

1 **Alcohol dependence promotes systemic IFN- $\gamma$  and IL-17 responses in mice**

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14 **Abstract**

15 Alcohol use disorder (AUD) is a chronic relapsing disorder characterized by an impaired ability  
16 to stop or control alcohol use despite adverse social, occupational, or health consequences. AUD  
17 is associated with a variety of physiological changes and is a substantial risk factor for numerous  
18 diseases. We aimed to characterize systemic alterations in immune responses using a mouse  
19 model of chronic intermittent alcohol exposure to induce alcohol dependence. We exposed mice  
20 to chronic intermittent ethanol vapor for 4 weeks and used multiparametric flow cytometry to  
21 analyze the expression of cytokines IFN- $\gamma$ , IL-4, IL-10, IL-12 and IL-17 by different immune  
22 cells in the blood, spleen and liver of alcohol dependent and non-dependent control mice. We  
23 found increases in IFN- $\gamma$  and IL-17 expression in a cell type- and organ-specific manner. Often,  
24 B cells and neutrophils are primary contributors to increased IFN- $\gamma$  and IL-17 levels while other  
25 cell types play a secondary role. We conclude that chronic alcohol exposure promotes systemic  
26 pro-inflammatory IFN- $\gamma$  and IL-17 responses in mice. These responses are likely important in the  
27 development of alcohol-related diseases, but further characterization is necessary to understand  
28 the initiation and effects of systemic inflammatory responses to chronic alcohol exposure.

## 29 **Introduction**

30           Chronic intermittent alcohol exposure is associated with increased risk of cancer, organ  
31 damage, and infection [1-3]. At the molecular level, chronic alcohol alters inflammatory  
32 processes which regulate immune function. This is often characterized by changes in cytokine  
33 expression and immune defense mechanisms of type 1—cell-mediated immunity associated with  
34 IFN- $\gamma$ , IL-12 and TNF- $\alpha$  expression—or type 2—humoral immunity associated with IL-4, IL-10  
35 and IgE expression—immune responses [4]. One mechanism through which chronic alcohol  
36 exposure leads to adverse clinical outcomes is well characterized: increased expression of TNF- $\alpha$   
37 by liver macrophages promotes inflammation leading to alcoholic liver disease (ALD)  
38 development in human alcoholics [5, 6].

39           Work in humans and rodents demonstrates that altered TNF- $\alpha$  expression is not limited to  
40 the liver; TNF- $\alpha$  levels also increase in the blood and spleen following chronic alcohol exposure  
41 [7, 8]. Furthermore TNF- $\alpha$  is not the only cytokine which exhibits increased expression  
42 following chronic alcohol exposure. IFN- $\gamma$  and IL-17, among others, are increased in systemic  
43 pro-inflammatory responses [9-12]. Notably, IL-17 is not a type 1 or type 2 cytokine; it mediates  
44 highly inflammatory type 17 responses [13, 14]. Macrophages may play a central role in TNF- $\alpha$   
45 production following chronic alcohol exposure, but a variety of other immune cell types—B  
46 cells, T cells, NK cells, NKT cells, neutrophils and dendritic cells—have also been implicated as  
47 mediators of alcohol-associated inflammation [10, 11, 15-17].

48           Despite the numerous studies addressing organ-specific inflammatory response  
49 mechanisms of individual cell types to chronic alcohol exposure, the field lacks knowledge of  
50 the interplay between these responses systemically. Furthermore, most studies analyze  
51 differences in the immune response following chronic alcohol exposure in the context of

52 infection or disease progression. Less is known about the effects of alcohol on immune function  
53 prior to infection or disease development.

54         Thus, in this study, we aimed to characterize changes in the immune system of alcohol  
55 dependent mice at steady state through multiparametric flow cytometry. Looking beyond TNF-  
56  $\alpha$ 's established functions, we analyzed changes in other pro- and anti-inflammatory cytokines—  
57 IFN- $\gamma$ , IL-4, IL-12, IL-17 and IL-10—and the cells which produce them—B cells, T cells, NK  
58 cells, NKT cells, macrophages, neutrophils and dendritic cells—in the blood, spleen and liver of  
59 alcohol dependent mice. We found significant systemic upregulation of IFN- $\gamma$  and IL-17 and  
60 identified B cells and neutrophils as major contributors to these responses. The results add new  
61 insight to the systemic effects of alcohol dependence on immunity and the organ-specific  
62 properties of the response.

## 63 **Materials and Methods**

### 64 *Animals*

65 Male IL10<sup>tm1Flv</sup> (stock no: 008379) mice were obtained from The Jackson Laboratory (ME) and  
66 bred in-house. Mice were group-housed in a temperature and humidity-controlled vivarium on a  
67 12 hour reversed light/dark cycle with food and water available *ad libitum*. All protocols  
68 involving the use of experimental animals in this study were approved by The Scripps Research  
69 Institute (TSRI) Institutional Animal Care and Use Committee and were consistent with the  
70 National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 71 *Chronic-intermittent ethanol vapor exposure*

72 To induce ethanol dependence, mice were exposed to chronic intermittent ethanol inhalation  
73 (CIE) as previously described [18]. Briefly, mice in the dependent group (n=8) were i.p. injected  
74 with 1.75 g/kg alcohol + 68.1 mg/kg pyrazole (alcohol dehydrogenase inhibitor) and placed in  
75 vapor chambers (La Jolla Alcohol Research, La Jolla, CA) for 4 days (16 hours vapor on, 8 hours  
76 off) followed by 72 hours of forced abstinence [19, 20]. This regimen was repeated for a total of  
77 4 full rounds. Non-dependent mice (n=5) were injected with 68.1 mg/kg pyrazole in saline and  
78 received only air in similar chambers for the same intermittent period as the dependent group. On  
79 the third day of vapor exposure tail blood was collected to determine blood ethanol levels  
80 (BELs). Alcohol drip rates in the vapor chambers were altered such that BELs progressively  
81 increased over the vapor rounds to a final target of 200-250 mg/dL. Before euthanasia, dependent  
82 mice were exposed to a single alcohol vapor exposure (16 hours), and experiments were  
83 conducted from dependent mice directly from the vapor chambers.

### 84 *Cell isolation and flow cytometry analysis*

85 Single cell suspensions were generated from blood, spleen, and liver of alcohol dependent and  
86 non-dependent mice. Cells were extracted from spleen and livers by mechanical disruption and  
87 washed with PBS through 40- $\mu$ m mesh filters. Immune cells were enriched through incubation  
88 with ACK buffer (spleen) or through density gradient centrifugation using Ficoll-Paque (GE  
89 Healthcare) following the manufacturer's protocol (blood and liver). Immune cells were washed  
90 with PBS containing 2% FBS and incubated with murine Fc Block at 4° C for 10 min prior to  
91 staining. Cells were stained with extracellular antibodies, washed, permeabilized, incubated with  
92 Fc Block again, and finally stained with intracellular antibodies. Permeabilization and  
93 intracellular staining steps were performed with the FoxP3 permeabilization buffer kit (Tonbo  
94 Biosciences, San Diego, CA) following the manufacturer's protocol. Flow cytometry data was  
95 acquired on a four-laser Aurora (Cytex, Fremont, CA) and analyzed with FlowJo v 10.6.2  
96 (Becton, Dickinson, and Company, Franklin Lakes, NJ). Graphing and statistical analyses were  
97 performed on Graphpad Prism 8.4.0 (San Diego, CA). **Supplementary Table 1** provides a list of  
98 the antibodies used for the experiment.

