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Pro-inflammatory role of monocyte-derived CX3CR1^{int} macrophages in *Helicobacter hepaticus*-induced colitis

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Running title: Myeloid cells in *H. hepaticus* colitis

21 **ABSTRACT**

22 Cells of the monocyte-macrophage lineage play important roles in the pathogenesis of
23 inflammatory bowel diseases, but they are also present in the normal healthy intestine, where
24 they are critical for maintaining homeostasis. It has been unclear whether the pro-
25 inflammatory roles of intestinal macrophages reflect altered behaviour of the existing resident
26 cells, or if they involve recruitment of a distinct cell type. Here we have explored these ideas
27 using the model of colitis induced by *Helicobacter hepaticus* (*Hh*) in the context of
28 neutralisation or deletion of interleukin 10 (IL-10). Granulocytes and monocytes made up
29 most of the inflammatory myeloid infiltrates found in the colon of *Hh*-infected colitic mice,
30 rising to a peak within 2 weeks of *Hh* inoculation, but taking several months to resolve
31 completely. The inflammatory response was dependent on the combined presence of *Hh* and
32 absence of IL-10, and was accompanied by increased production of inflammatory mediators
33 such as IL-1 β , TNF α , IL-6 and IL-23p19 by infiltrating myeloid cells, mostly relatively
34 immature cells of the macrophage lineage that express intermediate levels of CX3CR1. In
35 contrast, the population of mature CX3CR1^{hi} macrophages did not expand as markedly during
36 colitis, and these cells made little contribution to inflammatory mediator production. Taking
37 into account their numerical dominance in the myeloid compartment, we conclude that newly
38 recruited monocytes are the main source of pro-inflammatory mediators in colitis induced in
39 the absence of IL-10 signalling, and that altered behaviour of mature macrophages is not a
40 major component of this pathology.

41

42

43 INTRODUCTION

44 Inflammatory bowel diseases (IBD), comprising Crohn's disease and ulcerative colitis, are
45 growing health problems in the developed world. Although recent advances have helped
46 elucidate some of the mechanisms underlying IBD, treatment remains unsatisfactory and
47 individual regimes are seldom effective for all patients. Thus, a better understanding of the
48 processes and cells involved in the pathogenesis of IBD could help develop new targets for
49 therapy.

50 Macrophages (mø) have received considerable attention in recent years because of
51 their potential roles in both steady-state and inflamed intestine (1-3). Resident møs are
52 abundant in the healthy intestine, where they are involved in clearance of apoptotic cells and
53 play a crucial role in maintaining homeostasis, ingesting and killing commensal bacteria that
54 cross the epithelial barrier (reviewed in (2)). In contrast to other tissues, these processes do
55 not provoke overt inflammation in the intestine, due to powerful control mechanisms that
56 prevent local møy from producing pro-inflammatory mediators in response to stimuli such as
57 TLR ligands (4, 5). However, findings from both IBD and experimental models of the disease
58 have demonstrated that møs are also crucial components of the inflammatory infiltrate (4, 6-
59 9), raising the possibility of exploiting them as therapeutic targets.

60 We and others have shown recently that intestinal møy originate from Ly6C^{hi} blood
61 monocytes that continuously enter the colonic mucosa and differentiate locally through a
62 series of intermediaries. Although only present in small numbers, cells with the phenotypic
63 and morphological features of Ly6C^{hi} monocytes can be found in the steady state mucosa.
64 However, major transcriptional differences exist between these and Ly6C^{hi} monocytes found
65 in blood, suggesting that the differentiation process occurs immediately after monocytes enter
66 the colonic mucosa (6, 10). Importantly, as monocytes mature they progressively acquire anti-

67 inflammatory properties, such as constitutive production of IL-10 and hyporesponsiveness to
68 e.g. TLR ligands (6, 11, 12). However, this physiological process is disrupted during
69 inflammation (6, 12, 13), and understanding of how this change in mØ behaviour occurs would
70 be an important advance in our knowledge of how to prevent and treat IBD. Studies of the
71 colitis induced by feeding dextran sodium sulphate (DSS) suggest that the tissue pathology is
72 paralleled by the accumulation of highly pro-inflammatory monocytes, whereas the fully
73 differentiated mØs that remain do not alter their behaviour and retain their anti-inflammatory
74 characteristics (6, 12). However, it is not clear if this pattern extends to other forms of
75 intestinal inflammation, particularly under conditions where there are intrinsic defects in the
76 mechanisms that normally control resident mØ function.

77 The IL-10 – IL-10R axis is an important brake on mØ activation, particularly in the
78 intestine, where deletion of either the cytokine or its receptor leads to spontaneous onset of
79 inflammation in association with hyper-responsiveness of mØ (13-19). Furthermore, humans
80 with non-functional mutations in *IL10*, *IL10RA*, or *IL10RB* develop severe enterocolitis within
81 the first months of life (reviewed in (20)). Here we have examined the relationship between IL-
82 10 and the differentiation of intestinal mØ in inflammation in more depth by exploring mØ
83 behaviour during colitis induced by inoculation of mice with *Helicobacter hepaticus* (*Hh*) in the
84 absence of IL-10 signalling (21, 22). We report that *Hh*-induced colitis is characterized by the
85 accumulation of pro-inflammatory CD11b⁺ myeloid cells that are hyper-responsive to TLR
86 stimulation. Most of these cells are monocytes and their immediate progeny that express
87 intermediate levels of CX3CR1 and that have not differentiated fully into anti-inflammatory,
88 resident-type CX3CR1^{hi} mØ. We further show that the expansion of cells in the monocyte-mØ
89 compartment is maintained for several months before returning to normal levels as the
90 inflammation resolves.

91 MATERIALS AND METHODS

92 Infection and antibody treatment of mice

93 Female C57BL/6 (B6) *Il10*^{-/-}, B6 WT, B6 CD45.1⁺ *Cx3cr1*^{gfp/gfp} mice (obtained from Jackson
94 Laboratory), and B6 CD45.1⁺ *Cx3cr1*^{+/gfp} (generated by crossing *Cx3cr1*^{gfp/gfp} mice with B6
95 CD45.1⁺ WT animals) were bred and maintained in an accredited SPF facility, and the
96 experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act
97 1986 under a Project License authorized by the UK Home Office and approved by the
98 University of York Animal Welfare and Ethical Review Body. The mice tested negative for
99 antibodies to specific murine viruses, were free of *Helicobacter spp.* as assessed by PCR,
100 and were >6-weeks old when used.

101 Mice were allocated to treatment groups and inoculated i.g. with 1.5×10^7 *Hh* NCI-
102 Frederick isolate 1A (23), derived originally from the same mouse colony as isolate Hh-1 (24)
103 (American Type Culture Collection strain 51449). IL-10-sufficient mice were also treated i.p.
104 with 1 mg of anti-IL-10R (clone 1B1.3a) on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 of
105 *Hh* infection. Age- and sex-matched uninfected animals were included as controls. One week
106 after the last mAb injection, mice were sacrificed and intestines collected for analysis.

