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1 **PARP2 deficiency affects invariant-NKT-cell maturation and protects mice from**  
2 **Concanavalin A-induced liver injury.**

3

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6

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32 **List of abbreviations:**

33  $\alpha$ -GalCer:  $\alpha$ -Galactosylceramide, ConA: Concanavalin A, DAMPs: Damage  
34 Associated Molecular Patterns, DN: double negative, DR: death receptors, NKT:  
35 Natural Killer-T, NK: Natural Killer, PARP: poly(ADP) ribose polymerase.

36

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38

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46

47 b. Authors' contributions

48

- 49 Aveline Filliol : acquisition of data; analysis and interpretation of data, statistical analysis,  
50 drafting of the manuscript
- 51 Claire Piquet-Pellorce : acquisition of data, analysis and interpretation of data
- 52 Sarah Dion : acquisition of data, analysis and interpretation of data
- 53 Valentine Genet : acquisition of data and analysis
- 54 Catherine Lucas-Clerc : obtained technical or material support
- 55 Françoise Dantzer: drafting of the manuscript, critical revision of the manuscript for important  
56 intellectual content
- 57 Michel Samson: acquisition of data, analysis and interpretation of data, statistical analysis,  
58 drafting of the manuscript, study concept and design, study supervision
- 59

60 **Abstract (202 words)**

61 Excessive or persistent inflammation and hepatocyte death are the key triggers of  
62 liver diseases. The poly(ADP-ribose) polymerase (PARP) proteins induce cell death  
63 and inflammation. Chemical inhibition of PARP activity protects against liver injury  
64 during Concanavalin A (ConA)-induced hepatitis. In this mice model, ConA activates  
65 immune cells which promote inflammation and induce hepatocyte death, mediated by  
66 the activated invariant NKT-(iNKT) lymphocyte population. We analyzed immune cell  
67 populations in the liver and several lymphoid organs such as spleen, thymus, and  
68 bone marrow in *Parp2* deficient mice to better define the role of PARP proteins in  
69 liver immunity and inflammation at steady state and during ConA-induced hepatitis.  
70 We show that *i*) the genetic inactivation of *Parp2*, but not *Parp1*, protected mice from  
71 ConA hepatitis without deregulating cytokine expression and leucocyte recruitment;  
72 *ii*) cellularity was lower in the thymus, but not in spleen, liver, or bone marrow of  
73 *Parp2*<sup>-/-</sup> mice; *iii*) spleen and liver iNKT lymphocytes, as well as thymic T and NKT  
74 lymphocytes were reduced in *Parp2* knockout mice. In conclusion, our results  
75 suggest that the defect of T-lymphocyte maturation in *Parp2* knockout mice leads to a  
76 systemic reduction of iNKT cells, reducing hepatocyte death during ConA-mediated  
77 liver damage, thus protecting the mice from hepatitis.

78

## 79 INTRODUCTION

80 Hepatitis can be caused by various insults such as alcohol, a high fat diet, food-  
81 derived toxins, or infections, and is characterized by hepatocyte death and  
82 inflammation (5). Acute hepatitis is associated with massive hepatolysis which can  
83 lead to liver failure, whereas in chronic hepatitis, unresolved hepatolysis triggers the  
84 release of Damage Associated Molecular Patterns (DAMPs) which promote  
85 inflammation. This process induces an inflammation and cell death amplification loop  
86 that contributes to the progression of liver diseases (5, 18). Blocking the pro-  
87 inflammatory hepatocyte death response could be beneficial to in treating liver  
88 diseases.

89 During viral or auto-immune hepatitis, innate and adaptive lymphocytes kill  
90 hepatocytes which present proteins recognized as non-self-antigens, by the  
91 activation of death receptors (DR). In mice, Concanavalin A (ConA)-induced liver  
92 injury mimics immune cell-mediated hepatitis in humans (27) and is dependent on  
93 innate Natural Killer T (NKT) lymphocytes. NKT cells are involved in numerous  
94 immune responses and are particularly abundant in the liver and spleen of mice.  
95 They have the ability to recognize lipids presented by CD1d which allows there  
96 distinction into two categories of NKT cells. Type I or invariant-NKT (iNKT) cells  
97 express an invariant T-cell receptor  $\alpha$ -chain (TCR $\alpha$ ; V $\alpha$ 14-J $\alpha$ 18) and are able to  
98 recognize the  $\alpha$ -Galactosylceramide ( $\alpha$ -GalCer) presented by CD1d. Type II NKT  
99 cells express more diverse TCR V $\alpha$  chains that cannot recognize  $\alpha$ -GalCer and are  
100 characterized as CD4/CD8 double negative (DN). The iNKT are the major NKT  
101 subset in the liver and CD1d<sup>-/-</sup> (NKT deficient) mice as well as V $\alpha$ 14<sup>-/-</sup> (iNKT deficient)  
102 mice are protected from ConA-induced hepatitis (15, 26-28). ConA activates liver  
103 immune cells which promote hepatocyte death in two ways. First, activated immune

104 cells release large amounts of cytokines, such as TNF- $\alpha$  (17, 21), IFN- $\gamma$  (12, 25) and  
105 IL-4 (13) which play key roles in the hepatocyte death process (17, 27, 29). Second,  
106 activated NKT cells, potent producers of IFN-  $\gamma$ , kill hepatocytes by activation of the  
107 TNF-superfamily DR (3, 23, 26, 33) and the perforin/granzyme B system (31). Our  
108 group has extensively investigated the mechanisms of hepatocyte death during  
109 hepatitis. We and others have demonstrated a key role for PARP proteins in the liver.  
110 We have shown that inhibition of poly(ADP) ribose polymerase (PARP) 1 and 2  
111 activity with the PJ-34-inhibitor protects mice from ConA-mediated hepatolysis (2,  
112 14). Mukhopadhyay *et al.* have shown that genetic or chemical inhibition of PARP1  
113 also prevents liver inflammation and fibrosis induced by the hepatotoxic carbon  
114 tetrachloride (CCl<sub>4</sub>) or bile duct ligation (22). PARP1 and PARP2 are activated by  
115 DNA strand breaks and use NAD<sup>+</sup> as a substrate to synthesize chains of  
116 poly(ADPribose) onto various acceptor proteins, thereby inducing chromatin  
117 remodeling and the recruitment of DNA repair complexes (8). In the last decades,  
118 PARP1 and PARP2 have also been found to play an important role in inflammation  
119 (4) and cell death (30). In this study, we aimed to better define the roles of PARP1  
120 and PARP2 proteins in ConA-induced hepatitis using the *Parp1* and *Parp2* knockout  
121 mouse models.