## 99 **Results**

### 100 *Systemic immune responses are altered in alcohol dependent mice*

101 We observed organ-specific changes in immune cell and cytokine expression in alcohol  
102 dependent mice compared to non-dependent controls. **Supplementary Table 2** summarizes the  
103 gating strategy used to identify the various CD45<sup>+</sup> cell types highlighted throughout this analysis.  
104 We present cell type expression as percentages of total CD45<sup>+</sup> cells; an increase does not always  
105 translate to higher cell numbers but could be due to decreases in other cell types and less total  
106 CD45<sup>+</sup> cells. B cells, NK cells, T cells and NKT cells represent a higher proportion of CD45<sup>+</sup>  
107 cells than macrophages, neutrophils and dendritic cells in the blood, spleen and liver of  
108 dependent and non-dependent mice (Fig 1A-C).

109 **Figure 1: Alcohol dependent and non-dependent mice present with significant differences**  
110 **in immune cell compositions and cytokine expression.** (A-C) Immune cell expression as  
111 percentages of all CD45<sup>+</sup> cells in (A) blood, (B) spleen and (C) liver isolated from alcohol  
112 dependent (red, solid fill) and non-dependent (black outline, white fill) mice. (D-F) Expression  
113 of cytokines in (D) blood, (E) spleen and (F) liver CD45<sup>+</sup> cells isolated from dependent and non-  
114 dependent mice. \*, p<0.05; \*\*p<0.01 analyzed by Mann-Whitney U test; n=5-8.

115 The data demonstrate significant organ-specific differences in the distribution of immune  
116 cells in alcohol dependent mice compared to non-dependent controls: the blood exhibits changes  
117 in both lymphocyte and myeloid cell populations, whereas the spleen is mostly impacted by  
118 altered myeloid cell expression and changes in the liver are limited to lymphocytes. Of note, the  
119 relative abundance of neutrophils increases significantly in the blood and the spleen (Fig 1A,B).  
120 The liver exhibits significant changes in lymphocyte expression: a large decrease in liver B cells  
121 is complemented by an increase in T cells and NKT cells (Fig 1C). The changes in blood

122 lymphocyte populations did not reach significance, but the data resemble the dynamic changes of  
123 the liver more than the consistency of the spleen. Although, we did not investigate the cause of  
124 altered immune cell expression in alcohol dependent mice, proliferation, migration or cell death  
125 could contribute to these changes. Furthermore, we do not present cell numbers because the total  
126 cells analyzed from each mouse varied. Figures 1A-C primarily serve as a reference to  
127 contextualize cell specific-changes in the upcoming figures.

128 CD45<sup>+</sup> cells from the blood, spleen and liver also show differences in cytokine  
129 expression between dependent and non-dependent mice. We measured expression of type 1  
130 cytokines IFN- $\gamma$  and IL-12, type 2 cytokines IL-4 and IL-10, and type 17 cytokine IL-17. IFN- $\gamma$   
131 and IL-17 levels increase in all three organs, although the changes are not statistically significant  
132 in the liver. IL-12 expression did not change significantly in any organs despite its traditional  
133 role in stimulating IFN- $\gamma$  expression [21]. We did observe a significant increase in IL-10  
134 expression in the liver, but we question the physiological relevance because of its low frequency  
135 (Fig 1D-F). Nevertheless, we found strong evidence that chronic alcohol exposure promotes  
136 systemic IFN- $\gamma$  and IL-17 responses.

137 Next, we investigated further into expression of CD4<sup>+</sup> T cell subsets which selectively  
138 express these cytokines: Th1, Th2, Th17 and T regulatory (Treg) cells. They are often considered  
139 the main regulators of systemic type 1, 2, and 17 immunity and IL-10-mediated regulatory  
140 responses, respectively, but we found minimal differences in CD4<sup>+</sup> T cell cytokine expression  
141 between the two groups (S1A-F Fig). Th1 levels in the liver increase, however these cells are  
142 expressed at low frequency and are likely not the only contributor to increased IFN- $\gamma$  levels (S1F  
143 Fig). Although we observed clear increases in overall IFN- $\gamma$  and IL-17 levels, we do not see  
144 large changes in CD4<sup>+</sup> T cell expression patterns. It is unlikely that changes in T cell cytokine



145 expression alone are driving these type 1 and type 17 immune responses. Therefore, we  
146 hypothesized that other immune cell types are important in promoting type 1 and type 17  
147 immunity in alcohol dependent mice.

148

### 149 *Pro-inflammatory type 1 and type 17 responses are altered in alcohol dependent mice*

150 We analyzed the source of increased pro-inflammatory IFN- $\gamma$  and IL-17 cytokine  
151 responses in alcohol dependent mice compared to non-dependent controls. First, we investigated  
152 the cell types responsible for total IFN- $\gamma$  levels in the blood, spleen and liver (Fig 2A-C). We  
153 also analyzed the expression of IFN- $\gamma$  as a percentage of each individual cell type in all three  
154 organs (Fig 2D-J). Together, these analyses give a better understanding of the source(s) of  
155 increased IFN- $\gamma$  expression in each organ. Interestingly, the proportions of cell types which make  
156 up the total CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup> population do not mirror the distribution of all CD45<sup>+</sup> cells. For  
157 example, macrophages and neutrophils are among the largest contributors to IFN- $\gamma$  expression  
158 whereas they make up a minimal proportion of CD45<sup>+</sup> cells for all three organs (Fig 1A-C, 2A-  
159 C).

160 **Figure 2: IFN- $\gamma$  expression is increased in alcohol dependent compared to control mice.** (A-  
161 C) Immune cells as percentages of all IFN- $\gamma$  producing CD45<sup>+</sup> cells in (A) blood, (B) spleen and  
162 (C) liver isolated from alcohol dependent (red, solid fill) and non-dependent (black outline, white  
163 fill) mice. (D-J) Expression of IFN- $\gamma$  by (D) B cells, (E) NK cells, (F) T cells, (G) NKT cells, (H)  
164 macrophages, (I) neutrophils and (J) dendritic cells in dependent and non-dependent mice. \*,  
165 p<0.05; \*\*p<0.01 analyzed by Mann-Whitney U test; n=5-8.

166 In the blood, the distribution of CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cell types are mostly consistent between  
167 dependent and non-dependent mice. Neutrophils are responsible for 50-60% of IFN- $\gamma$  production

168 in both groups (Fig 2A). Despite minimal changes between the two groups in the composition of  
169 IFN- $\gamma$ <sup>+</sup> cells in the blood, the percent of B cells and neutrophils which produce IFN- $\gamma$  is  
170 increased significantly; B cell expression increases more dramatically than neutrophil  
171 expression, but a much higher percentage of neutrophils produce IFN- $\gamma$  overall (Fig 2D, I).  
172 Neutrophils are likely the main source of increased IFN- $\gamma$  in the blood of the alcohol dependent  
173 mice, while B cells are a secondary source.

174 We also found that CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells in the spleen of dependent mice are characterized  
175 by a lower proportion of B cells and higher proportion of neutrophils compared to non-dependent  
176 mice. Although not statistically significant, these changes are evident in the data (Fig 2B). Like  
177 in the blood, splenic B cells are characterized by increased IFN- $\gamma$  expression (Fig 2D); the  
178 expression level is low, but B cells comprise about 60% of total CD45<sup>+</sup> cells and 20-30% of  
179 CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells in the spleen (Fig 1B, 2B). Increased IFN- $\gamma$  expression in splenic  
180 macrophages complements the increase in B cell IFN- $\gamma$  expression (Fig 2H). Despite  
181 representing a smaller percentage of CD45<sup>+</sup> cells, they contribute similarly to total CD45<sup>+</sup> IFN-  
182  $\gamma$ <sup>+</sup> cells because about 20% of them produce IFN- $\gamma$  (Fig 1B, 2B). NK cells and DCs also show  
183 increased expression of IFN- $\gamma$  in the spleen, although their minimal contributions to overall IFN-  
184  $\gamma$  levels may render this change less important to overall IFN- $\gamma$  responses (Fig 2B, E, J).