107

108 Lamina propria (LP) cell isolation

109 For the experiments shown in Figures 1 and 2, colon and caecum were pooled from individual
110 mice and digested with Liberase CI (0.42 mg/ml, Roche, Burgess Hill, UK) and DNase I (125
111 U/ml; Sigma-Aldrich, Gillingham, UK), followed by enrichment of LP cells on 40/80% Percoll
112 gradients as described previously (25). For the experiments shown in Figures 3-5 and in
113 Supplementary Figures, colonic LP cells were isolated as described previously without the
114 use of Percoll gradients (4, 26).

115

116 Flow cytometric analysis and FACS

117 After blocking Fc receptors with anti-Fc γ RII/III mAb (2.4G2; BD Biosciences), LP cells were
118 stained at 4°C with fluorochrome-conjugated antibodies (see Table S1) for 20-30 min in the
119 dark before being washed in FACS buffer (2% FCS and 1mM EDTA in PBS) and then
120 analyzed on a CyAn ADP (Beckman Coulter, High Wycombe, UK) or LSRII/FACS Aria I (BD
121 Bioscience) flow cytometer. Data were analyzed using FlowJo software (Tree Star Inc. OR,
122 USA). Myeloid cell populations (as defined in figure legends) were sorted using a MoFlo
123 Astrios or a FACS Aria I sorter, with purities of >96%. For flow cytometric analyses on BD
124 instruments, automatic compensation was performed in FACSDiva using UltraComp or
125 OneComp beads together with fluorescence minus one controls.

126

127 Intracellular cytokine staining (ICS)

128 Large intestinal LP cells (2×10^6 /ml) from uninfected and 2-week *Hh*-infected *Il10*^{-/-} mice
129 were cultured in medium alone or in the presence of 10 μ g/ml Pam3CSK4 (Invivogen,
130 Toulouse, France) for 4 hr at 37°C with 10 μ g/ml brefeldin A during the last 3 hr. Thereafter,
131 cells were stained for surface markers (CD45 and CD11b) and intracellular TNF- α as
132 described previously (25).

133

134 Analysis of cytokine protein and mRNA expression

135 For cytokine protein analysis, FACS-purified CD45⁺CD11b⁺ cells were cultured in 96-well
136 round-bottomed plates (5×10^4 /ml; 0.2ml/well) at 37°C and 5% CO₂ in medium alone or with 10
137 μ g/ml ultrapure *E. coli* LPS, 10 μ g/ml Pam3CSK4 (both from Invivogen, Toulouse, France) or
138 10 μ g/ml soluble *Hh* antigen (SHelAg) prepared as described (21, 27). After 24 hr,

139 supernatants were collected and analyzed by ELISA for IL-12p40 (Mabtech, Nacka Strand,
140 Sweden) and IL-6 (R&D Systems), and by FlowCytomix for TNF- α (Bender MedSystems,
141 Vienna, Austria).

142 For cytokine RT-qPCR analysis in Fig. 2, FACS-purified CD45⁺CD11b⁺ cells were
143 homogenized in TRIzol, and total RNA isolated by chloroform extraction and reverse
144 transcribed using SuperScript II and random hexamers. cDNA was amplified using SYBR
145 green reagents and an ABI Prism RT-PCR system (Applied Biosystems). Cytokine expression
146 levels for each individual sample (run in duplicates) were normalized to HPRT using Δ Ct
147 calculations and the 7000 system SDS software (Applied Biosystems). For myeloid cell
148 populations in Fig. 5, FACS-purified cells were lysed in RLT buffer (Qiagen) and
149 homogenised using Qias shredders (Qiagen). RNA was then isolated and purified using an
150 RNeasy micro kit (Qiagen) and reverse transcribed using a High Capacity RNA-to-cDNA kit
151 (Life Technologies) and random hexamers. cDNA was amplified using SYBR green reagents
152 and an ABI 7900HT Prism RT-PCR system (Applied Biosystems). Cytokine expression levels
153 for each individual sample (run in triplicates) were normalized to TBP using Δ Ct calculations.
154 Specific primers pairs are detailed in Table S2.

155

156 **Statistical analysis**

157 Multiple group comparisons were performed by one-way ANOVA, while Student's t-test and
158 Mann Whitney test was used to compare two groups. Differences were considered
159 statistically significant with $P < 0.05$.

160

161 RESULTS

162 Expansion of myeloid cells in the large intestine of *Hh*-infected colitic *Il10*^{-/-} mice.

163 To begin to characterize the innate immune response in the large intestine following *Hh*
164 inoculation, WT and *Il10*^{-/-} mice were inoculated i.g. with the bacterium, and the cellular
165 composition of LP cells from pooled ceca and colons was examined 2 weeks later, the time
166 point at which pathology peaks in *Il10*^{-/-} hosts (25). As expected, *Hh*-infected *Il10*^{-/-} mice
167 displayed enhanced cellularity of the large intestinal LP compared with uninfected controls
168 (Fig. 1A, left panel), with greatly expanded proportions and numbers of CD45⁺ hematopoietic
169 cells (Fig. 1A, middle and right panels), consisting of B cells, CD4⁺ T cells, and CD11b⁺
170 myeloid cells (Fig. 1B). Only minor increases were observed in the proportion and number of
171 LP CD45⁺ cells in *Hh*-infected WT mice (Fig. 1A), and the size of CD11b⁺ myeloid
172 compartment was unaffected in these hosts (Fig. 1B, right panel). More detailed analysis of
173 the early cellular kinetics following *Hh* inoculation of *Il10*^{-/-} animals revealed that the numbers
174 of total CD45⁺ cells and of CD11b⁺ myeloid cells were significantly increased in *Hh*-infected
175 *Il10*^{-/-} mice on day 5 pi, and expanded steadily until day 11 pi (Fig. 1C). Thus, *Hh*-driven
176 typhlocolitis in *Il10*^{-/-} mice is associated with massive infiltration by both lymphoid and
177 myeloid cells.

178

179 Myeloid cells from *Hh*-infected *Il10*^{-/-} mice display a pro-inflammatory phenotype

180 To understand how the expansion of the myeloid compartment might contribute to the colitis
181 in *Il10*^{-/-} hosts, we examined the cytokine secretion profile of CD11b⁺ cells from uninfected
182 and *Hh*-infected *Il10*^{-/-} mice. To this end, large intestinal LP cells were stimulated with the
183 TLR ligand Pam3CSK4, and TNF α production examined by intracellular cytokine staining
184 (ICS). TLR ligation of LP cells from *Hh*-infected *Il10*^{-/-} animals resulted in ~3-fold increase in

185 the proportion of CD11b⁺ cells producing TNF α compared with cells cultured in medium alone
186 (Fig. 2A, bottom panels), and this translated to a highly significant increase in the absolute
187 number of TNF α ⁺CD11b⁺ cells in the LP of infected *Il10*^{-/-} mice compared with uninfected
188 controls (Fig. 2B). In contrast, the proportion of TNF α ⁺ CD11b⁺ cells from uninfected *Il10*^{-/-}
189 animals was unaffected by Pam3CSK4 stimulation and remained at levels similar to those
190 seen in naïve WT colon (Fig. 2A, upper panels and Fig. 2B).