122

## 123 **METHODS**

### 124 **Animals and treatment protocols**

125 C57BL/6 *Parp1* and *Parp2* genetically modified mice (*Parp1*<sup>-/-</sup> and *Parp2*<sup>-/-</sup>,  
126 respectively) were provided by Dr. F. Dantzer and generated as described previously  
127 (7, 32). Heterozygous mice were crossed to obtain knockout and littermate WT mice.  
128 Adult male and female mice, of 10 to 12 weeks of age, were used for each

129 experiment with the ConA model. ConA (C2010 Sigma-Aldrich, St. Louis, MO) was  
130 prepared at 3 mg/mL in PBS supplemented with 0.31 mM MnCl<sub>2</sub> and 0.75 mM CaCl<sub>2</sub>  
131 and administered by intravenous (i.v.) injection at a dose of 10 or 12 mg/kg body  
132 weight. For histopathological and biochemical studies, mice treated with ConA were  
133 compared to mice which received PBS supplemented with 0.31 mM MnCl<sub>2</sub> and 0.75  
134 mM CaCl<sub>2</sub>. For all phenotypic immune cell analyses, adult (10 to 12 weeks old) or  
135 early adult (five- week-old) mice were either not treated, to analyze the population at  
136 steady state, or treated with ConA. Animals were housed in individual cages and  
137 bred in specific pathogen-free conditions in the local animal house facilities. All  
138 treatment protocols were in accordance with the French laws and the institution's  
139 guidelines for animal welfare (agreement of M. Samson # R-2012-CPP-01).

140

#### 141 **Histopathological and biochemical studies**

142 Mouse liver biopsies were fixed in 4% paraformaldehyde and embedded in paraffin  
143 for IHC and hematoxylin and eosin (H&E) staining. For histopathology, H&E staining  
144 of liver tissues was carried out to investigate liver injury. Serum ALT and AST  
145 transaminases were measured according to the IFCC primary reference procedures  
146 using an Olympus AU2700 Autoanalyser<sup>®</sup> (Olympus Optical, Tokyo, Japan).

147

#### 148 **RNA analysis**

149 Total RNA was extracted from mouse livers using TRIzol reagent (Invitrogen). First-  
150 strand cDNA was synthesized using the SuperScript<sup>™</sup> II Reverse Transcriptase  
151 (Invitrogen). Real-time quantitative PCR was performed using the fluorescent dye



152 SYBR Green with the double-strand specific SYBR<sup>®</sup> Green system (Applied  
153 Biosystems) and the ABI 7000 Prism sequence detector (Applied Biosystems) or the  
154 CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). cDNA was used as  
155 template for amplification using specific primer pairs (Table 1). Each measurement  
156 was performed in triplicate. The relative gene expression was normalized against  
157 18S rRNA gene expression. The control mice in each treatment group served as a  
158 reference for messenger RNA (mRNA) expression (the control mRNA level was  
159 arbitrarily set to 1).

160

#### 161 **Immune cell analysis by flow cytometry**

162 Immune cells were prepared from spleen, thymus, or liver crushed on a 70 µm filter.  
163 Liver immune cells were isolated after sedimentation and cell fractionation on a 35%  
164 Percoll layer. For each organ, red blood cells were lysed using ammonium-chloride-  
165 potassium (ACK) buffer. Bone marrow immune cells were isolated by flushing two  
166 femurs with RPMI medium (Gibco). The number of immune cells was determined by  
167 counting in a Malassez counting chamber.

168 Cell suspensions were labeled for 30 min with LIVE/DEAD fixable yellow stain (Life  
169 technologies, L34959) to exclude dead cells from the analysis. Cells were also pre-  
170 incubated with an anti-CD16/32 antibody (BD Pharmingen) to block non-specific  
171 binding before incubation with the appropriate fluorochrome-conjugated antibodies  
172 (BD Pharmingen, eBioscience): anti-CD3-FITC (clone 17A2), anti-TCRβ-V450 (clone  
173 H57-597), anti-CD69-PE (clone H1.2F3), anti-CD19-APC or anti-CD19-PE (clone  
174 1D3), anti-NK1.1-PerCP-Cy-5.5 (clone PK136), anti-CD4-PE-Cy7 (clone RM4-5),  
175 anti-CD8-APC-Cy7 (clone 53-6.7), anti-GR1-eFluor450 (clone FB6-8C5), anti-

176 CD11b-PE-Cy7 (clone M1/70), anti-Sca1-PE (clone D7), anti-c-kit-PerCP-eFluor710  
177 (clone 2B8), anti-Ter119-eFluor780 (clone Ter119), and empty CD1d tetramer or  
178 CD1d tetramer loaded with  $\alpha$ -galactosylceramide (GalCer)-PE (provided by Dr. M.  
179 Leite de Moraes). The stained cells were analyzed on a FACS Aria™ II flow  
180 cytometer and data were analyzed using CXP software (Beckman Coulter). Doublets  
181 and dead cells were excluded on the basis of forward/side scatter and LIVE/DEAD  
182 labeling, respectively. The immuno-phenotyping used was as follows: B-lymphocytes:  
183 CD19+/CD3- cells; T-lymphocytes: CD3+/TCRV $\beta$ + /NK1.1-; NKT cells:  
184 CD3+/TCRV $\beta$ + /NK1.1+; NK cells: CD3-/NK1.1+; and granulocytes: GR1+CD11b+.  
185 Lymphoid activation was studied by analyzing the expression of CD69. We calculated  
186 the percentage of each immune cell population, by considering the sum of events of  
187 all immune cell populations analyzed (sum of T, NK, NKT, B cells and myeloid cells)  
188 as 100% of the total immune cells. The absolute number in each immune cell  
189 population was calculated by multiplying the percentage of each population by the  
190 total number of immune cells.

191

### 192 **Serum cytokine immunoassay by flow cytometry**

193 Murine TNF- $\alpha$ , IFN- $\gamma$  and IL-6 cytokines were quantified by bead-based  
194 immunoassays according to manufacturer protocol, using a filter plate and a vacuum  
195 filtration system for washing steps (BioLegend's LEGENDPLEX, multi-analyte flow  
196 assay kit). Samples were analyzed on a LSR Fortessa cytometer (BD Biosciences).

197

### 198 **Statistical analysis**

199 Data are expressed as the means +/- SEM for all mice treated similarly. Kruskal–  
200 Wallis one-way analysis of variance (ANOVA) was performed, and mean differences  
201 between experimental groups were assessed using the non-parametric Mann–  
202 Whitney *U*-test using GraphPad Prism5 software.

203

## 204 **RESULTS**

205

### 206 **PARP2 but not PARP1 deficiency protects mice from ConA-induced hepatitis**

207 We investigated the role of PARP1 and PARP2 proteins in hepatitis using adult  
208 knockout mice for PARP1 (*Parp1*<sup>-/-</sup>) and PARP2 (*Parp2*<sup>-/-</sup>) and comparing the results  
209 to those obtained using wild-type mice (WT). We challenged mice with ConA to  
210 induce hepatitis, or with PBS as a control, and evaluated liver damage using serum  
211 transaminase (ALT and AST) levels and hematoxylin coloration. In ConA induced-  
212 hepatitis, the peak of hepatolysis occurs 11 h after ConA treatment.(29) *Parp1*<sup>-/-</sup> mice  
213 displayed visibly, but non-significantly, lower ALT and AST transaminase levels than  
214 WT mice at this time point. In contrast, *Parp2*<sup>-/-</sup> mice were highly protected from  
215 ConA-induced hepatitis as they had significantly lower (4-fold) levels of both AST and  
216 ALT than WT mice (Fig. 1A). Accordingly, H&E coloration of liver slides from Con A-  
217 treated WT and *Parp2*<sup>-/-</sup> mice, revealed fewer areas of necrosis in *Parp2* deficient  
218 mice than in WT mice (Fig. 1B). Moreover, the absence of PARP2 appeared to  
219 protect mice from hepatolysis and did not simply delay liver injury as serum  
220 transaminase levels remained significantly lower in *Parp2*<sup>-/-</sup> mice than in WT mice,  
221 even 24 h after ConA administration (Fig. 1C). We next evaluated the level of  
222 inflammation induced by ConA, by measuring the mRNA levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-  
223 1 $\beta$ , and IL-4, all known to play key roles during ConA-induced hepatitis. TNF- $\alpha$ , IFN-