185 The role of neutrophils is unclear in the spleen. They do not show increases in IFN- $\gamma$   
186 expression on a per cell basis (Fig 2I), however they increase as a proportion of CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup>  
187 cells in the spleen in dependent mice (Fig 2B). This could be explained by their significant  
188 increase as a percentage of CD45<sup>+</sup> cells (Fig 1B): increased abundance of neutrophils contributes  
189 to increased IFN- $\gamma$  expression without significant changes in the percent of neutrophils  
190 producing IFN- $\gamma$ . Increases in spleen B cell and macrophage IFN- $\gamma$  levels—and potentially

191 increases in neutrophil abundance—are the main source of observed increases in IFN- $\gamma$  in the  
192 spleen of alcohol dependent mice.

193         Dynamic changes in CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cell type expression occur in the liver. NK cells  
194 make up about 20% of IFN- $\gamma$  producing cells in non-dependent mice but become a strikingly  
195 lower contributor to total IFN- $\gamma$ <sup>+</sup> cells in dependent mice. This is accompanied by trends towards  
196 decreases in B cell and increases in T cell and macrophage contributions to total IFN- $\gamma$  levels  
197 (Fig 2C). Liver B cells and NKT cells have significant increases in IFN- $\gamma$  expression (Fig 2D,  
198 G), however non-significant trends towards increased IFN- $\gamma$  in T cells, macrophages and  
199 neutrophils (Fig 2F, H, I) should be considered due to their higher relative contributions to the  
200 total IFN- $\gamma$  pool in the liver. Although the trend towards increased total expression of IFN- $\gamma$  in  
201 the liver was not significant (Fig 1F), the data here show a variety of changes in the production  
202 of IFN- $\gamma$  across nearly every cell type. Altogether, **Figure 2** demonstrates that activation of the  
203 type 1 immune response is a prominent aspect of chronic alcohol exposure's immunomodulatory  
204 effects: the liver, spleen and blood each exhibit activation of type 1 responses in an organ-  
205 specific manner.

206         We then completed a parallel analysis to understand increased IL-17 expression in  
207 alcohol dependent mice compared to non-dependent controls (Fig 1D-F). Like CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup>  
208 cells, CD45<sup>+</sup> IL-17<sup>+</sup> cells do not mirror the distribution of all CD45<sup>+</sup> cells: macrophages and  
209 neutrophils represent a much larger proportion of IL-17 producing CD45<sup>+</sup> cell than all CD45<sup>+</sup>  
210 cells (Fig 1A-C, 3A-C). In the blood of alcohol dependent mice, the B cell population increases  
211 significantly as a percentage of all CD45<sup>+</sup> IL-17<sup>+</sup> cells, accompanied by trends towards  
212 increasing NK cell and decreasing NKT cell percentages. Neutrophils are the dominant CD45<sup>+</sup>

213 IL-17<sup>+</sup> cell type, while expression of other cells is relatively evenly distributed within this  
214 population (Fig 3A).

215 **Figure 3: IL-17 expression is increased in alcohol dependent compared to control mice.** (A-  
216 C) Immune cells as percentages of all IL-17 producing CD45<sup>+</sup> cells in (A) blood, (B) spleen and  
217 (C) liver isolated from alcohol dependent (red, solid fill) and non-dependent (black outline, white  
218 fill) mice. (D-J) Expression of IL-17 by (D) B cells, (E) NK cells, (F) T cells, (G) NKT cells, (H)  
219 macrophages, (I) neutrophils and (J) dendritic cells in dependent and non-dependent mice. \*,  
220  $p < 0.05$ ; \*\* $p < 0.01$  analyzed by Mann-Whitney U test;  $n = 5-8$ .

221 The percentage of B cells, NK cells and neutrophils expressing IL-17 increase  
222 significantly in the blood of dependent mice (Fig 3D, E, I). Of the lymphocytes, NK cells may be  
223 more important contributors to increased IL-17 levels than B cells: NK cell expression levels  
224 increase in the blood of dependent mice whereas B cells decrease (Fig 1A) and NK cells  
225 contribute more significantly to the total blood CD45<sup>+</sup> IL-17<sup>+</sup> cell population (Fig 3A). NK cells,  
226 B cells and neutrophils together are the major sources of increased IL-17 expression in the blood  
227 of dependent mice.

228 In the spleen, B cells, macrophages and neutrophils make up the vast majority of CD45<sup>+</sup>  
229 IL-17<sup>+</sup> cells; this distribution does not vary between dependent and non-dependent groups (Fig  
230 3B). B cell IL-17 expression increases significantly in dependent spleen, although the frequency  
231 of IL-17<sup>+</sup> B cells is quite low (Fig 3D). Like for IFN- $\gamma$ , this is counterbalanced by an abundance  
232 of B cells in the spleen; a small increase in the percent of IL-17<sup>+</sup> splenic B cells could still  
233 promote type 17 responses. T cells also show significantly increased IL-17 expression (Fig 3F),  
234 but the frequency is low—around 2% in dependent mice—and the contribution of T cells to total  
235 IL-17<sup>+</sup> CD45<sup>+</sup> cells is minimal (Fig 3B). Non-significant trends towards increased IL-17

236 expression in macrophages and neutrophils, the other major contributors to total CD45<sup>+</sup> IL-17<sup>+</sup>  
237 cells besides B cells, are likely important in the overall increases in IL-17 observed in the  
238 dependent spleen (3H, I).

239 In the liver, the proportion of neutrophils in the CD45<sup>+</sup> IL-17<sup>+</sup> population increases  
240 significantly. T cells also show increasing trends which are offset by decreased contributions by  
241 NKT cells, B cells and macrophages to total IL-17<sup>+</sup> cells (Fig 3C). Despite a potential decrease  
242 in macrophages, they still represent the largest proportion of CD45<sup>+</sup> IL-17<sup>+</sup> cells in the liver. No  
243 specific cell populations show significant increases in IL-17 expression, so it is difficult to  
244 attribute the overall increase in IL-17<sup>+</sup> cells to one cell type (Fig 3D-J). However, each of them  
245 exhibits a trend towards increasing IL-17 expression which likely add up to the overall increase  
246 in IL-17 levels in the liver. This, considered with changes in the distribution of CD45<sup>+</sup> IL-17<sup>+</sup>  
247 cells, suggests a dynamic type 17 immune response in the liver: interactions between a variety of  
248 cell types contribute to excess IL-17 production and inflammation.

249

### 250 *Anti-inflammatory immune responses are not altered in alcohol dependent mice*

251 We also observed a significant increase in IL-10 levels in the liver of alcohol dependent  
252 mice compared to controls (Fig 1F). Although in both groups IL-10 was only expressed in about  
253 0.1% of CD45<sup>+</sup> cells, we investigated the potential for increased anti-inflammatory responses in  
254 alcohol dependent mice. The abundance of IL-10 was too low to accurately analyze which cell  
255 types produce notable amounts, but we analyzed the expression of the IL-10 receptor (IL-10R)  
256 and found a significant increase in the spleen and a trend towards increased IL-10R expression in  
257 the blood of dependent mice compared to non-dependent controls (S2A Fig). It is unclear

258 whether changes in IL-10R expression are important in this context as very few cells express IL-  
259 10 in the organs analyzed from both groups.