191 To extend these analyses, we next FACS-purified CD11b⁺ cells from the large
192 intestinal LP of uninfected and 2-week *Hh*-infected *Il10*^{-/-} animals and cultured them overnight
193 with LPS, Pam3CSK4, or soluble *Hh* antigen (SHelAg), before assessing the levels of a wider
194 range of cytokines using ELISA and FlowCytoMix. CD11b⁺ cells from *Hh*-infected *Il10*^{-/-} mice
195 secreted higher amounts of IL-12p40, IL-6, and TNF α after all forms of stimulation compared
196 with CD11b⁺ cells from uninfected *Il10*^{-/-} hosts (Fig. 2C). When analysed directly *ex vivo*,
197 FACS-purified CD11b⁺ cells from *Hh*-infected *Il10*^{-/-} mice also contained higher levels of *Il12a*
198 and *Il23a* transcripts compared with CD11b⁺ cells from uninfected controls, whereas the
199 levels of *Il12b* accumulation were similar in the two populations (Fig. 2D). Thus, the expanded
200 myeloid cell compartment in the large intestine of *Hh*-infected colitic *Il10*^{-/-} mice displays pro-
201 inflammatory characteristics.

202

203 **Composition of the myeloid compartment in *Hh*-infected *Il10*^{-/-} mice**

204 We next set out to explore what cell types accounted for the change within the CD11b⁺
205 compartment during *Hh* colitis. To do this, we exploited multiparameter flow cytometry and
206 rigorous gating strategies we have developed recently to characterise the myeloid
207 compartment of the intestinal LP, allowing precise identification of monocytes, m ϕ ,
208 eosinophils, neutrophils and dendritic cells (DC) ((6); Fig. S1). We also omitted the Percoll

209 gradient step during the purification to exclude the possibility of selective loss of individual cell
210 types. These approaches confirmed marked changes in the composition of the myeloid cell
211 compartment in the colon 2 weeks after *Hh* inoculation of *Il10*^{-/-} mice, compared with
212 uninfected *Il10*^{-/-} mice or *Hh*-infected WT mice (Fig. 3A). Ly6G⁺ neutrophils and SSC^{hi}
213 (MHCII⁻) eosinophils (Figs. 3A, B and C) accounted for a substantial part of the infiltration
214 found in *Hh*-infected *Il10*^{-/-} mice and no significant differences were seen in these granulocyte
215 populations in *Hh*-infected WT mice, or in uninfected *Il10*^{-/-} mice compared with uninfected
216 WT mice (Figs. 3A, B, and C).

217 *Hh*-infected *Il10*^{-/-} mice also showed marked expansion in the numbers of non-
218 granulocytic myeloid cells (Figs. 3A, D and E), and we therefore explored the contribution of
219 monocytes and mØ in more detail. To do this, we focused on cells expressing the pan-mØ
220 markers F4/80 and/or CD64 (Fig. S1) and examined the monocyte-mØ differentiation
221 continuum that we and others have defined in the colonic LP (6, 28, 29). This so-called
222 'monocyte waterfall' consists of newly arrived Ly6C^{hi}MHCII⁻ monocytes, differentiating
223 Ly6C⁺MHCII⁺ monocytes, and more mature Ly6C⁻MHCII⁺ cells that include tissue-resident mØ
224 (Fig. 3D). *Hh*-infected *Il10*^{-/-} mice showed marked increases in the proportions and absolute
225 numbers of Ly6C^{hi}MHCII⁻ and Ly6C⁺MHCII⁺ cells, which were increased by ~125-fold and
226 155-fold respectively compared with their numbers in uninfected *Il10*^{-/-} mice (Figs. 3D and E).
227 There was also a modest, but significant increase in the number of more mature Ly6C⁻
228 MHCII⁺ cells in *Hh*-infected *Il10*^{-/-} mice (Fig. 3E, right panel). *Hh*-infected WT mice, which do
229 not develop colitis, showed some evidence of increased infiltration by Ly6C^{hi}MHCII⁻,
230 Ly6C⁺MHCII⁺ and Ly6C⁻ cells, while uninfected *Il10*^{-/-} mice had slightly higher numbers of
231 Ly6C⁺MHCII⁺ cells compared with their naïve WT counterparts; however, these differences
232 were modest and did not attain statistical significance (Fig. 3E).

233 DCs were identified as CD11c⁺MHCII⁺CD64⁻ cells amongst LP leukocytes (30) and
234 their numbers increased in *Hh*-infected compared to control *Il10*^{-/-} mice (Fig. S2A). The
235 frequency of DCs among LP cells of *Hh*-infected *Il10*^{-/-} mice decreased compared to
236 uninfected controls (data not shown), most likely reflecting the increase in other leukocytes.
237 Finally, there were minor changes in the proportion of DC subsets identified on the basis of
238 CD11b and CD103 expression, although none of these changes reached statistical
239 significance (Fig. S2B).

240

241 **Altered monocyte - mØ differentiation in *Hh*-infected colitic mice**

242 The accumulation of monocytes we found in *Hh* colitis is reminiscent of our own and other
243 results from DSS and T-cell-mediated colitis, where there appeared to be an arrest in the
244 local differentiation continuum that normally generates anti-inflammatory, resident mØ (6, 12,
245 28). To examine whether a similar block was present during *Hh*-induced colitis, we used
246 *Cx3cr1*^{+/gfp} reporter mice in which one allele of the *Cx3cr1* gene has been replaced with the
247 gene encoding green fluorescent protein (GFP) (31). This allows fully differentiated resident
248 CX3CR1^{hi} mØ to be distinguished from cells in the earlier CX3CR1^{int} stages in the
249 developmental continuum, some of which would have been included amongst the Ly6C⁻
250 MHCII⁺ population we defined earlier. In this way, we could examine the relative roles of
251 resident and recently recruited mØ in *Hh*-induced inflammation, as well as explore how these
252 cells behave during the resolution of pathology that occurs at later stages after bacterial
253 inoculation (25). Because the *Cx3cr1*^{+/gfp} reporter mice are IL-10 sufficient, we had to induce
254 colitis by *Hh* inoculation plus weekly administration of anti-IL-10R mAb (22). Consistent with
255 our studies in *Il10*^{-/-} mice, this resulted in massive accumulation of total leukocytes and
256 CD11b⁺ myeloid cells in the colonic mucosa by day 14 pi (Figs. 4A and B). However, by day

257 41 pi, both compartments had contracted significantly, and by day 77 pi they were both
258 reduced almost to baseline levels (Figs. 4A and B).