224  $\gamma$ , IL-1 $\beta$ , and IL-4 transcript levels were higher in all genotypes after ConA treatment.  
225 However, we observed no significant differences between the IFN- $\gamma$  or IL-4 mRNA  
226 levels in *Parp1*<sup>-/-</sup>, *Parp2*<sup>-/-</sup>, or WT mice, whereas TNF- $\alpha$  transcript levels were  
227 significantly lower in *Parp2*<sup>-/-</sup> mice than in the other two mouse strains. In contrast, IL-  
228 1 $\beta$  mRNA levels were higher in both *Parp2*<sup>-/-</sup> and *Parp1*<sup>-/-</sup> mice than in WT mice.  
229 Next, we analyze serum cytokines from WT and *Parp2*<sup>-/-</sup> mice. TNF- $\alpha$ , IFN- $\gamma$  and IL-  
230 6 were up-regulated in both genotypes after ConA challenge. However, TNF- $\alpha$  and  
231 IFN- $\gamma$  levels were similar between WT and *Parp2*<sup>-/-</sup> mice, whereas IL-6 rate  
232 decreased in *Parp2*<sup>-/-</sup> mice (Fig. 1E).

233

#### 234 ***Parp2*<sup>-/-</sup> mice display a substantial reduction in the number of liver iNKT cells**

235 We performed all further experiments using only the *Parp2*<sup>-/-</sup> mouse model, as  
236 PARP2 protein deficiency protected mice from ConA-induced liver damage, whereas  
237 that of PARP1 did not. ConA induces liver injury in an immune cell-dependent  
238 manner. Thus we next measured the steady-state, basal levels of the different liver  
239 immune cell subsets in *Parp2*<sup>-/-</sup> mice of two different ages: early adult (five weeks)  
240 and adult (10 to 12 weeks) by flow cytometry. Cell doublets and dead cells were  
241 excluded and the phenotypic analysis of liver immune cells was determined using the  
242 gating strategy presented in Figure 2A.

243 *Parp2*<sup>-/-</sup> mice displayed a similar number of liver immune cells as WT mice at the two  
244 different ages (Fig. 2B). The absolute number of total T lymphocytes and the ratio of  
245 CD4:CD8 T-lymphocytes were also similar in the livers of both genotypes (Fig. 2C).  
246 We next analyzed the NKT-lymphocyte population, defined as CD3, TCRV $\beta$ , and  
247 NK1.1 positive cells. We used the CD1d tetramer loaded with  $\alpha$ GalCer to analyze the  
248 invariant-NKT (iNKT) subset (CD3<sup>+</sup> TCRV $\beta$ <sup>+</sup> NK1.1<sup>+</sup>  $\alpha$ GalCer<sup>+</sup>) and the anti-CD4

249 antibody to study NKT DN cells (CD3<sup>+</sup> TCRV $\beta$ <sup>+</sup> NK1.1<sup>+</sup>  $\alpha$ GalCer<sup>-</sup> CD4<sup>-</sup>). *Parp2*<sup>-/-</sup>  
250 mice displayed a reduction in the percentage of total liver NKT cells of two fold in  
251 five-week-old mice and of 1.5 fold in 10-12-week-old mice (Fig. 2D). However,  
252 PARP2 deficiency did not impair the increase in the percentage of liver NKT cells as  
253 the mice aged (Fig 2D). We also observed that only the percentage of iNKT cells, but  
254 not NKT DN cells, was significantly lower in the *Parp2*<sup>-/-</sup> mice at both ages (Fig. 2E).  
255 The absolute number of liver immune cells was similar between both genotypes,  
256 whereas the number of iNKT cells was lower in *Parp2*<sup>-/-</sup> mice. We thus aimed to  
257 determine which immune cell subsets compensated this reduction. The percentage of  
258 NK-cells (NK1.1<sup>+</sup> CD3<sup>-</sup>) and B-lymphocytes (CD19<sup>+</sup> CD11b<sup>-</sup>) were higher in five-  
259 week-old *Parp2*<sup>-/-</sup> mice, but only the percentage of NK-cells was higher in adult *Parp2*  
260 <sup>-/-</sup> mice (Fig. 2F and 2G). The liver myeloid compartment, based on GR1 and CD11b  
261 labelling, also appeared to be similar in *Parp2*<sup>-/-</sup> and WT mice, regardless of age (Fig.  
262 2H). In conclusion, PARP2 deficiency induced a significant reduction in the number of  
263 iNKT cells in the liver which was not compensated by an increase of all immune cell  
264 subsets, but by a gain of B and NK-lymphocytes.

265

266 **PARP2 deficiency does not affect the recruitment and activation of immune**  
267 **cells during ConA-induced hepatitis.**

268 We next examined the impact of PARP2 deficiency on ConA-induced cell recruitment  
269 and activation. ConA-induced inflammation and cell death induced a similarly high  
270 level of leucocyte recruitment to the liver in mice of both genotypes as seen by the  
271 large increase in the absolute number of liver immune cells 11 h after injection, which  
272 then decreased with the resolution of the hepatitis 24 h after ConA treatment (Fig.  
273 3A). We evaluated the NKT-cell subset and found a reduction in the number of NKT

274 cells in the livers of mice of both genotypes upon ConA treatment. However, this  
275 reduction was 3.7 fold in WT mice, relative to untreated mice, but only 1.7 fold in  
276 *Parp2*<sup>-/-</sup> mice, (Fig. 3B). The absence of PARP2 protein did not seem to affect the  
277 recruitment of other leucocyte populations, as T, B, and NK-lymphocytes, and  
278 myeloid cells were similarly recruited for both genotypes 11 h after ConA  
279 administration (Fig. 3C). Finally, we analyzed the early activation marker CD69 on  
280 lymphocytes and observed that more than 90% of T-lymphocytes in both *Parp2*<sup>-/-</sup> and  
281 WT mice were activated after ConA treatment (Fig. 3D).