260 Nevertheless, we analyzed IL-10R expression in immune cells from the blood, spleen and  
261 liver. The cell types which make up the total CD45<sup>+</sup> IL-10R<sup>+</sup> population vary by organ and  
262 exhibit subtle changes in alcohol dependent mice compared to non-dependent controls (S2B-D  
263 Fig). The most notable change between the two groups is an increase in B cells as a percentage  
264 of the total IL-10R<sup>+</sup> population in the blood (S2B Fig). This is reflected by a significantly higher  
265 expression of IL-10R as a percentage of total blood B cells (S2E Fig). We observe consistent  
266 increases in B cell activation in our data; increased IL-10R expression could be a measure to  
267 prevent overactivation, but with low levels of circulating IL-10 a higher IL-10R expression  
268 would have minimal effects (S2D Fig).

269 In general, lymphocyte IL-10R expression in alcohol dependent mice increases whereas  
270 myeloid cell IL-10R expression decreases (S2E-K Fig). In line with increases in IL-10R  
271 expression blood, spleen and liver lymphocytes, we also found increases in PD-1 expression in  
272 lymphocytes overall (S3A-D Fig). These increases are most pronounced in B cells, however  
273 trends towards increased expression are evident in other lymphocytes as well. Further  
274 investigation of anti-inflammatory responses and B cell regulation in response to chronic alcohol  
275 exposure would be beneficial to understand the physiological relevance of these changes.

276

### 277 ***Co-expression of IFN- $\gamma$ and IL-17 in alcohol dependent mice***

278 We observed that the cell types responsible for both IFN- $\gamma$  and IL-17 expression are  
279 distributed similarly in the blood, spleen and liver (Fig 2A-C, 3A-C). Thus, we investigated this  
280 further by analyzing the co-expression of these inflammatory cytokines. The percent of CD45<sup>+</sup>

281 cells which co-express IFN- $\gamma$  and IL-17 increase significantly in both the blood and liver of  
282 alcohol dependent mice compared to non-dependent controls. Co-expression of these cytokines  
283 in the spleen of dependent mice also trends towards an increase which is not significant (Fig 4A).  
284 The contribution of different cell types to total CD45<sup>+</sup> cells co-expressing IFN- $\gamma$  and IL-17  
285 differs from expression of each cytokine individually in one major aspect: B cells do not make  
286 up a large percentage of these cells in any of the analyzed organs. Macrophages and neutrophils,  
287 however, remain prominent contributors to total cytokine expression levels (Fig 4B-D).

288 **Figure 4: Immune cells co-expressing IFN- $\gamma$  and IL-17 are significantly increased in alcohol**  
289 **dependent mice.** (A) Co-expression of IFN- $\gamma$  and IL-17 in blood, spleen and liver CD45<sup>+</sup> cells  
290 isolated from alcohol dependent (red, solid fill) and non-dependent (black outline, white fill)  
291 mice. (B-D) Immune cells as percentages of all IFN- $\gamma$ <sup>+</sup> IL-17 $\gamma$ <sup>+</sup> CD45<sup>+</sup> cells in (B) blood, (C)  
292 spleen and (D) liver isolated from dependent and non-dependent mice. (E-K) Expression of IL-  
293 17 by (E) B cells, (F) NK cells, (G) T cells, (H) NKT cells, (I) macrophages, (J) neutrophils and  
294 (K) dendritic cells in dependent and non-dependent mice. \*, p<0.05; \*\*p<0.01 analyzed by  
295 Mann-Whitney U test; n=5-8.

296 In the blood—which sees the largest increase in IFN- $\gamma$  and IL-17 co-expressing cells in  
297 dependent mice compared to non-dependent controls—neutrophils account for the highest  
298 percent of CD45<sup>+</sup> IFN- $\gamma$ <sup>+</sup> IL-17<sup>+</sup> cells. In dependent mice, they are further separated from other  
299 cell types because NK cells become less prominent within this group (Fig 4B). The percentage of  
300 IFN- $\gamma$ <sup>+</sup> IL-17<sup>+</sup> neutrophils increases significantly, whereas the percentage of NK cells co-  
301 expressing these cytokines remains relatively consistent (Fig 4F, 4J). B cells, T cells and NKT  
302 cells increase in percent co-expressing IFN- $\gamma$  and IL-17, however the frequency is much lower

303 than neutrophils (Fig 4E, G, H). Neutrophils in the blood co-expressing IFN- $\gamma$  and IL-17 could  
304 be a major instigator of inflammatory immune responses to chronic alcohol exposure.

305 The IFN- $\gamma^+$  IL-17 $^+$  cells in the spleen are also mostly neutrophils, however co-expression  
306 of these cytokines does not change in splenic neutrophils of dependent mice compared to non-  
307 dependent controls (Fig 4C, J). The next most abundant IFN- $\gamma^+$  IL-17 $^+$  cell type in the spleen is  
308 the macrophage (Fig 4C). Macrophages are characterized by significant increases in IFN- $\gamma^+$   
309 IL17 $^+$  cells in dependent mice. B cells, T cells, NKT cells and dendritic cells also show  
310 significant increases in co-expression in the spleen (Fig 4B, G, H, K). These cell-specific  
311 changes suggest that IFN- $\gamma$  and IL-17 co-expression is relevant in a variety of splenic cell types  
312 in alcohol dependent mice, but altogether does not amount to significantly higher levels of IFN-  
313  $\gamma^+$  IL-17 $^+$  cells overall within the total CD45 $^+$  population in the spleen (Fig 4A).

314 In the liver, a significant decrease in the NK cell proportion of CD45 $^+$  IFN- $\gamma^+$  IL-17 $^+$  cells  
315 is accompanied by trends towards increased percentages of T cells, macrophages and neutrophils  
316 in response to chronic alcohol exposure (Fig 4D). The percentages of these cells largely varied  
317 across samples, so it is difficult to say there is a meaningful increase despite higher average  
318 values in dependent mice. Within individual cell types, there were not significant differences in  
319 the percentage of IFN- $\gamma^+$  IL-17 $^+$  cells, although T cells, macrophages and neutrophils look to  
320 have increases which do not reach statistical significance. These three cell types may  
321 complement each other in contributing to the overall increase of IFN- $\gamma$  and IL-17 co-expressing  
322 cells in dependent mice: T cells are the most highly expressed cell type in the liver but a lower  
323 percentage are IFN- $\gamma^+$  IL-17 $^+$  compared to macrophages and neutrophils; macrophages and  
324 neutrophils are less populous in the liver but have a higher percentage are IFN- $\gamma^+$  IL-17 $^+$   
325 compared to T cells (Fig 1C, 4G, I, J).



326 **Discussion**

327           In the present study, we found that alcohol dependent mice express higher levels of IFN- $\gamma$   
328 and IL-17 compared to non-dependent controls in their blood, spleen and liver. The data suggest  
329 that neutrophils and B cells are the major contributors to systemic pro-inflammatory type 1 and  
330 type 17 cytokine responses in alcohol dependent mice, although the importance of individual cell  
331 types varies in an organ-specific and cytokine-specific manner.