259 As in colitic *Il10*^{-/-} mice, the composition of the myeloid compartment was markedly
260 altered in anti-IL-10R-treated *Hh*-infected *Cx3cr1*^{+/gfp} mice, with a large expansion of
261 granulocytes and monocyte-derived cells at day 14 pi, followed by resolution at later times
262 (Fig. 4C). Moreover, the non-granulocyte component of the myeloid infiltrate contained large
263 numbers of Ly6C^{hi}MHCII⁻ and Ly6C⁺MHCII⁺ cells at day 14 pi (Figs. 4C-E). The Ly6C⁻MHCII⁺
264 population also expanded in number early in colitis, but most of this expansion was accounted
265 for by CX3CR1^{int} cells (Fig. 4F), a population that we have shown previously to be a further
266 intermediary stage in the local differentiation of monocytes (6). In parallel, there was a
267 substantial decrease in the proportion of fully-mature CX3CR1^{hi} mØ amongst the Ly6C⁻
268 MHCII⁺ cells at this time (Fig. 4F), although their absolute numbers were increased and
269 remained so throughout the experiment (Fig. 4G). By day 41 pi, the proportions and numbers
270 of CX3CR1^{int} cells amongst Ly6C⁻MHCII⁺ cells had contracted significantly, and by day 77 pi,
271 CX3CR1^{hi} cells had again come to dominate the Ly6C⁻ mature mØ compartment (Fig. 4F &
272 G). At this time the numbers of Ly6C^{hi}MHCII⁻ and Ly6C⁺MHCII⁺ cells had also returned to
273 baseline levels (Fig. 4E).

274 The numbers of Ly6G⁺ neutrophils and CD11c⁺CD11b⁺F4/80⁻ DC were greatly
275 increased by day 14 pi in anti-IL-10R-treated *Hh*-infected *Cx3cr1*^{+/gfp} mice, before falling at
276 later times in parallel with the reduction in monocytes and mØ (Fig. S3A and B). Interestingly
277 however, the numbers of colonic eosinophils remained high in these mice until the end of the
278 experiment on day 77 pi (Fig. S3C). All these *Hh*-induced changes in myeloid cells were seen
279 only when *Hh*-infected *Cx3cr1*^{+/gfp} mice were also co-administered with anti-IL-10R (Fig. 4 and
280 data not shown). Anti-IL-10R treatment alone of uninfected *Cx3cr1*^{+/gfp} mice did not result in

281 leukocyte accumulation in the colonic mucosa (Fig. S4), findings that are in agreement with
282 those we previously reported for uninfected WT mice (22).

283 Together our results demonstrate that the acute phase of *Hh*-induced colitis is
284 associated with accumulation of CX3CR1^{int} monocytes and early-stage mØ, consistent with
285 the idea that there may be a block in the normal differentiation process.

286
287 **Colonic CX3CR1^{int} monocytes/mØs are the major producers of pro-inflammatory**
288 **cytokines during *Hh* colitis**

289 Finally we explored whether elicited CX3CR1^{int} cells were responsible for the production of
290 pro-inflammatory mediators by myeloid cells during colitis, or if this reflected altered behaviour
291 of the more mature CX3CR1^{hi} mØ. RT-qPCR analysis showed marked upregulation of mRNA
292 transcripts for *Il1b*, *Nos2* (iNOS), *Il23p19* and *Il12p35* in CX3CR1^{int} cells from anti-IL-10R-
293 treated *Hh*-infected mice at the peak of inflammation on day 14 compared with CX3CR1^{int}
294 cells from control mice (Fig. 5A-D). In contrast, colonic CX3CR1^{hi} mØ from anti-IL-10R-treated
295 *Hh*-infected mice showed only minor changes in mRNA levels of pro-inflammatory mediators
296 (Fig. 5). Thus, taking into account their numerical dominance in the myeloid compartment,
297 newly recruited CX3CR1^{int} monocytes/mØs form the predominant pro-inflammatory population
298 in this model of colitis.

299
300 **DISCUSSION**

301
302 The results presented here underline the importance of the myeloid compartment in the
303 inflammatory colitis that occurs in mice infected with *Helicobacter hepaticus* when IL-10-
304 mediated signalling is absent. Using a variety of approaches to identify myeloid lineages,

305 including *Cx3cr1*^{+/gfp} mice, we show that an intense infiltrate of CD11b⁺ cells appears early
306 during infection and that this is made up of neutrophils, eosinophils, Ly6C^{hi}MHC^{-/+} monocytes
307 and CX3CR1^{int} mφ at the time of peak disease at 2 weeks after *Hh* inoculation. While this
308 confirms other work (32), we also show here that this expansion of the monocyte-mφ
309 compartment is sustained for up to 11 weeks after infection, by which time other parameters
310 of inflammation, such as changes in myeloid cell subset composition, have returned to
311 steady-state levels. Importantly, the inflammatory changes required both infection with *Hh* and
312 neutralisation of IL-10R signalling, with very few changes being seen in mice with *Hh* infection
313 alone or loss of IL-10 alone. Similarly, the greatly heightened production of pro-inflammatory
314 mediators in response to activation *in vitro* by intestinal myeloid cells was fully dependent on
315 these factors operating together. These results confirm previous conclusions that *Hh* plays a
316 crucial role in provoking intestinal inflammation in the absence of IL-10-mediated
317 immunoregulation and that pro-inflammatory responses to this organism are normally
318 restrained by IL-10 (21, 22, 33).

319 In previous work, we have shown that resident mφ in normal intestinal mucosa are
320 replenished continuously by Ly6C^{hi} monocytes that differentiate via a number of intermediary
321 stages into mature, resident mφ (6, 11, 28). At early times after inoculation with *Hh*, this so-
322 called monocyte-mφ “waterfall” expanded dramatically, and consisted mostly of Ly6C^{hi}MHCII⁻
323 ^{+/+} monocytes and CX3CR1^{int} mφ, with less expansion of fully mature CX3CR1^{hi} mφ. Although
324 these findings are consistent with previous work, e.g. in chemically-induced colitis (6), and
325 suggest that the normal process of monocyte-mφ differentiation is arrested, it is important to
326 note that the absolute numbers of mature mφ were actually increased in our mice with *Hh*-
327 induced colitis and remained so for the duration of the 11-week experiment. Thus, although
328 the majority of infiltrating monocytes may be short-lived in the colitic mucosa, a proportion of

329 these cells may still develop into mature CX3CR1^{hi} m ϕ , even in the face of inflammation. How
330 the m ϕ recruited under these conditions might contribute to the inflammation or its resolution
331 and if they eventually acquire all the properties of the normal, resident population remain to
332 be determined, as do the relative contributions of newly recruited and pre-existing cells to the
333 “resident” m ϕ compartment.