282

### 283 ***Parp2*<sup>-/-</sup> mice have fewer spleen iNKT cells than WT mice**

284 We demonstrated that *Parp2*<sup>-/-</sup> mice were protected from ConA-hepatitis and had  
285 fewer hepatic NKT cells, without affecting ConA-induced inflammation. We next  
286 analyzed the immune cell subsets in the various lymphoid compartments such as  
287 spleen, bone marrow, and thymus. Gating strategies used for splenocyte analysis are  
288 depicted in Supplementary Figures 1 and 2. PARP2 deficiency did not affect the  
289 absolute number of splenocytes in mature mice but they were slightly, but not  
290 significantly, higher in number in early adult mice (Fig. 4A). We then evaluated the  
291 percentage cells in each immune cell subset in the spleen. The number of T-  
292 lymphocytes (CD3<sup>+</sup> NK1.1<sup>-</sup> TCRVβ<sup>+</sup>) were lower only in early adult *Parp2*<sup>-/-</sup> mice  
293 (Fig. 4B) and was compensated by an increase in the number of B-lymphocytes  
294 (CD19<sup>+</sup> CD11b<sup>-</sup>) (Fig. 4C). Similar to our observations in the liver, the spleens of  
295 five- and 10-12-week-old *Parp2*<sup>-/-</sup> mice had significantly fewer NKT cells than those of  
296 WT mice, and only the iNKT cells were affected (Fig. 4D). In contrast to the liver, the  
297 number of spleen NK cells was only slightly, but not significantly, higher in *Parp2*<sup>-/-</sup>  
298 than in WT mice (Fig. 4E).

299

300 ***Parp2*<sup>-/-</sup> mice have fewer thymus iNKT cells than WT mice.**

301 We next investigated the thymocyte population of five-week-old mice to understand  
302 the cause of the reduction in NKT-cell number in the liver and spleen of *Parp2*<sup>-/-</sup> mice.  
303 The absolute number of thymocytes was lower in *Parp2*<sup>-/-</sup> mice than in WT mice, and  
304 was associated with a reduction in the absolute number of immature CD4/CD8  
305 double positive (DP) and CD4 or CD8 simple positive (SP) thymocytes (Fig. 5A and  
306 5B). The absolute number of iNKT cells ( $\alpha$ GalCer<sup>+</sup>) was also lower in *Parp2*<sup>-/-</sup> mice  
307 (Fig. 5C). However, the remaining iNKT cells expressed wild-type levels of mature T  
308 lymphocyte markers such as CD3 and TCRV $\beta$  (Fig. 5D).

309

310 ***Parp2*<sup>-/-</sup> mice have more Ter119<sup>+</sup>-cells than WT mice.**

311 Finally, we eliminated doublets and dead cells, analyzed BM immune cells by flow  
312 cytometry to analyze the different immune cell subsets, as depicted in Figure 3A,  
313 then we investigated the immune cell subsets in the bone marrow (BM) of *Parp2*<sup>-/-</sup>  
314 mice. As observed in the spleen and the liver, BM cellularity was similar between  
315 *Parp2*<sup>-/-</sup> and WT adult mice (Fig. 6B). The absolute numbers of the different mature  
316 BM cell subsets (CD19<sup>+</sup>, GR1<sup>high</sup> CD11b<sup>+</sup>, GR1<sup>high</sup> CD11b<sup>+</sup> and CD3<sup>+</sup>) were  
317 similar with slight variations between the two genotypes, except for Ter119 positive  
318 cells which were significantly more numerous in *Parp2*<sup>-/-</sup> mice (Fig. 6C). Similarly, the  
319 number of Lin<sup>-</sup> cells (Ter119<sup>-</sup> CD19<sup>-</sup> CD3<sup>-</sup> GR1<sup>-</sup> CD11b<sup>-</sup>) was slightly, but not  
320 significantly higher in the *Parp2*<sup>-/-</sup> mice, and was associated with a small increase in  
321 the number of the myeloid progenitor (MP) (Lin<sup>-</sup> ckit<sup>+</sup> Sca<sup>-</sup>) and LSK populations  
322 (Lin<sup>-</sup> ckit<sup>+</sup> Sca<sup>+</sup>) (Fig. 6D).

323

324 **DISCUSSION**

325 We previously reported that inhibition of PARP protein activity with the PJ-34 inhibitor  
326 protected mice from ConA-induced hepatolysis (2, 14). PARP proteins mediate the  
327 activation of various transcription factors, such as NF- $\kappa$ B, to promote inflammation in  
328 several inflammatory diseases, including CCl<sub>4</sub> induced chronic liver injury.  
329 Inactivation or inhibition of PARP proteins prevents inflammation-induced tissue  
330 damage (4). Inflammation induced by ConA plays a key role in the induction of  
331 hepatolysis (12, 13, 17, 21, 25). However, we previously demonstrated that PJ-34  
332 does not inhibit inflammation during ConA-induced hepatitis, as cytokine release and  
333 liver leucocyte recruitment occurred efficiently, suggesting a more direct role for  
334 PARP proteins in the induction of hepatocyte death (2, 14). Here, we show that the  
335 genetic inactivation of *Parp2*, but not *Parp1*, protects mice from ConA-induced  
336 hepatitis without deregulating inflammation, as leucocyte recruitment and pro-  
337 inflammatory cytokine expression were not affected. Analysis of liver and spleen  
338 immune populations revealed a substantial reduction in the percentage of iNKT cells,  
339 normally enriched in both organs (19). The reduction in the number of iNKT in *Parp2*<sup>-/-</sup>  
340 mice may explain the reduced hepatolysis and protection from ConA-induced  
341 hepatitis observed in these mice. Indeed, rapid elimination of NKT cells after ConA  
342 treatment is a marker of NKT-induced liver injury following their activation (26). We  
343 also observed the elimination of NKT cells after ConA treatment in both *Parp2*<sup>-/-</sup> and  
344 WT mice, but as fewer NKT cells were present in the untreated *Parp2*<sup>-/-</sup> mice relative  
345 to WT mice, there were fewer NKT cells available to induce hepatocyte cell death  
346 after ConA treatment in these mice. Surprisingly, whereas IFN- $\gamma$  is highly produced  
347 by NKT cells, we did not observed a reduction of both its expression and its serum  
348 level in *Parp2*<sup>-/-</sup> mice. However in the liver, the reduction of NKT-cell number was



349 compensated by an increase in the number of NK cells which are potent producers of  
350 IFN- $\gamma$ . As a consequence, in *Parp2*<sup>-/-</sup> mouse liver, the increase of NK cells could  
351 compensated the NKT-cell reduction in the inflammatory process. However, the  
352 increase in NK-cell number was not sufficient to restore hepatocyte cell death, as  
353 ConA-induced liver injury is strictly NKT-dependent and NK-independent (26, 28).  
354 Our study using *Parp1* and *Parp2* knockout mice did not allow us to clarify the effect  
355 of the PJ-34 inhibitor. Moreover, the fact that *Parp1*<sup>-/-</sup> mice were not protected against  
356 ConA-induced hepatitis, suggests that PARP2 participates in ConA-mediated liver  
357 injury, as the two proteins share common functions (1, 8). *Parp1* and *Parp2* double  
358 knockout mice are lethal (20). It may be informative to use conditional knockout mice  
359 to further decipher the role of both proteins in the liver.

360 We analyzed the thymus and bone marrow of *Parp2*<sup>-/-</sup> mice to identify the cause of  
361 the systemic reduction of iNKT cells detected in these mice. As previously described,  
362 *Parp2*<sup>-/-</sup> mice displayed an increase of the erythroid subset (10), but no T-lymphocyte  
363 deregulation. iNKT cells mature in the thymus and are derived from the DP  
364 population (11). In agreement with others, we observed a reduction in the number of  
365 DP-thymocytes in the absence of PARP2 protein (32) and revealed a substantial  
366 reduction in the number of thymic iNKT cells. PARP2 deficiency leads to inefficient  
367 V $\alpha$  to J $\alpha$  rearrangement inducing the death of DP-thymocytes (32), resulting in a  
368 decrease in the number of mature SP T-lymphocytes and iNKT cells. However, only  
369 iNKT-cell numbers were lower in the liver and spleens of adult mice, but not those of  
370 conventional T-lymphocytes.