332           In the blood and spleen, neutrophils and B cells are primarily responsible for increased  
333 IFN- $\gamma$  and IL-17 expression in alcohol dependent mice. Blood NK cells and splenic macrophages  
334 are additional contributors to increased IL-17 and IFN- $\gamma$  expression, respectively. In the liver, we  
335 did not find any cell types to be majorly responsible for increased cytokine expression, but we  
336 hypothesize that non-significant trends towards increases in many cell types have cumulative  
337 effects on overall IFN- $\gamma$  and IL-17 levels in alcohol dependent mice. While trends towards  
338 increases in IFN- $\gamma$  and IL-17 individually in the liver are not significant, there is a significant  
339 increase in the overall co-expression of these cytokines due to T cells, macrophages and  
340 neutrophils. We also observed co-expression of IFN- $\gamma$  and IL-17 in blood neutrophils.

341           Our analysis reveals mechanisms through which cytokine expression by various cells  
342 promotes systemic type 1 and type 17 immune responses in alcohol dependent mice. Some of  
343 these inflammatory mechanisms have been addressed previously using *in vitro* or *in vivo* models  
344 of chronic alcohol exposure, but no other studies have investigated a similarly large range of  
345 cytokines, cell types and organs simultaneously [9-12, 15-17]. Most analyze one specific  
346 inflammatory process and focus on its role in the development of alcoholic liver disease (ALD),  
347 a leading cause of alcohol-related deaths [22]. In our analysis, we observed dynamic

348 inflammatory processes which occur independent of ALD and identified the key cellular and  
349 molecular mediators of this systemic inflammation.

350 We observed an increase in IFN- $\gamma$  expression but no change in IL-4 expression and  
351 concluded that chronic intermittent alcohol exposure in mice skews immunity towards systemic  
352 type 1 responses. The literature provides evidence both in support and opposition of these results.  
353 Excessive TNF- $\alpha$  production—evidence of type 1 responses— and increased IgE levels—  
354 evidence of type 2 responses—are hallmarks of alcoholism [4, 7, 23]. Studies of IFN- $\gamma$  and IL-4  
355 expression provide less consistent conclusions. Some data show increased IFN- $\gamma$ /IL-4 ratios  
356 whereas others find the opposite [4, 10, 11, 24-26]. Based on this, it is still unclear how IFN- $\gamma$   
357 and IL-4 production is altered in response to chronic alcohol use.

358 Although many of these studies report T cell cytokine secretion, we observed that T cells  
359 are not the main drivers of differential cytokine responses in alcohol dependent mice (S1 Fig). In  
360 fact, for every cell type besides T cells we saw significant differences in IFN- $\gamma$  expression in at  
361 least one organ. B cell IFN- $\gamma$  expression increased in all three organs. Neutrophils and  
362 macrophages also contribute to increased IFN- $\gamma$  levels: neutrophil IFN- $\gamma$  expression increases  
363 significantly in the blood, macrophage IFN- $\gamma$  expression increases significantly in the spleen, and  
364 both show non-significant trends towards higher IFN- $\gamma$  expression in the liver. There is limited  
365 evidence that B cells and neutrophils produce IFN- $\gamma$  in response to infection or stimulation [27-  
366 35]. Furthermore, macrophages are known to secrete IFN- $\gamma$  in vitro, and lung-resident alveolar  
367 have also been described to secrete IFN- $\gamma$  in response to pulmonary infection in vivo [36-40].

368 There are no studies of chronic alcohol use which address IFN- $\gamma$  production by any of  
369 these cell types, despite extensive studies of T cells in this context. Future studies should not  
370 depend on T cell cytokine secretion to understand the balance between type 1 and type 2

371 cytokines in response to chronic alcohol use. Experiments with T cells may overlook meaningful  
372 alterations in immunity due to cytokine production by other cells. Instead, studies should always  
373 analyze overall cytokine levels and consider neutrophils, B cells and macrophages as better  
374 measures of these changes.

375         We also observed systemic increases in IL-17 levels in alcohol dependent mice. Type 17  
376 responses to chronic alcohol exposure are less studied than type 1 and type 2 responses and most  
377 research addresses their role in alcoholic liver disease (ALD) development. Patients with ALD  
378 have increased IL-17 levels in the blood and liver which correlate with disease severity. In these  
379 patients, IL-17<sup>+</sup> liver-infiltrating cells are mostly T cells and neutrophils [9]. IL-17 blockade can  
380 reverse alcohol dependence and liver damage in mice [41]. Indeed, we found increases in  
381 neutrophil abundance and expression of IL-17 in the liver of alcohol dependent mice. Previous  
382 research found IL-17 is involved in neutrophil recruitment to the liver in ALD [9]. Our data  
383 support an additional ALD-independent role for IL-17<sup>+</sup> neutrophil infiltration in the liver during  
384 the early stages of alcohol-induced inflammation.

385         Beyond the liver, systemic IL-17-mediated inflammation due to chronic alcohol exposure  
386 has not been thoroughly investigated. Our data show IL-17 expression also increases in the blood  
387 and spleen in response to chronic intermittent alcohol exposure in mice. It is known that IL-17  
388 expression in the serum is increased in ALD patients [9]. We found that IL-17 expression in  
389 neutrophils, NK cells, and B cells increased dramatically in the blood of alcohol dependent mice  
390 compared to non-dependent controls. However, we are the first to report increased splenic B cell  
391 IL-17 expression that drives increases in spleen IL-17 levels.

392         It is well established that chronic alcohol exposure inhibits NK cell cytotoxic functions  
393 and modulates B cell antibody production [42-47]. Although neither cell type has been

394 implicated in alcohol-induced IL-17 production, there is evidence that both can contribute  
395 substantially to type 17 responses in infection and autoimmune disorders [48-50]. Here, we  
396 demonstrate that increased IL-17 expression by B cells, NK cells and neutrophils in the blood  
397 and spleen promote relevant type 17 responses beyond the liver in alcohol dependent mice.

398         The mechanisms behind altered IL-17 expression in alcoholics are not understood. Our  
399 data demonstrate that previously described increases in IL-17 in the liver of ALD patients are not  
400 exclusively a consequence of ALD: IL-17 expression increases systemically in the blood, spleen  
401 and liver after chronic intermittent alcohol exposure. These changes occur prior to any clinical  
402 manifestations of chronic alcohol use in mice. More studies are necessary to understand the  
403 molecular mechanisms which promote systemic type 17 responses to chronic alcohol exposure,  
404 the cell types which initiate this response, and the long-term effects on host health.

405         We also found increases in co-expression of IFN- $\gamma$  and IL-17 contributed to the skewing  
406 of type 1 and type 17 responses. IFN- $\gamma^+$  IL-17 $^+$  cells are not commonly studied, but there is  
407 evidence of T cell subsets which co-express these cytokines in chronic infections or autoimmune  
408 disorders [51-55]. Similarly, in dependent mice we did observe increases in IFN- $\gamma^+$  IL-17 $^+$  T  
409 cells in the blood and spleen, as well as evidence of B cells, NKT cells, macrophages,  
410 neutrophils and dendritic cells with increased IFN- $\gamma$  IL-17 co-expression in at least one organ.  
411 Co-expression of IFN- $\gamma$  and IL-17 is not documented beyond T cells. Even in T cells, it is  
412 unclear what mechanisms promote expression of IFN- $\gamma^+$  IL-17 $^+$  cells and what role they play in  
413 host defense. Our data suggest that co-expression of IFN- $\gamma$  and IL-17 should be considered in  
414 future studies as a relevant component of systemic inflammation due to chronic alcohol use.

415         In this analysis, we give a snapshot of diverse immune processes which are altered in  
416 alcohol dependent mice. The study's strength lies in the wide range of immune processes

417 investigated as we analyzed a variety of organs, cell types and cytokine secretion profiles to  
418 understand shifts in immune system characteristics in alcohol dependent mice. We did not  
419 perturb the immune system with other stimuli or pathogens, gaining insight into how immunity is  
420 altered at resting state.

