender (%female)	52	50
Bilirubin (µmol/L)	288 (166)	102 (73)
Albumin (g/L)	25.7 (7.1)	30.1 (5.1)
INR	1.6 (0.5)	1.6 (0.5)
Creatinine (µmol/L)	68.4 (40.1)	70.1 (20.0)
Sodium (µmol/L)	133 (53)	128.3 (9.7)
DF	60.8 (23.9)	28.1 (28.4)
MELD	18.1 (6.6)	15.1 (5.4)
28 day mortality (%)	14	10
90 day mortality (%)	24	30

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Figure 1

A

Monocytes x 10^9/L

Baseline monocytes

B

HV

AH

Non-classical

Intermediate

Classical

CD16

CD14

C

Classical

Intermediate

Non classical

% monocytes

HV AH ALD

ns

D

HV

AH

CCR2

CCR5

CX_3CR1

HLA-DR

CD206

CD80

Fold difference v HV Classical

Classical Intermediate Non-classical

Figure 2

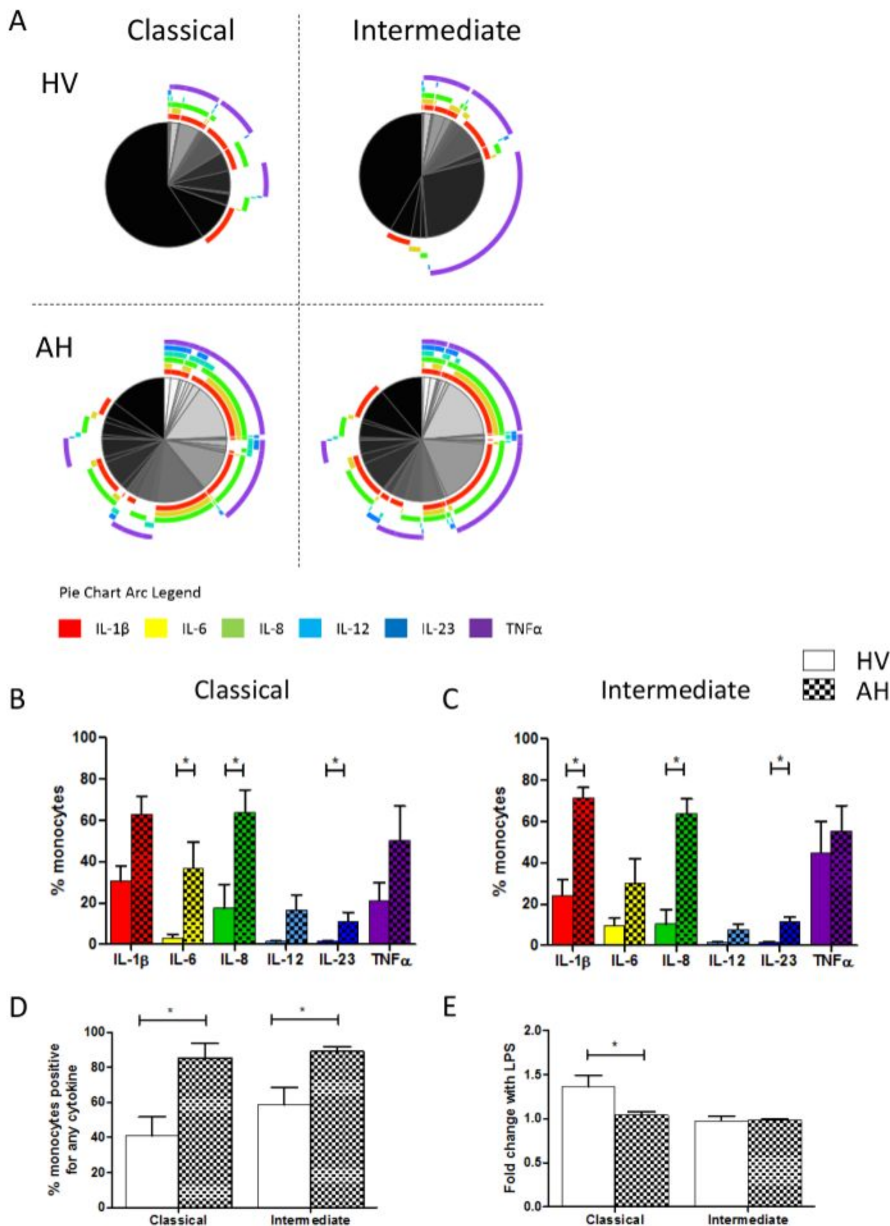


Figure 3

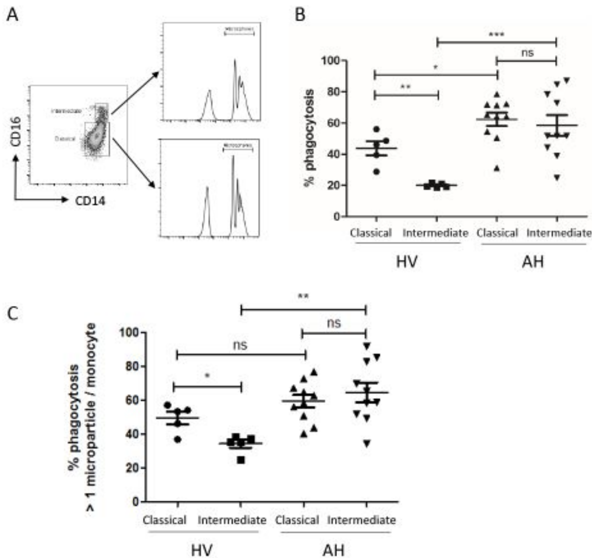
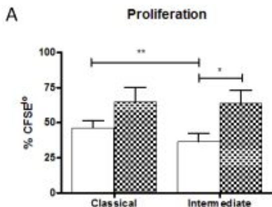
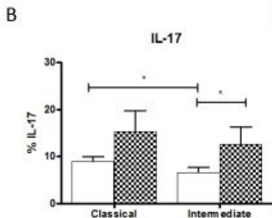
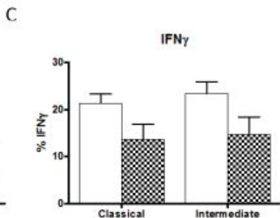
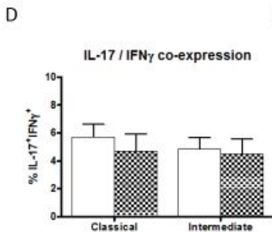


Figure 4









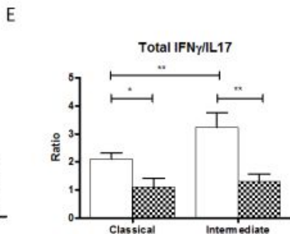


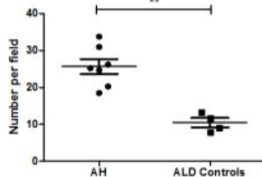
Figure 5

A

DAPI
CD14
CD16
100 µm
AH
ALD

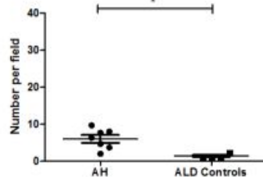
B

Total CD14⁺ monocytes



C

CD14⁺CD16⁺ monocytes



D

CD14⁺CD16⁻ monocytes