334 Although methods were not available to examine and track infiltrating monocytes
335 directly following their arrival in the gut, we were able to explore whether the total mature m ϕ
336 population was altered in function during *Hh* colitis. In steady-state intestine, resident m ϕ
337 exhibit an anti-inflammatory phenotype characterised by constitutive production of IL-10 and
338 low levels of TNF α , together with an inability to respond to stimuli such as TLR ligands, but it
339 has been unclear whether these properties can change during inflammation (34). In contrast
340 to their CX3CR1^{hi} counterparts, as we show here, cells within the CX3CR1^{int} compartment are
341 actively pro-inflammatory, expressing much higher levels of mRNA for pro-inflammatory
342 mediators such as IL1 β , iNOS, IL-23, and IL-12 during *Hh* colitis than their control
343 counterparts. In parallel, CX3CR1^{hi} m ϕ taken at the peak of inflammation showed no
344 increased expression of IL-1 β or IL-12 mRNA, and only a modest increase in iNOS and IL-23
345 mRNA, suggesting some, but limited ability to adopt a pro-inflammatory phenotype under
346 these circumstances. In DSS colitis, we and others have shown that resident m ϕ do not
347 acquire pro-inflammatory characteristics (6, 12), but this has been reported in models of colitis
348 where IL-10-mediated signalling is absent, with wider alterations in gene expression than we
349 tested here (18). While this contrasting behaviour in the presence and absence of IL-10 is yet
350 to be confirmed in other models of inflammation, the results are consistent with recent
351 evidence that IL-10 drives permanent, epigenetic silencing of pro-inflammatory genes (35). As
352 a result, the absence of IL-10R-mediated signalling profoundly alters the genetic landscape of

353 mature intestinal m ϕ , allowing them to respond to stimuli to which they are normally resistant
354 (18). Whether IL-10 also controls other aspects of intestinal m ϕ differentiation is unclear,
355 although it has been reported that their expression of the scavenger receptor CD163 is
356 dependent on IL-10 (14). Arnold *et al.* showed reduced levels of CD206 on CX3CR1^{hi} m ϕ in
357 *Hh* colitis (32), but it is not clear whether this reflects an intrinsic effect of IL-10, or is
358 secondary to the effects of inflammation. That resident m ϕ retain a substantial proportion of
359 their homeostatic properties in the absence of IL-10 is suggested by our finding that sustained
360 expansion of this population is not accompanied by maintenance of *Hh*-dependent
361 immunopathology.

362 The colitis induced by *Hh* is dependent on IL-23 (22) and upregulation of this cytokine
363 was one of the major changes we and others observed in CX3CR1^{int}CD11b⁺ myeloid cells in
364 this model (32). It has often been difficult to identify precisely the cell responsible for
365 producing IL-23 in infection or inflammation, in great part because m ϕ and DC share many
366 phenotypic features such as CD11c, MHCII, CX3CR1 and CD11b. Therefore it is important to
367 note that by using rigorous gating strategies based on CD64 as a m ϕ marker, both we and
368 Arnold *et al.* (32) show that IL-23 was derived from the m ϕ lineage, which is consistent with
369 work in other models (7, 36, 37). Notably, however, we detected increased IL-12p35 and IL-
370 23p19 transcript levels in elicited CX3CR1^{int} monocyte/m ϕ s, suggesting that these cells
371 produce both IL-12 and IL-23 during *Hh*-induced intestinal inflammation. Thus we propose
372 that the T_H17 response and subsequent phenotype switching of Th17 cells into IFN- γ ⁺IL-17A⁺
373 lymphocytes in *Hh* colitis (24) is driven by IL-23 derived from m ϕ , presumably activated
374 directly by products of the organism. Nevertheless it should be noted that both
375 CD103⁺CD11b⁺ and CD103⁻CD11b⁺ DC from the intestine have been shown to be capable of
376 driving T_H17 responses *in vivo* and *in vitro* respectively, via production of IL-23 and/or IL-6

377 (30, 38-40). Thus there may be flexibility between the $m\phi$ and DC lineages in their ability to
378 stimulate T_H17 activity depending on the stimulus. Alternatively, these cell types may
379 cooperate in driving such T-cell responses, perhaps with migratory DC being responsible for
380 priming T_H17 cells in the draining lymph node, while tissue resident $m\phi$ may sustain the
381 survival of these cells once they migrate back to the mucosa (41).

382 Together our results show that elicited monocyte-derived $CX3CR1^{int}$ macrophages
383 form the predominant pro-inflammatory macrophage population in *Hh*-induced colitis. Given
384 that these cells appear to derive from the same $Ly6C^{hi}$ blood monocytes as their homeostatic
385 counterparts, identifying the factors that govern monocyte fate in the colon during
386 homeostasis versus inflammation could provide new therapeutic targets for the treatment for
387 IBD.

388

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394

395 **AUTHOR CONTRIBUTIONS**

396 CCB, CJO, CAT, and MCK performed the experiments, analysed the data, and assisted with
397 the manuscript. CCB, MCK, and AMM designed and coordinated the research, interpreted the
398 data, and wrote the manuscript.

399

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554 **FIGURE LEGENDS**

555 **Figure 1. Expansion of myeloid cells in the large intestine of *Hh*-infected colitic *Il10*^{-/-}**
556 **mice.** WT and *Il10*^{-/-} mice were inoculated with *Hh*, and LP cells were isolated from pooled
557 caecum and colon 2 weeks (**A-B**) or 1, 3, 5, 8, and 11 days (**C**) later and examined by flow
558 cytometry. Uninfected mice were included as controls. (**A**) Total numbers of LP cells (left
559 panel), percentage (middle panel) and total numbers (right panel) of CD45⁺ hematopoietic
560 cells from uninfected (white bars) and 2-week *Hh*-infected (black bars) WT and *Il10*^{-/-} mice.
561 (**B**) Numbers of B220⁺CD19⁺ B cells (left panel), CD3⁺CD4⁺ T cells (middle panel), and
562 CD11b⁺ myeloid cells (right panel) from uninfected (white bars) and 2-week *Hh*-infected
563 (black bars) WT and *Il10*^{-/-} mice. Data in **A-B** are from one representative experiment out of
564 at least three performed, and bars represent mean + SD of 3 mice per group. (**C**) Total
565 numbers of LP cells (left panel), CD45⁺ hematopoietic cells (middle panel) and CD11b⁺ cells
566 (right panel) from pooled caecum and colon of 1-, 3-, 5-, 8-, and 11-day *Hh*-infected *Il10*^{-/-}
567 mice. Data are pooled from two individual experiments, and are the mean + SD of 5-6 LP cell
568 preparations (each pooled from 1-2 animals) from *Hh*-infected mice and 8 cell preparations
569 (each pooled from 2-3 animals) from uninfected mice. One-way ANOVA followed by Tukey's
570 multiple comparison test. ***P<0.001, ****P<0.0001 when comparing *Hh*-infected and
571 uninfected mice (A-B) or when compared to uninfected mice (C).

572

573 **Figure 2. Large intestinal CD11b⁺ myeloid cells from *Hh*-infected *Il10*^{-/-} mice secrete**
574 **elevated amounts of pro-inflammatory cytokines following TLR stimulation.** *Il10*^{-/-} mice
575 were inoculated with *Hh*, and LP cells were isolated from pooled caecum and colon 2 weeks
576 later. Uninfected mice were included as controls. (**A-B**) Large intestinal LP cells were cultured