421 In our model of alcohol dependence, mice were exposed to ethanol vapor intermittently  
422 for 4 weeks [18]. Of note this ethanol model is most commonly used for studies of addiction as it  
423 results in higher blood alcohol levels but does not induce the same levels of liver damage as  
424 alcohol drinking. Furthermore, rodent models that more closely mimic harmful inflammatory  
425 processes like ALD in humans may use an alcohol exposure time of 2-3 months [56]. The  
426 purpose of this study was not to understand ALD, rather to describe the systemic inflammatory  
427 processes that occur as mice develop dependence on alcohol. We cannot rule out that the  
428 inflammatory processes observed here lead directly to liver damage. Regardless, altered type 1  
429 and type 17 responses are worth further investigation for their role in altered systemic immunity  
430 and initiation of potentially harmful inflammation.

431 Our main goal was to investigate as many immune processes as possible which limited  
432 precision in differentiating between cell types. In distinguishing macrophage, neutrophil and  
433 dendritic cell populations, we may have misclassified some of the rarer populations. Our  
434 phenotypic definition of neutrophils excluded CD11c<sup>+</sup> neutrophils and that of macrophages  
435 excluded F4/80- macrophages. F4/80- CD11c- macrophages specifically would be identified as  
436 neutrophils in our analysis (Supplemental Table 2). We did not have the neutrophil-specific  
437 marker Ly6G included in the experiment which would have provided a clearer way to distinguish  
438 these populations [57].

439 Furthermore, defining type 1 and type 2 responses by only IFN- $\gamma$  and IL-4 simplifies  
440 these dynamic processes. We did not observe an increase in IL-12, a stimulator of type 1  
441 responses, in dependent mice despite higher IFN- $\gamma$  levels [21]. It would have been beneficial to  
442 compare our results to previous studies if we had measurements of type 1 and type 2 markers  
443 TNF- $\alpha$  and IgE, respectively, which are commonly seen to be elevated in models of alcohol  
444 dependence. Nevertheless, we observed clear increases in systemic IFN- $\gamma$  and IL-17 expression  
445 and believe future studies of chronic alcohol use would benefit from including these cytokines in  
446 their analyses.

447 The data provide insight into dynamic systemic responses that are underappreciated in  
448 alcohol research. Through our approach, we observed diverse immune processes which are  
449 altered in alcohol dependent mice. Future studies will assess whether different models of chronic  
450 alcohol exposure in mice such as liquid ethanol diet or a longer time period of alcohol exposure  
451 would provide a more human-like pattern of alcohol consumption and be more physiologically  
452 relevant to how alcohol affects the human body. A time course experiment analyzing the  
453 expression of inflammatory cytokines at various time points would be particularly insightful.

454 While most studies address the effects of chronic alcohol consumption on ALD, our data  
455 highlight systemic inflammatory processes which are activated prior to liver disease. These  
456 systemic changes could be important initiators of the severe long-term effects where the field  
457 currently focuses. We identify neutrophils and B cells as substantial contributors to early  
458 inflammatory processes of chronic alcohol exposure. Future studies are needed for a better  
459 understanding of mechanisms through which neutrophils and B cells are activated and how their  
460 altered functionalities contribute to the adverse effects of AUD.

461           In conclusion, we found that chronic intermittent alcohol exposure in mice induces  
462 systemic IFN- $\gamma$  and IL-17 inflammatory responses. A variety of cell types are responsible for  
463 these responses in a cytokine- and organ-specific manner, but neutrophil and B cell cytokine  
464 secretion patterns are the most commonly dysregulated across all organs studied. We observed  
465 these changes *in vivo* without additional stimulation suggesting that alcohol dependence alters  
466 immune system functions at steady state. The data we present provide valuable insight into  
467 systemic inflammatory responses in alcohol dependent mice and serve as a starting point for  
468 future studies to probe these alcohol-induced inflammatory mechanisms.

469 **Conflict of Interest Statement**

470 The authors have declared that no conflict of interest exists.

471

472 **Author Contributions**

473 Conceptualization – SP

474 Formal analysis – KF, SP

475 Funding acquisition – MR, SP

476 Investigation – KF, SA, RN, RRP, AJR

477 Methodology – SA, RN, RRP, AJR, MR, SP

478 Project administration – AJR, MR, SP

479 Resources –AJR, MR, SP

480 Supervision – SP

481 Validation – RRP, MR, SP

482 Visualization – KF, SP

483 Writing (original draft) – KF

484 Writing (review and editing) – RRP, MR, SP

485

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665 **Supporting Table 1:** Antibodies used for the multiparametric flow cytometry experiment.

666 **Supporting Table 2:** Gating strategy to identify individual cell types isolated from blood, spleen  
667 and liver of dependent and non-dependent mice.

668 **Supporting Figure 1: Alcohol dependent mice exhibit minor tissue specific changes in their**  
669 **T cell subset ratios.** (A-C) CD4<sup>+</sup> and CD8<sup>+</sup> T cell expression as a percentage of CD3<sup>+</sup> cells  
670 isolated from (A) blood, (B) spleen and (C) liver of alcohol dependent (red, solid fill) and non-  
671 dependent (black outline, white fill) mice. (D-F) Th1 (CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup>), Th2 (CD4<sup>+</sup> IL-4<sup>+</sup>), Th17  
672 (CD4<sup>+</sup> IL-17<sup>+</sup>), and Treg (CD4<sup>+</sup> FoxP3<sup>+</sup>) expression as a percentage of CD4<sup>+</sup> T helper cells  
673 isolated from (D) blood, (E) spleen and (F) liver of dependent and non-dependent mice. \*,  
674 p<0.05; \*\*p<0.01 analyzed by Mann-Whitney U test; n=5-8.

675 **Supporting Figure 2: IL-10 receptor expression is increased in alcohol dependent mice**  
676 **compared to controls.** (A) Expression of IL-10 receptor (IL-10R) blood, spleen and liver  
677 CD45<sup>+</sup> cells isolated from alcohol dependent (red, solid fill) and non-dependent (black outline,  
678 white fill) mice. (B-D) Immune cells as percentages of all IL-10R<sup>+</sup> CD45<sup>+</sup> cells in (B) blood, (C)  
679 spleen and (D) liver isolated from dependent and non-dependent mice. (E-K) Expression of IL-  
680 10R by (E) B cells, (F) NK cells, (G) T cells, (H) NKT cells, (I) macrophages, (J) neutrophils  
681 and (K) dendritic cells in dependent and non-dependent mice. \*, p<0.05; \*\*p<0.01 analyzed by  
682 Mann-Whitney U test; n=5-8.

683 **Supporting Figure 3: PD-1 expression is minimally altered in lymphocytes of alcohol**  
684 **dependent mice compared to controls.** (A-D) Expression of PD-1 by (E) B cells, (F) NK cells,  
685 (G) T cells, and (H) NKT cells in alcohol dependent (red, solid fill) and non-dependent (black  
686 outline, white fill) mice. \*, p<0.05; \*\*p<0.01 analyzed by Mann-Whitney U test; n=5-8.