371 The rearrangement of TCR occurs at the DP-thymocyte stage and involves DNA-  
372 break and DNA-repair mechanisms. PARP2 is sensitized to DNA damage (8) and  
373 has been highlighted to play a role in the TCR $\alpha$  rearrangement process during

374 thymocyte development, as *Parp2*<sup>-/-</sup> DP thymocytes display a skewed TCRα  
375 repertoire and a decrease of cell survival (32). The rearrangement of the TCRα locus  
376 occurs in a sequential manner from proximal to distal regions (16) and *Parp2*<sup>-/-</sup> DP-  
377 thymocytes present a reduction of the distal segments Jα42 to Jα4 (32). Interestingly,  
378 iNKT cell lineage differentiates at the DP stage and is characterized by expression of  
379 an unique TCRα chain with a Vα14-Jα18 rearrangement. As the Jα18 segment is  
380 located between the segments Jα42 and Jα4, this strongly suggests that use of Jα18  
381 is reduced in DP-thymocyte *Parp2*<sup>-/-</sup>, and could explain the reduction of iNKT-  
382 lymphocyte subset. This is supported by the fact that a default of TCRα  
383 rearrangement is known to induce DP-thymocyte cell death (16) and that *Parp2*<sup>-/-</sup>  
384 thymocytes present a shorter lifespan (32).

385

386 In conclusion, previous phenotypic investigations of *Parp2*<sup>-/-</sup> mice revealed an  
387 important role of the PARP2 protein in the regulation of lipid metabolism (24),  
388 erythropoiesis (9), thymopoiesis (32) and spermatogenesis (6). Here, we highlight a  
389 new phenotype in *Parp2*<sup>-/-</sup> mice associated with the deregulation of thymopoiesis,  
390 resulting in a systemic iNKT deficiency, leading to protection against ConA-induced  
391 liver injury.

392

393

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400 and animal house platforms) of SFR BIOSIT, University of Rennes 1, France.

401

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499

500

501 **Figures legends**

502 **Figure 1: The absence of PARP2 but not PARP1 protects mice from ConA**  
503 **induced-hepatitis.** (A) Serum levels of serum AST and ALT in WT, *Parp1*<sup>-/-</sup>, and  
504 *Parp2*<sup>-/-</sup> mice, 11 h after PBS (n = 4 to 6) or ConA injection (n =1 3 for WT, 8 for  
505 *Parp1*<sup>-/-</sup>, and 24 for *Parp2*<sup>-/-</sup>). (B) Representative images of H&E stained liver tissue  
506 sections, necrosis areas are outlined by the white dotted line. (C) Serum levels of  
507 ALT of WT or *Parp2*<sup>-/-</sup> mice 24 h after ConA treatment. (D) Levels of liver TNF- $\alpha$ , IFN- $\gamma$ ,  
508 IL-1 $\beta$ , and IL-4 transcripts from WT, *Parp1*<sup>-/-</sup>, and *Parp2*<sup>-/-</sup> mice 11 h after ConA  
509 administration. (E) Serum levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-6 in WT and *Parp2*<sup>-/-</sup> mice, 11  
510 h after PBS (n = 4 to 6) or ConA injection (n= 6 to 11). For the scatter plots, errors  
511 bars represent + SEM. (\* #p < 0.05; \*\* ## p < 0.01; \*\*\* ### p < 0.001; ns: non-  
512 significant).

513

514 **Figure 2: PARP2 deficiency results in a decrease in the number of liver iNKT**  
515 **cells.** (A) Gating strategy used to analyze lymphocyte subpopulations (NKT, NK, T  
516 cells, B cells) and myeloid cells in the liver and spleen. The gating strategy for liver  
517 immune cells is presented. (B) Absolute number of liver immune cells. (C) Absolute  
518 number of T lymphocytes (CD3+ TCRVb+NK1.1-), CD4-positive cells (in black) and  
519 CD8-positive cells (in white). (D) Absolute number of NKT cells (CD3+  
520 TCRVb+NK1.1-) in the liver (E) Representative dot plot showing NKT subsets in the  
521 liver (left panel) and absolute number of liver iNKT cells (corresponding to  
522 aGalCer+CD4+ and aGalCer+CD4- cells) (middle panel) and liver NKT DN-cells  
523 (aGalCer-CD4-) (right panel). (F) Representative dot plot showing liver NK-cell  
524 population (left part) and absolute number of liver NK cells (NK1.1+CD3-) (right  
525 panel). (G) Absolute number of liver B-lymphocytes (CD19+CD11b) and (H) myeloid

526 cells (GR1<sup>int</sup>CD11b+ and GR1<sup>high</sup>CD11b+). All suspensions of liver immune cells from  
527 non-treated mice, aged 5 or 10 to 12 weeks, were obtained from the livers of WT or  
528 *Parp2*<sup>-/-</sup> mice. Each dot in the graph represents the number of cells recovered from  
529 one liver. The line and error bars represent the mean +/- SEM. For the histograms,  
530 the errors bars represent + SD. (\* #p < 0.05; \*\* ## p < 0.01; \*\*\* ### p < 0.001; ns:  
531 non-significant).

532

533 **Figure 3: PARP2 deficiency does not affect ConA-induced liver-leucocyte**  
534 **recruitment or activation.** (A) Absolute number of liver immune cells from WT or  
535 *Parp2*<sup>-/-</sup> adult mice, non-treated (Ctl) or treated with ConA. Immune cells were  
536 isolated 11 or 24 h after ConA treatment. (B) Absolute number of NKT-cells from the  
537 livers of WT or *Parp2*<sup>-/-</sup> mice at steady state (Ctl) or 11 h after ConA treatment. (C)  
538 Absolute number of lymphocytes: T cells, B cells, and NK cells (upper panel) and  
539 myeloid cells (lower part) from liver of WT or *Parp2*<sup>-/-</sup> mice at steady state or 11 h  
540 after ConA treatment. (D) Representation of the activation marker (CD69) of T-  
541 lymphocytes CD8+ (NK1.1-TCRVb+CD3+CD8+) in the liver of WT or *Parp2*<sup>-/-</sup> mice  
542 treated or not with ConA. The line and error bars represent the mean + SD (\* #p <  
543 0.05; \*\* ## p < 0.01; \*\*\* ### p < 0.001; ns: non-significant).

544

545 **Figure 4: PARP2 deficiency results in a reduction in the number of iNKT cells in**  
546 **the spleen.** (A) Number of splenocytes of non-treated WT and *Parp2*<sup>-/-</sup> mice, aged 5  
547 (early adult mice) or 10 at 12 weeks (adult mice). (B) Percentage of T lymphocytes  
548 (CD3+), TCRVb+NK1.1-), (C) B-ymphocytes (CD19+CD11b-), (D) total NKT cells  
549 (CD3+TCRVb+NK1.1+) (left panel), and NKT subsets: iNKT (NKT, α-GalCer+) (middle  
550 panel) and NKT-DN (NKT, α-GalCer- CD4-) (right panel), (E) NK cells (CD3-NK1.1+)

551 determined by flow cytometry analysis from spleens of early-adult or adult WT and  
552 *Parp2*<sup>-/-</sup> mice. Each dot in the graph represents the number of cells recovered from  
553 one liver. The lines and error bars represent the mean +/- SEM. (\* p <0.05; \*\* p <  
554 0.01; \*\*\* p < 0.001; ns: non-significant).