577 in medium alone or stimulated with 10 μ g/ml Pam3CSK4 for 4 hours with 10 μ g/ml brefeldin A
578 during the last 3 hours, and then stained for CD45, CD11b, and TNF α or appropriate isotype
579 control. Dot plots in **A** are gated on CD45⁺CD11b⁺ LP cells from uninfected (Uninf.; upper
580 panels) and 2-week *Hh*-infected *Il10*^{-/-} mice (lower panels), and numbers (+ SD) of TNF α ⁺
581 CD11b⁺ cells per mouse in **B** were calculated from the percentages in A and the total number
582 of cells isolated from each mouse. Data in **A-B** are representative of two independent
583 experiments performed with 3 individual mice per group. One-way ANOVA followed by
584 Tukey's multiple comparison test. **P<0.01 and ****P<0.0001. **(C)** Large intestinal LP CD11b⁺
585 myeloid cells were FACS-purified from uninfected (white bars) and 2-week *Hh*-infected (black
586 bars) *Il10*^{-/-} mice and stimulated *in vitro* with LPS (10 μ g/ml), Pam3CSK4 (10 μ g/ml), or
587 SHelAg (10 μ g/ml) or cultured in medium alone. After 24 hours, supernatants were collected
588 and analyzed for the presence of IL-12p40, IL-6, and TNF α . Bars represent mean + SD of
589 quadruplicate (IL-12p40 and IL-6) or duplicate (TNF α) ELISA values (where each value
590 represents a separate culture) combined from two independent experiments.
591 **(D)** RT-qPCR analysis of IL-12p35 (*Il12a*), IL-12p40 (*Il12b*), and IL-23p19 (*Il23a*) transcripts
592 relative to HPRT in sorted CD11b⁺ cells from uninfected (white bars) or 2-week *Hh*-infected
593 (black bars) *Il10*^{-/-} mice. Bars represent means + SD of three (for uninfected mice) and four
594 (for *Hh*-infected mice) individual experiments, each consisting of cells pooled from 5-6 mice.
595 Mann Whitney test. *P<0.05.

596
597 **Figure 3. Composition of the myeloid compartment in *Hh*-infected *Il10*^{-/-} mice.** WT and
598 *Il10*^{-/-} mice were inoculated with *Hh* and colonic LP cells were isolated 2 weeks later and
599 examined by flow cytometry. Uninfected mice were included as controls. **(A)** Relative
600 frequencies amongst total colonic CD11b⁺ cells of Ly6G⁺ neutrophils, SSC^{hi} MHCII⁻

601 eosinophils, CD64⁻ CD11c⁺ MHCII⁺ CD11b⁺ DC and Ly6C/MHCII-defined cells of the CD64⁺
602 monocyte/macrophage compartment (Ly6C^{hi}MHCII⁻, Ly6C⁺MHCII⁺, Ly6C⁻MHCII⁺) from
603 uninfected or *Hh*-infected WT and *Il10*^{-/-} mice. **(B-C)** Absolute numbers of Ly6G⁺ neutrophils
604 **(B)** and SSC^{hi} MHCII⁻ eosinophils **(C)** per colon of uninfected or *Hh*-infected WT and *Il10*^{-/-}
605 mice. **(D)** Representative expression of Ly6C and MHCII by CD11b⁺Ly6G⁻SSC^{lo}CD64⁺ cells
606 from uninfected or *Hh*-infected WT and *Il10*^{-/-} mice. Bar graph shows the frequency amongst
607 CD11b⁺Ly6G⁻SSC^{lo}CD64⁺ cells of Ly6C^{hi}MHCII⁻, Ly6C⁺MHCII⁺ and Ly6C⁻MHCII⁺ cells. **(E)**
608 Absolute numbers of Ly6C^{hi}MHCII⁻, Ly6C⁺MHCII⁺ and Ly6C⁻MHCII⁺ cells per colon of
609 uninfected or *Hh*-infected WT and *Il10*^{-/-} mice. Data are from one of two independent
610 experiments performed. Bars represent the mean + SD of 4 individual mice per group. One-
611 way ANOVA followed by Tukey's multiple comparison test. ****P<0.0001

612
613 **Figure 4. Monocyte differentiation is dysregulated in anti-IL-10R-treated *Hh*-infected**
614 **colitic *Cx3cr1*^{+/gfp} mice.** *Cx3cr1*^{+/gfp} mice were inoculated with *Hh* and treated weekly with
615 anti-IL-10R to induce colitis. The composition of the colonic myeloid compartment was then
616 examined at 14, 41 and 77 days post infection and compared to uninfected mice or mice
617 given *Hh* alone. **(A-B)** Absolute numbers of total CD45⁺ **(A)** and CD11b⁺ cells **(B)** per colon of
618 *Hh*/anti-IL-10R-treated mice 14, 41 and 77 days after infection, compared with control mice
619 (uninfected and *Hh* alone; data pooled from day 14-77 for these groups). **(C)** Relative
620 frequencies amongst total colonic CD11b⁺ cells of Ly6G⁺ neutrophils, SSC^{hi}MHCII⁻
621 eosinophils, F4/80⁻CD11c⁺MHCII⁺CD11b⁺ DC and Ly6C/MHCII-defined cells of the F4/80⁺
622 monocyte/mφ compartment (Ly6C^{hi}MHCII⁻, Ly6C⁺MHCII⁺, Ly6C⁻MHCII⁺) of mice as in **A**. **(D-**
623 **E)** Representative expression of Ly6C and MHCII by CD11b⁺Ly6G⁻SSC^{lo}F4/80⁺ cells **(D)** and
624 absolute numbers of Ly6C^{hi}MHCII⁻, Ly6C⁺MHCII⁺ and Ly6C⁻MHCII⁺ cells per colon **(E)** of

625 mice as in **A**. **(F)** Representative expression of CX3CR1-GFP by Ly6C⁺MHCII⁺ cells from
626 uninfected mice, mice inoculated with *Hh* alone, and mice receiving *Hh* plus anti-IL-10R when
627 analysed at 14, 41 and 77 post inoculation. The histograms for the uninfected and *Hh* alone
628 are taken from the day 14 experiment. **(G)** Absolute numbers of CX3CR1^{int} (top panel) and
629 CX3CR1^{hi} (bottom panel) Ly6C⁺ MHCII⁺ cells per colon of mice as in **A**. Data are from one
630 experiment and bars represent the mean + SD of 4 individual mice per group. One-way
631 ANOVA followed by Tukey's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001.

632

633 **Figure 5. Colonic CX3CR1^{int} monocytes/mφs produce pro-inflammatory mediators**
634 **during *Hh* colitis.** *Cx3cr1^{+/-gfp}* mice were inoculated with *Hh* and treated weekly with anti-IL-
635 10R to induce colitis. Two weeks later colonic LP CD64⁺CX3CR1^{int} and CD64⁺CX3CR1^{hi} cells
636 (both pre-gated on CD45⁺CD11b⁺Ly6G⁺SiglecF⁺) were FACS-purified and processed for RT-
637 qPCR analysis of pro-inflammatory mediators. Uninfected mice, mice given *Hh* alone, and
638 mice given anti-IL-10R alone were included as controls. Transcript levels of IL-1β (*Il1b*) (**A**),
639 iNOS (*Nos2*) (**B**), IL-23 (*Il23a*) (**C**) and IL-12p35 (*Il12a*) (**D**) relative to TATA-binding protein
640 (TBP) in uninfected, *Hh* alone, anti-IL-10R alone, and *Hh* plus anti-IL-10R groups. Each
641 symbol represents a pool of 3 mice (for uninfected, *Hh* alone, and anti-IL-10R alone) or
642 individual mice (for *Hh* plus anti-IL-10R). Data are pooled from two individual experiments.
643 Unpaired Student's t test. *P<0.05, **P<0.01, ***P<0.001.