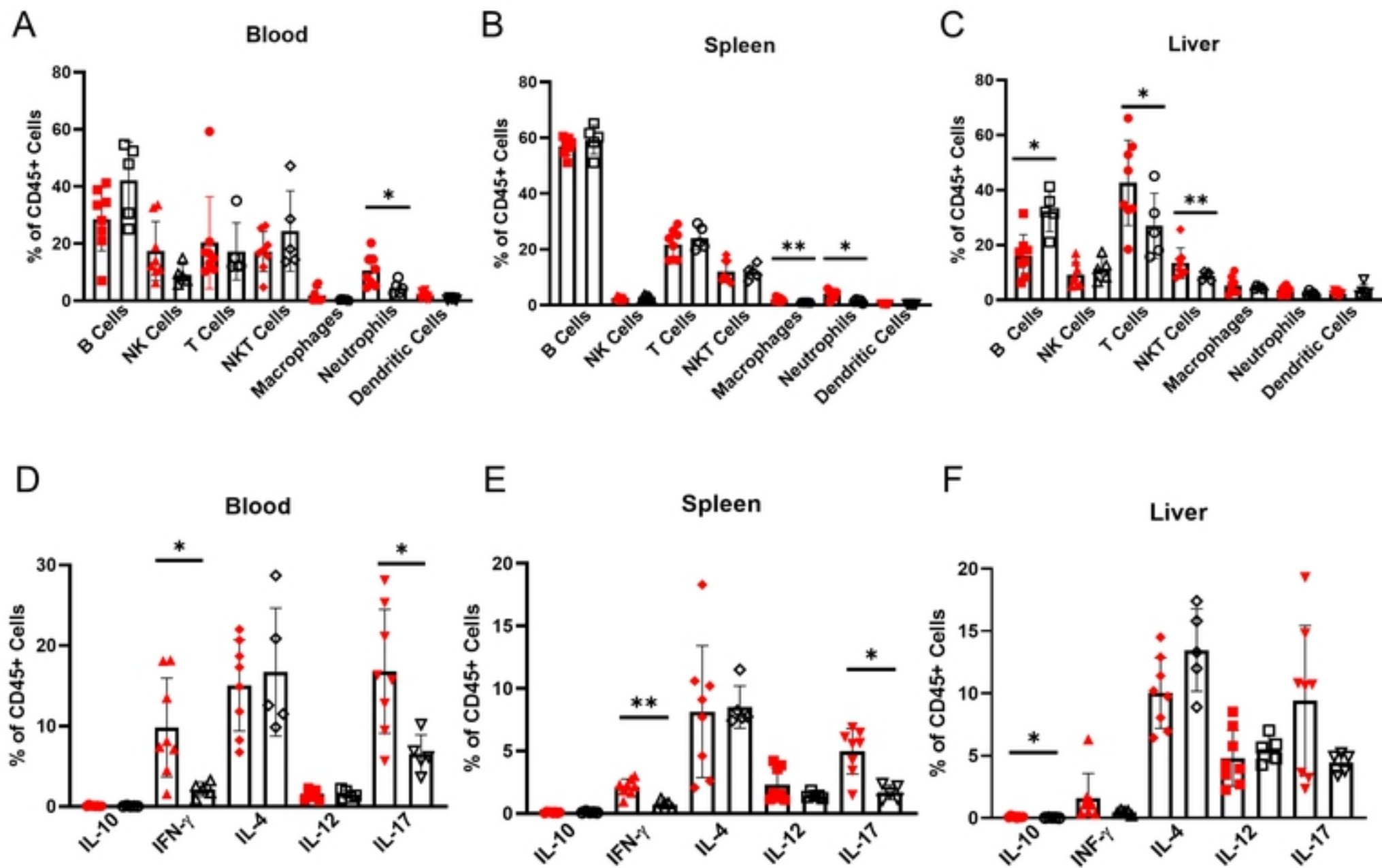


Figure 1

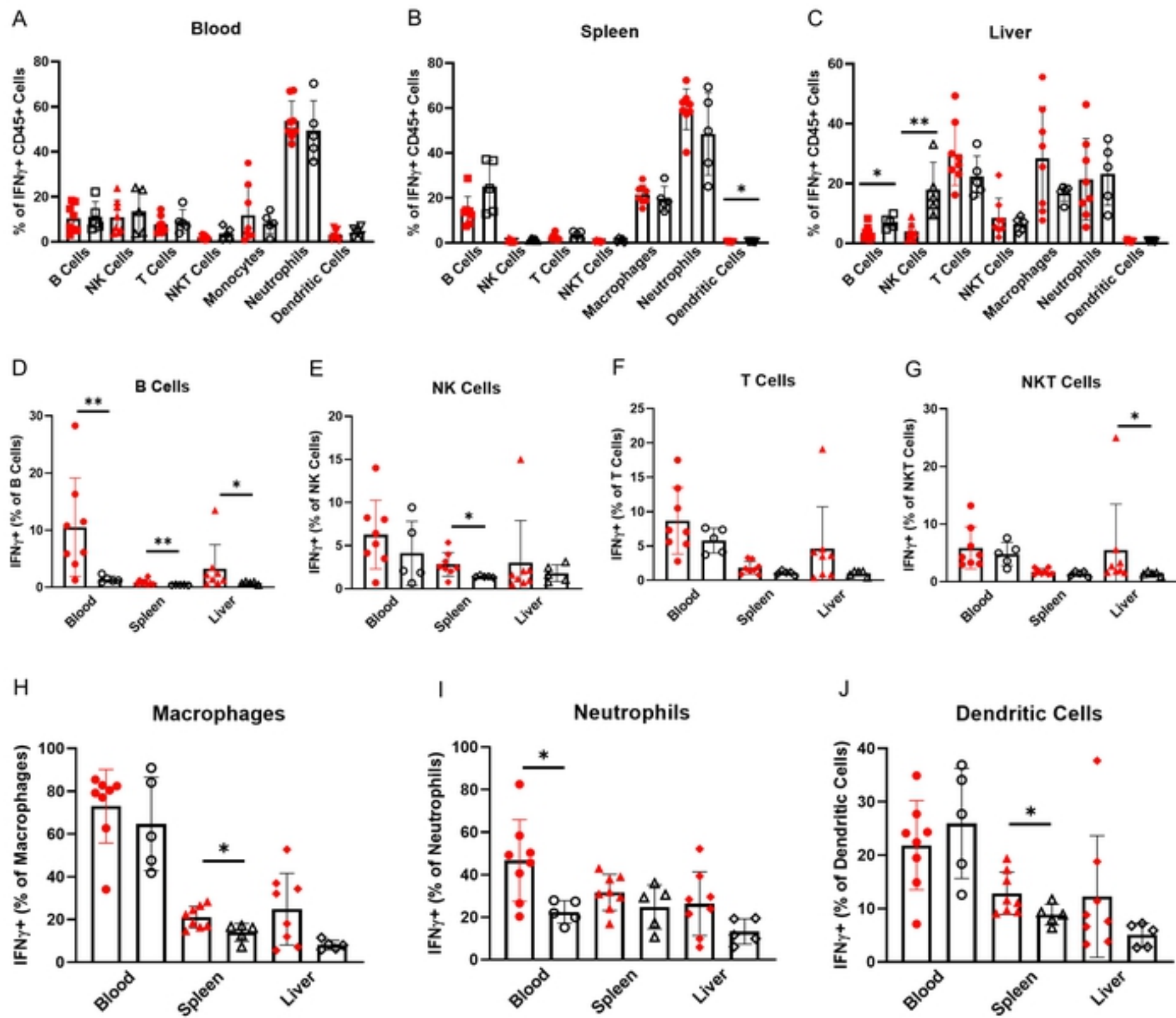


Figure 2