555

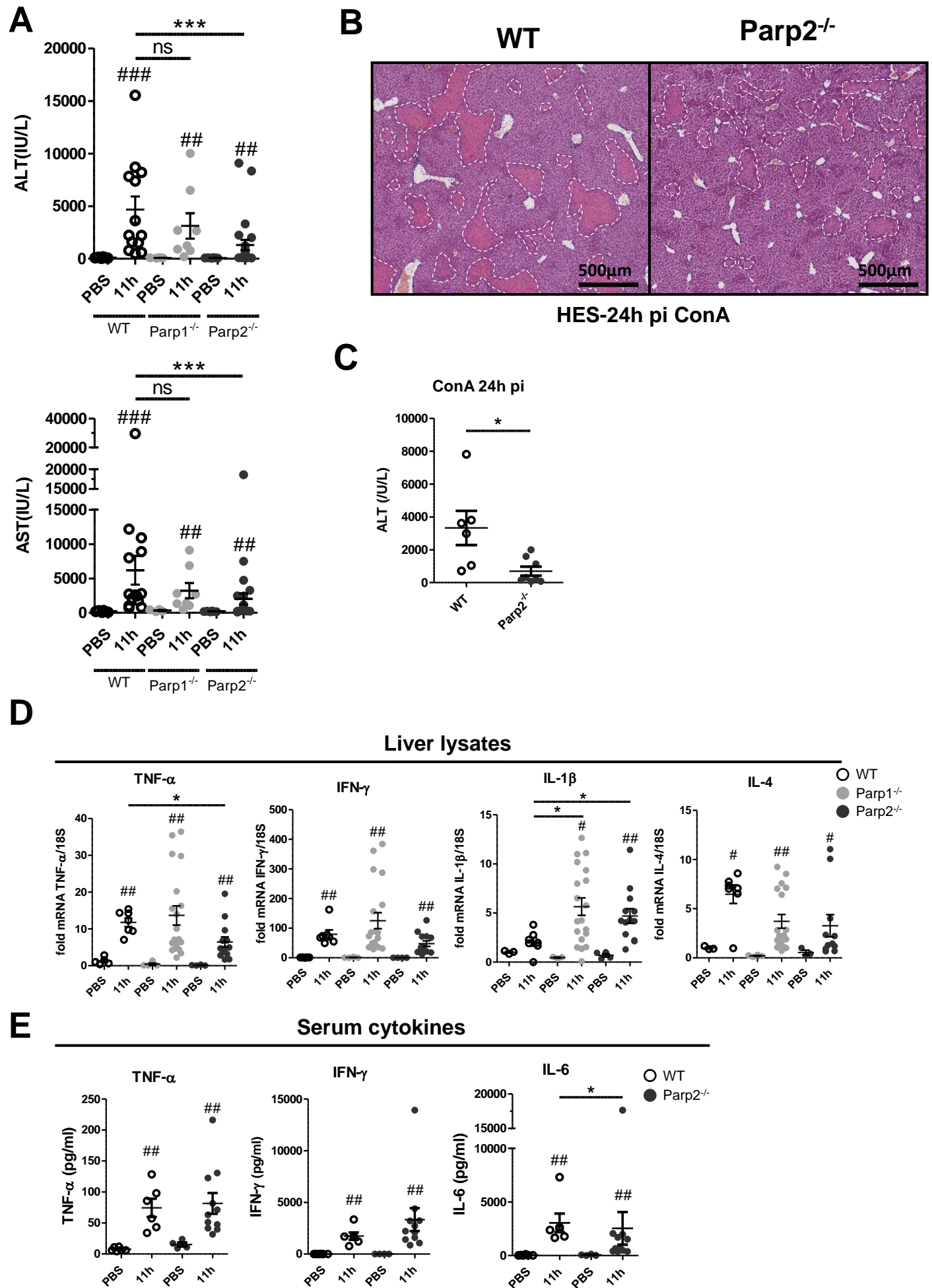
556 **Figure 5: PARP2 deficiency results in a decreased number of CD4/CD8 DP and**  
557 **SP cells as well as iNKT cells.** (A) Representative staining profiles of CD4 and CD8  
558 single or double positive cells from the thymus of non-treated five-week-old WT or  
559 *Parp2*<sup>-/-</sup> mice. (B) Graph showing total number and the number of CD4<sup>+</sup>CD8<sup>+</sup> double  
560 positive thymocytes. (C) Representative staining profile of  $\alpha$ GalCer positive  
561 thymocytes. (D) Representation of mature T-cell markers (CD3 and TCRV $\beta$ ) of  
562  $\alpha$ GalCer positive thymocytes. Each dot in the graph represent the number of cells  
563 recovered from one liver. The lines and errors bars represent the mean +/- SEM. (\*  
564 p<0.05; \*\* p<0.01; \*\*\* p<0.001; ns: non-significant).