Figure 1

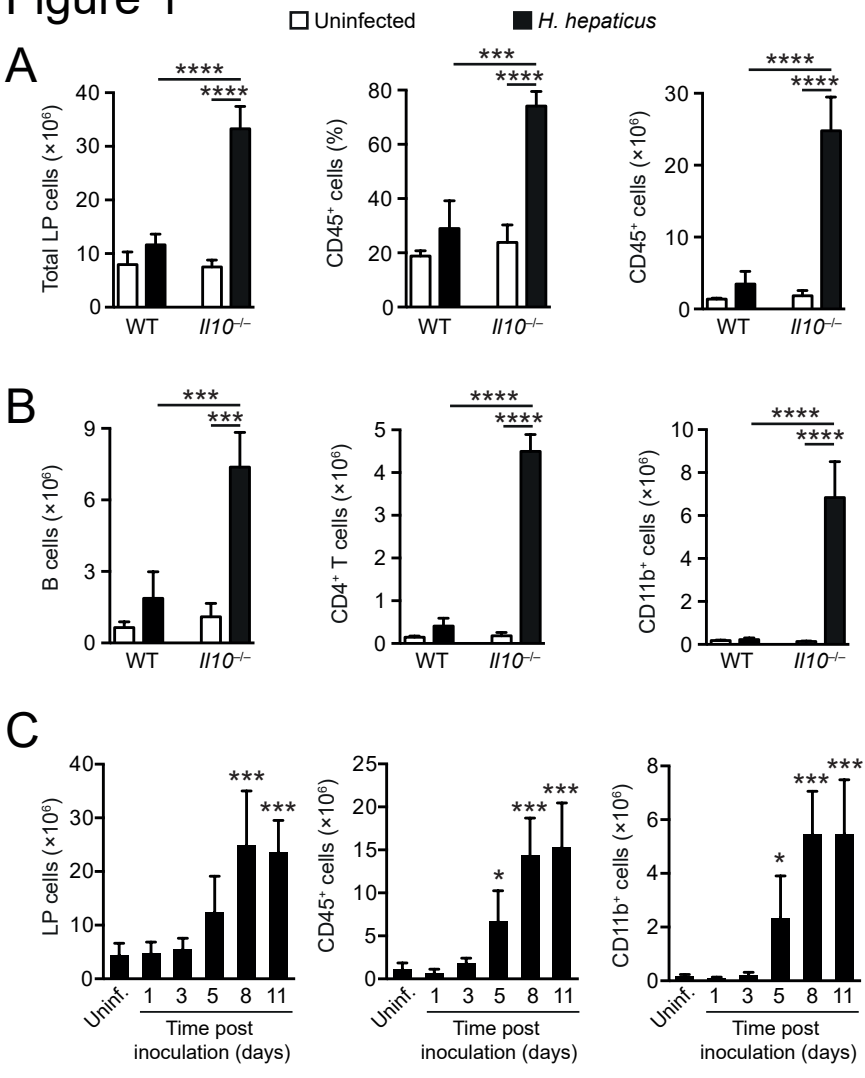


Figure 2

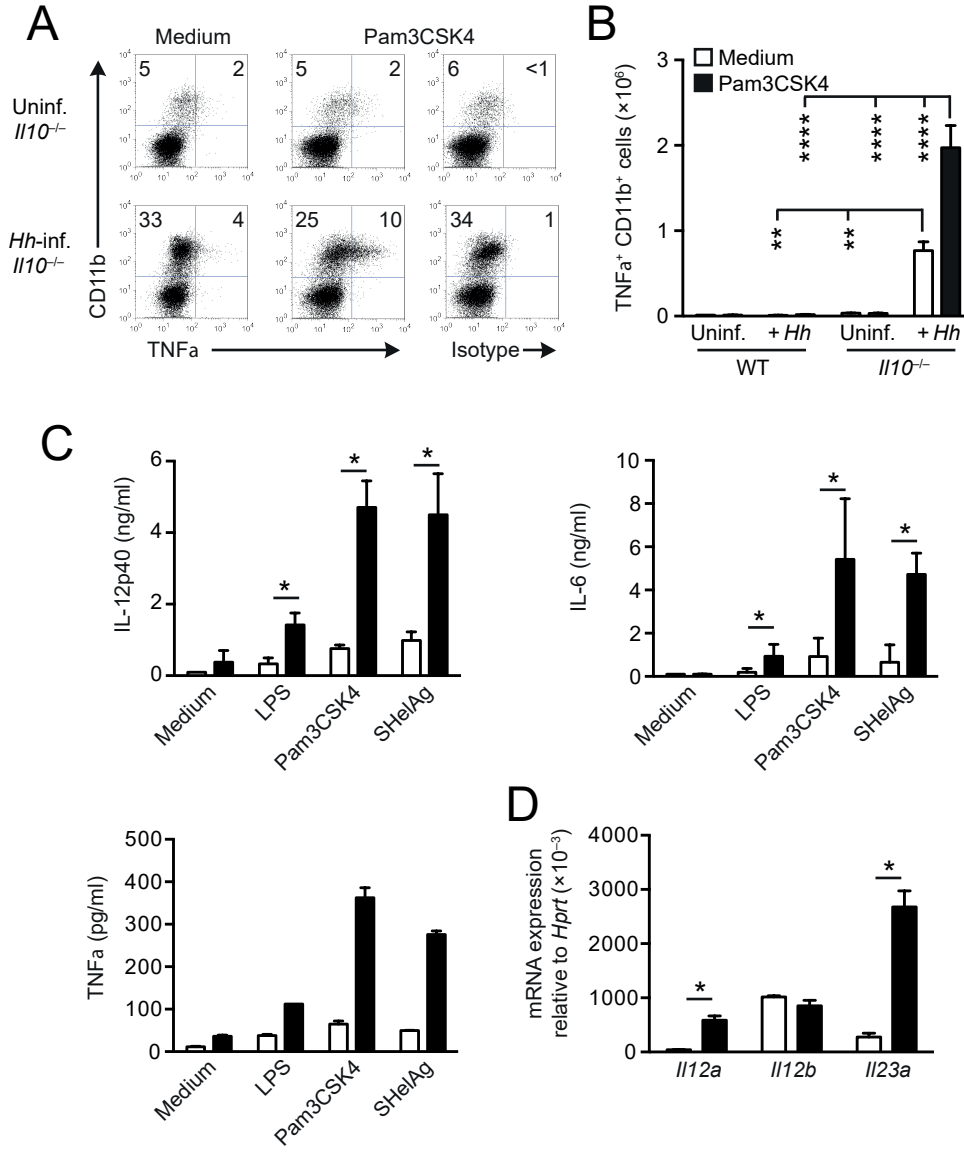