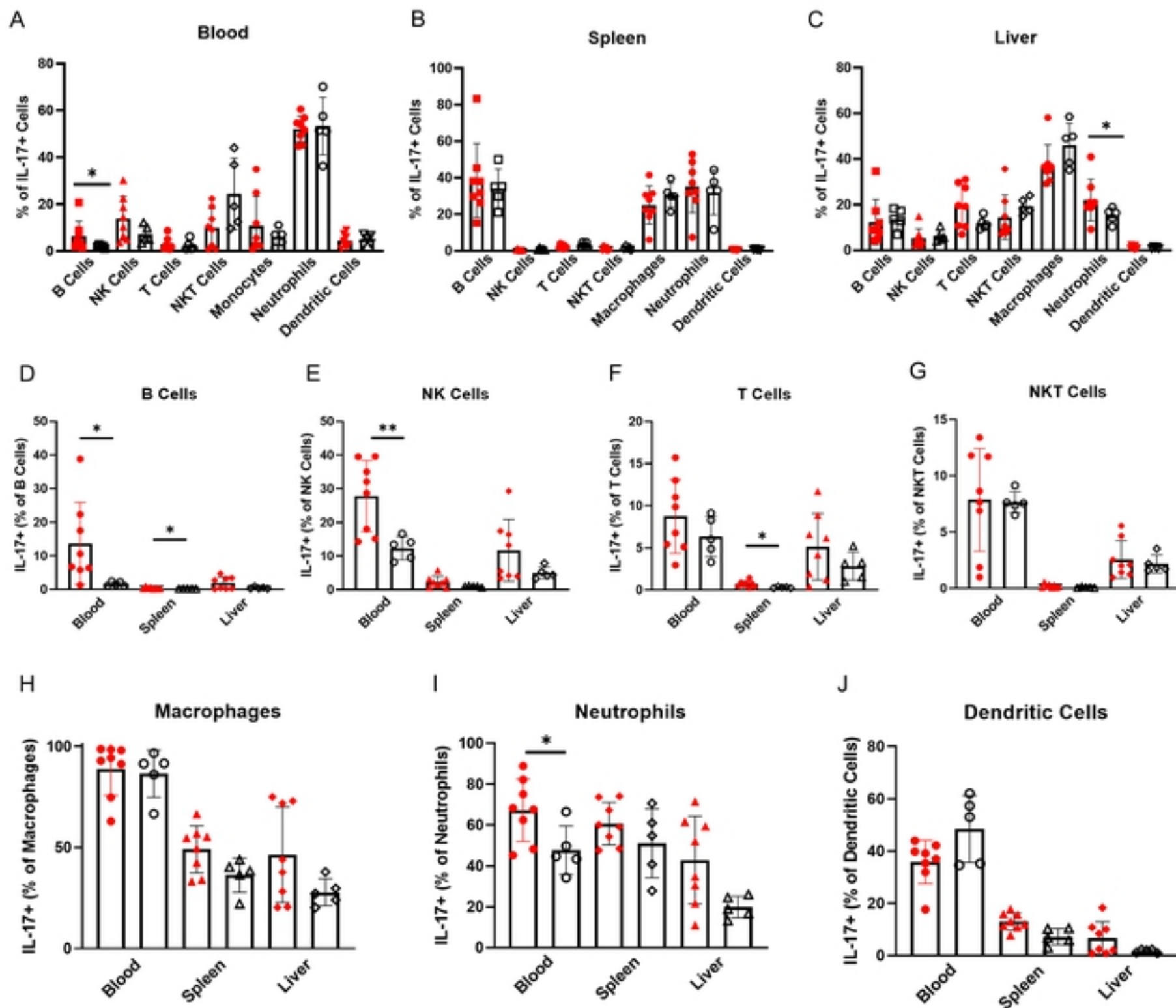


Figure 3



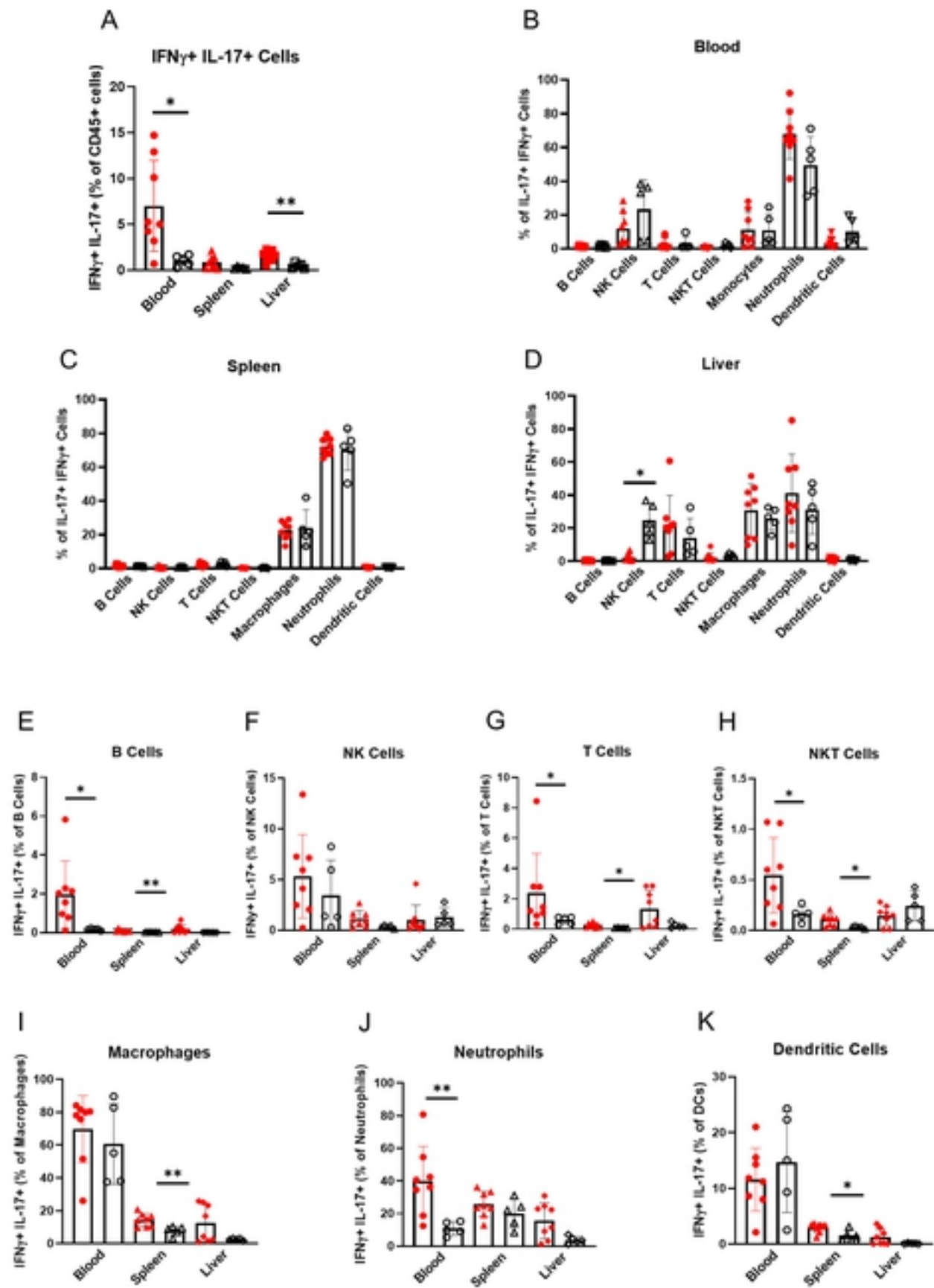


Figure 4