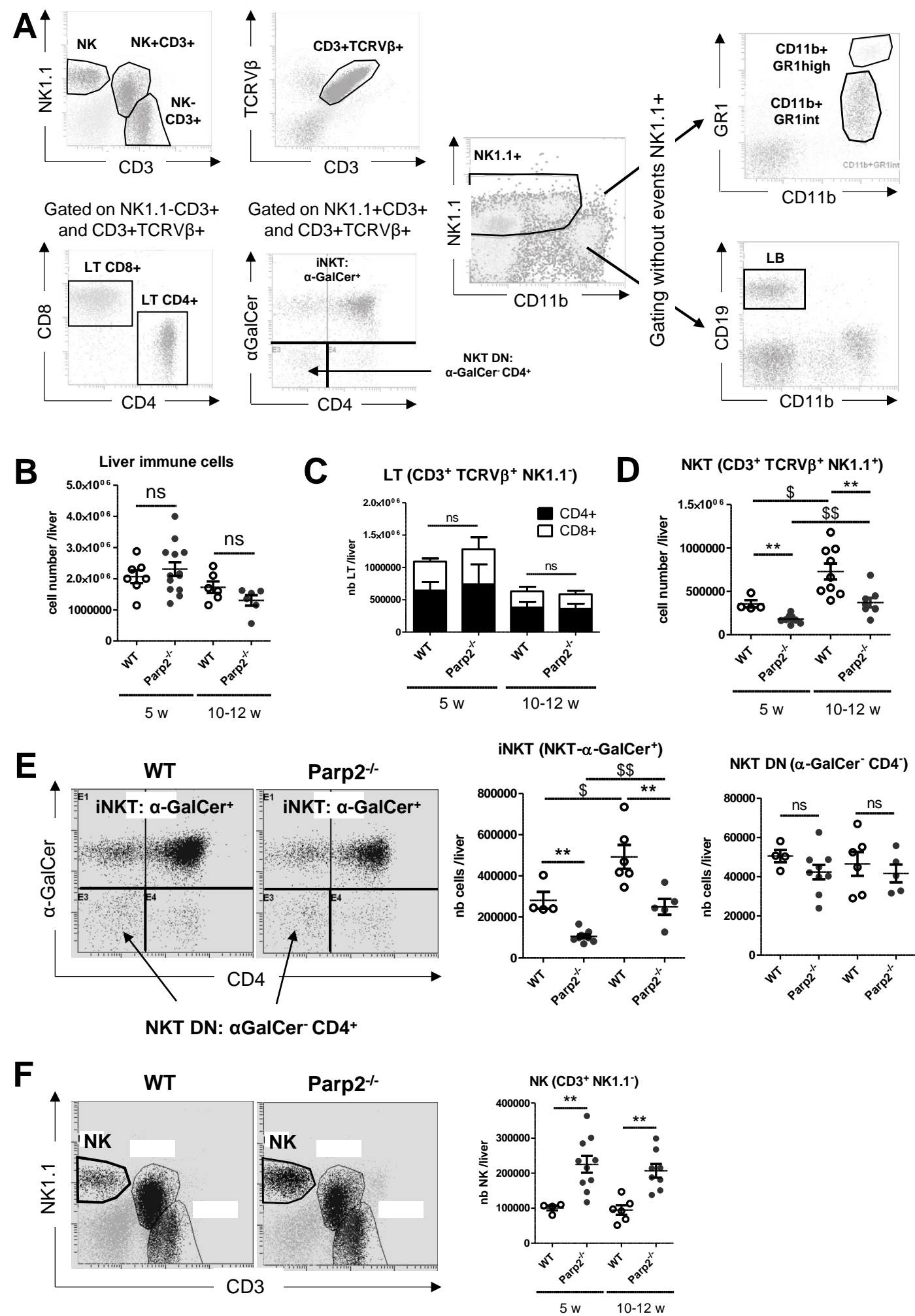
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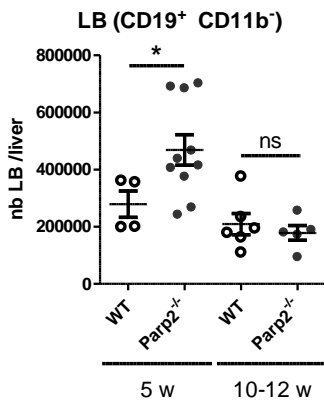
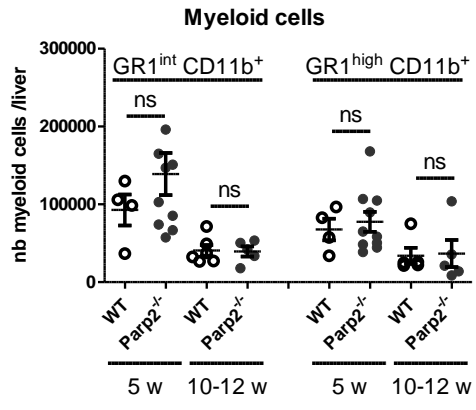
566 **Figure 6: *Parp2*<sup>-/-</sup> mice display an increased number of Ter119<sup>+</sup>-cells but not of**  
567 **other mature or immature subsets.** (A) Gating strategy used to analyze bone  
568 marrow immune cell subsets. (B) Absolute number of cells recovered from two  
569 femurs of WT or *Parp2*<sup>-/-</sup> adult (10 at 12 weeks old) mice. (C) Absolute cell number  
570 for each mature bone marrow immune cells subset: Ter119<sup>+</sup>, CD19<sup>+</sup>, CD3<sup>+</sup>,  
571 GR1<sup>high</sup>CD11b<sup>+</sup>, and GR1<sup>int</sup>CD11b<sup>+</sup>. (D) Absolute number of immature Lin<sup>-</sup> bone  
572 marrow immune cells (Ter119<sup>-</sup> CD19<sup>-</sup> CD3<sup>-</sup> GR1<sup>-</sup> CD11b<sup>-</sup>) (left panel) and the specific  
573 MP (Lin<sup>-</sup>ckit<sup>+</sup>Sca<sup>-</sup>) and LSK (Lin<sup>-</sup>ckit<sup>+</sup>Sca<sup>+</sup>) subsets (right panel). Each dot in the  
574 graph represents the number of cells recovered from one liver. The lines and errors



575 bars represent the mean +/- SEM. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; ns: non-  
576 significant).

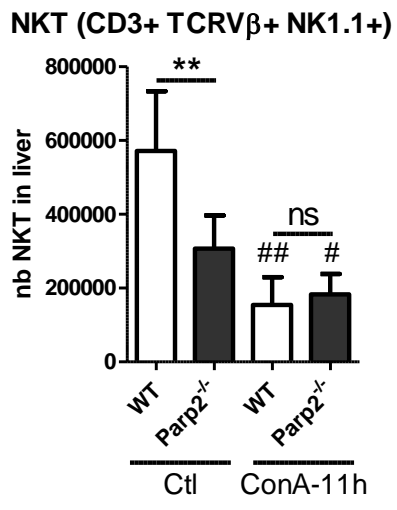
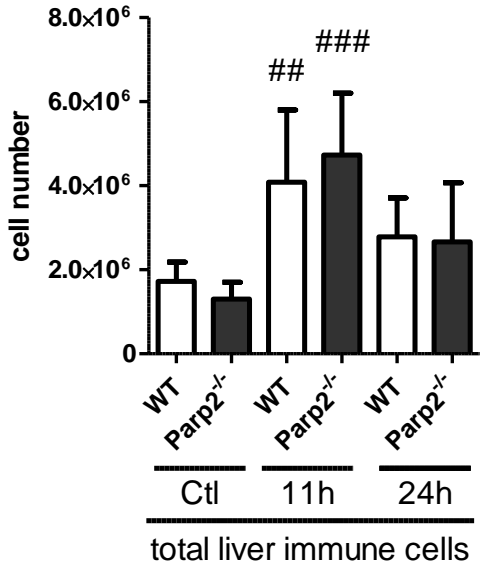
**Figure 1**

**Figure 2**

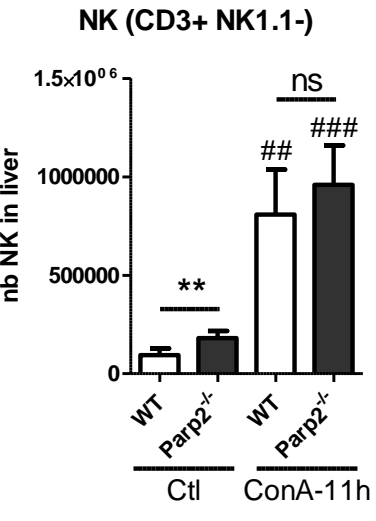
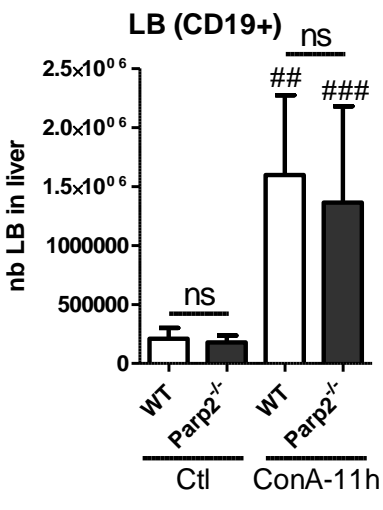
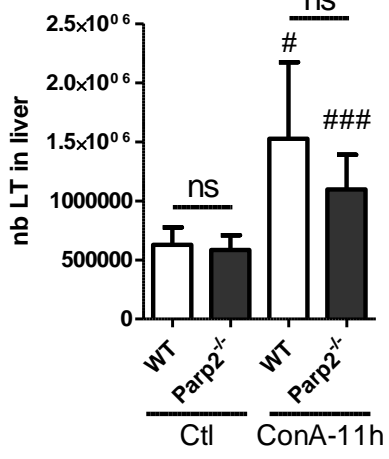
**G****H**