Figure 3

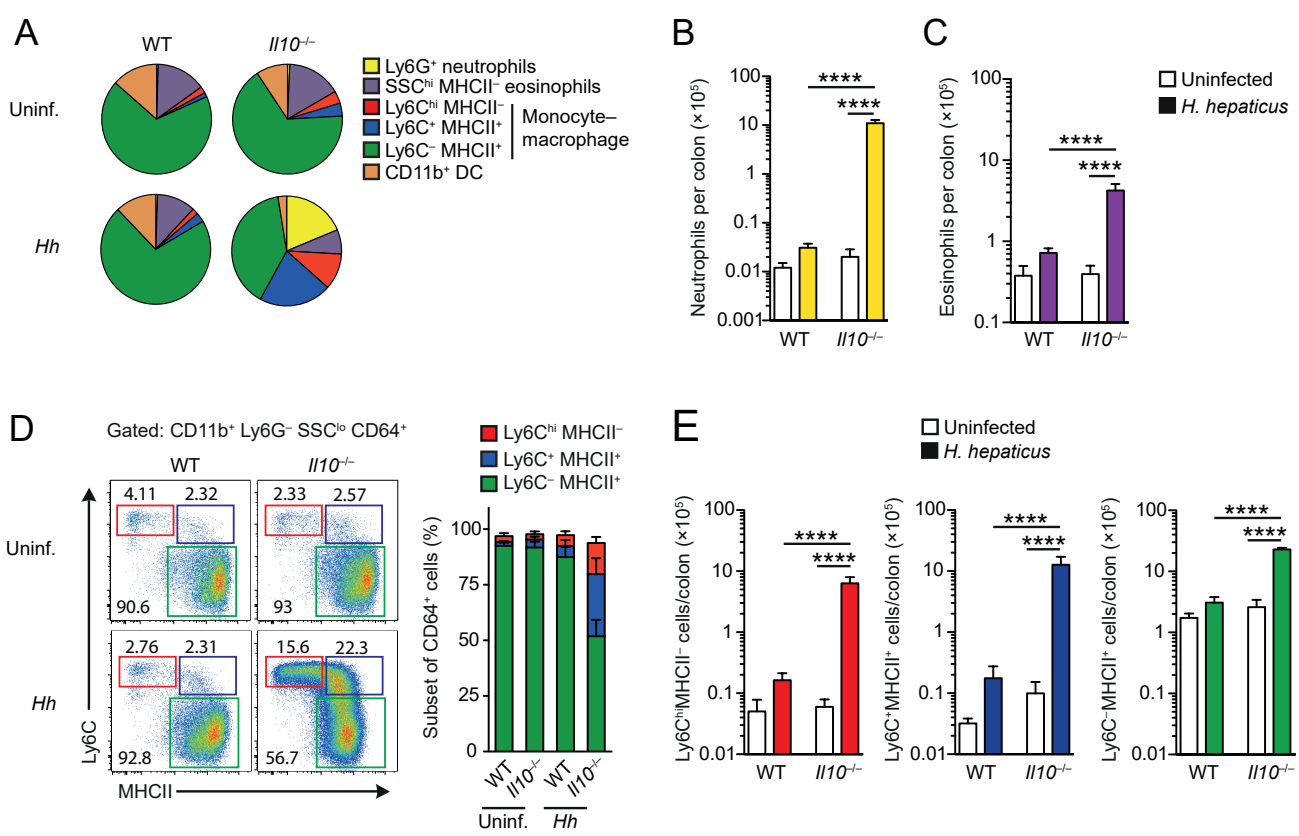


Figure 4

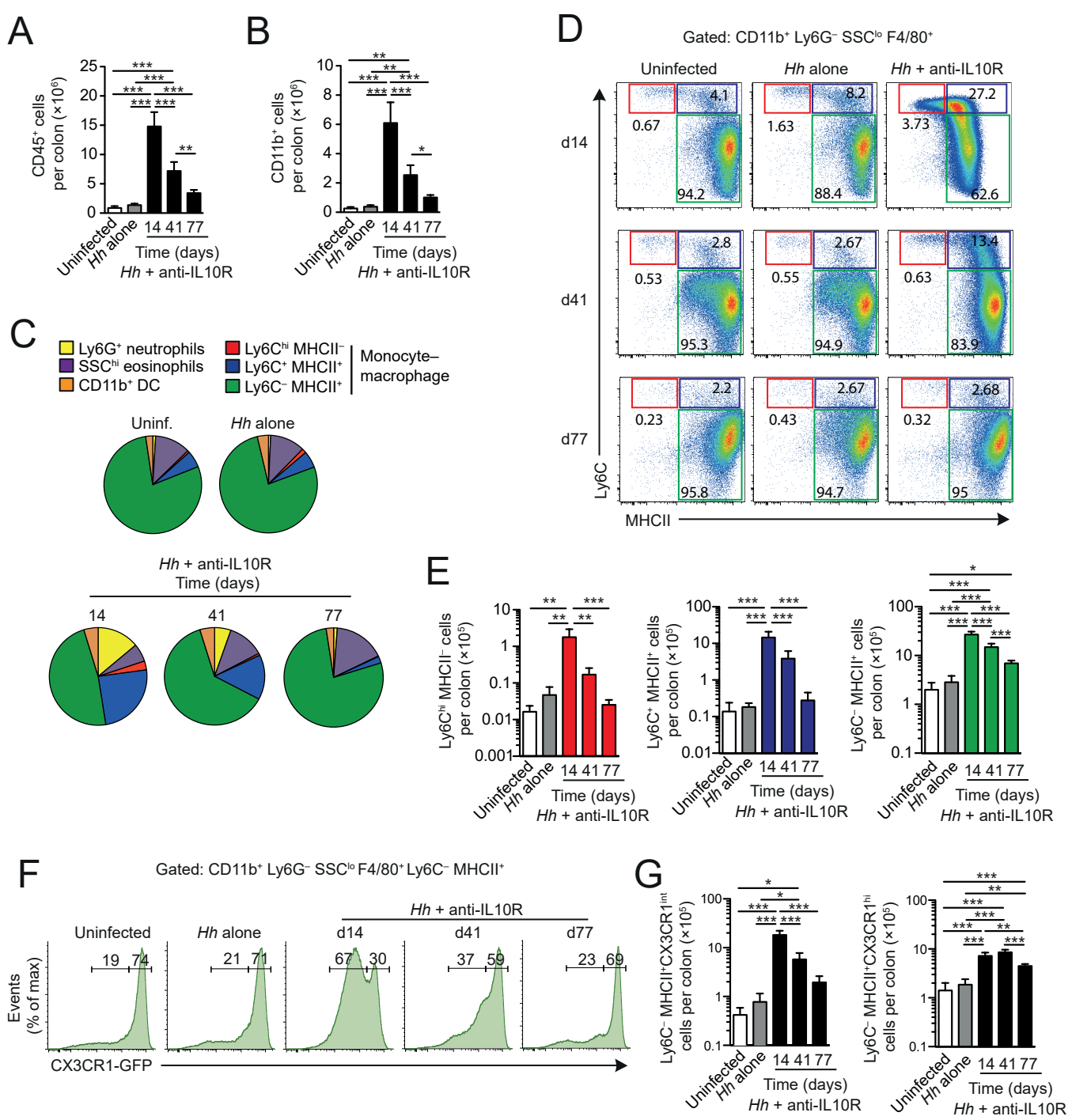


Figure 5