**Figure 3**

**A ConA (12 mg/kg) B**



**C LT (CD3+ TCRVβ+ NK1.1-) LB (CD19+) NK (CD3+ NK1.1-)**



**GR1<sup>int</sup> CD11b+ GR1<sup>high</sup> CD11b+**

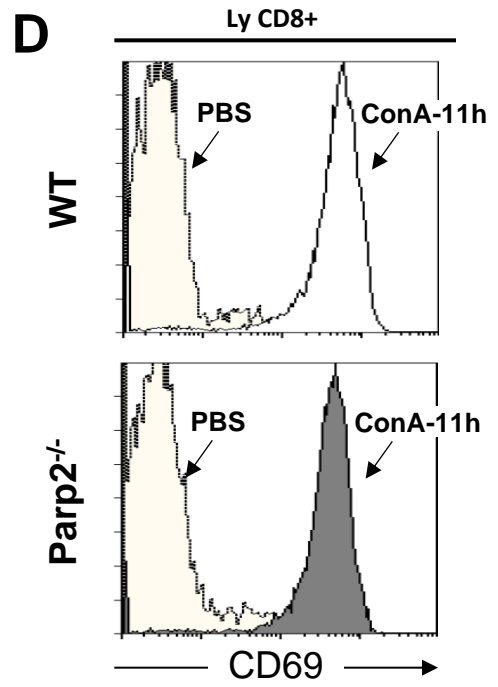
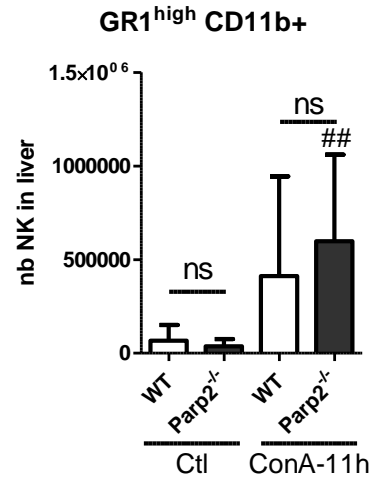
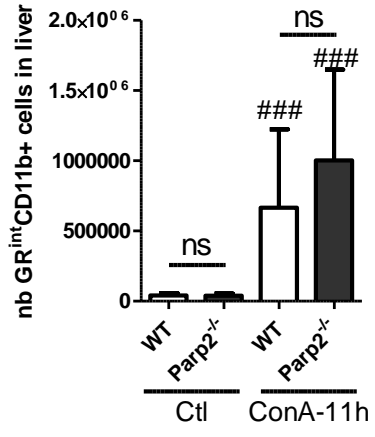
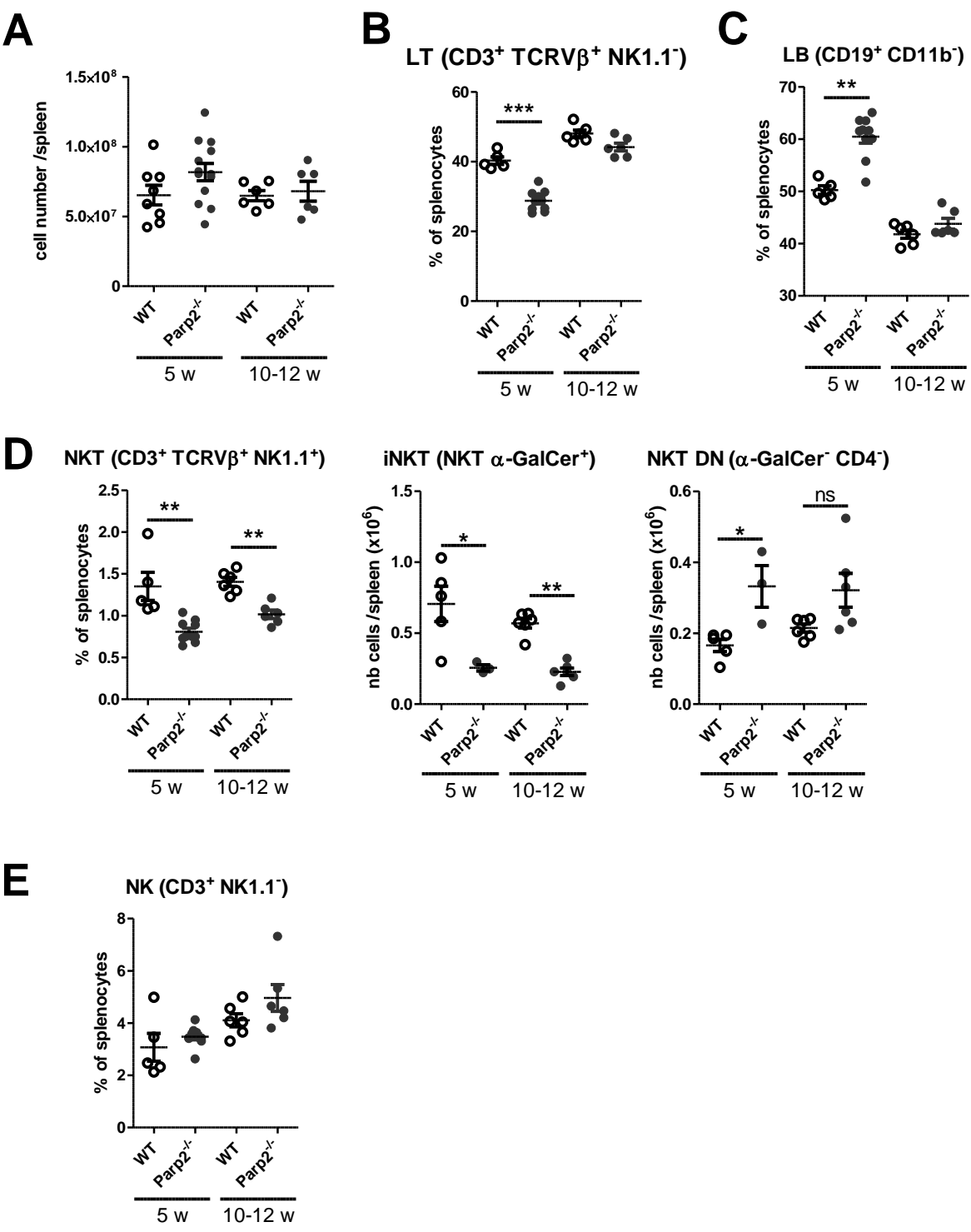
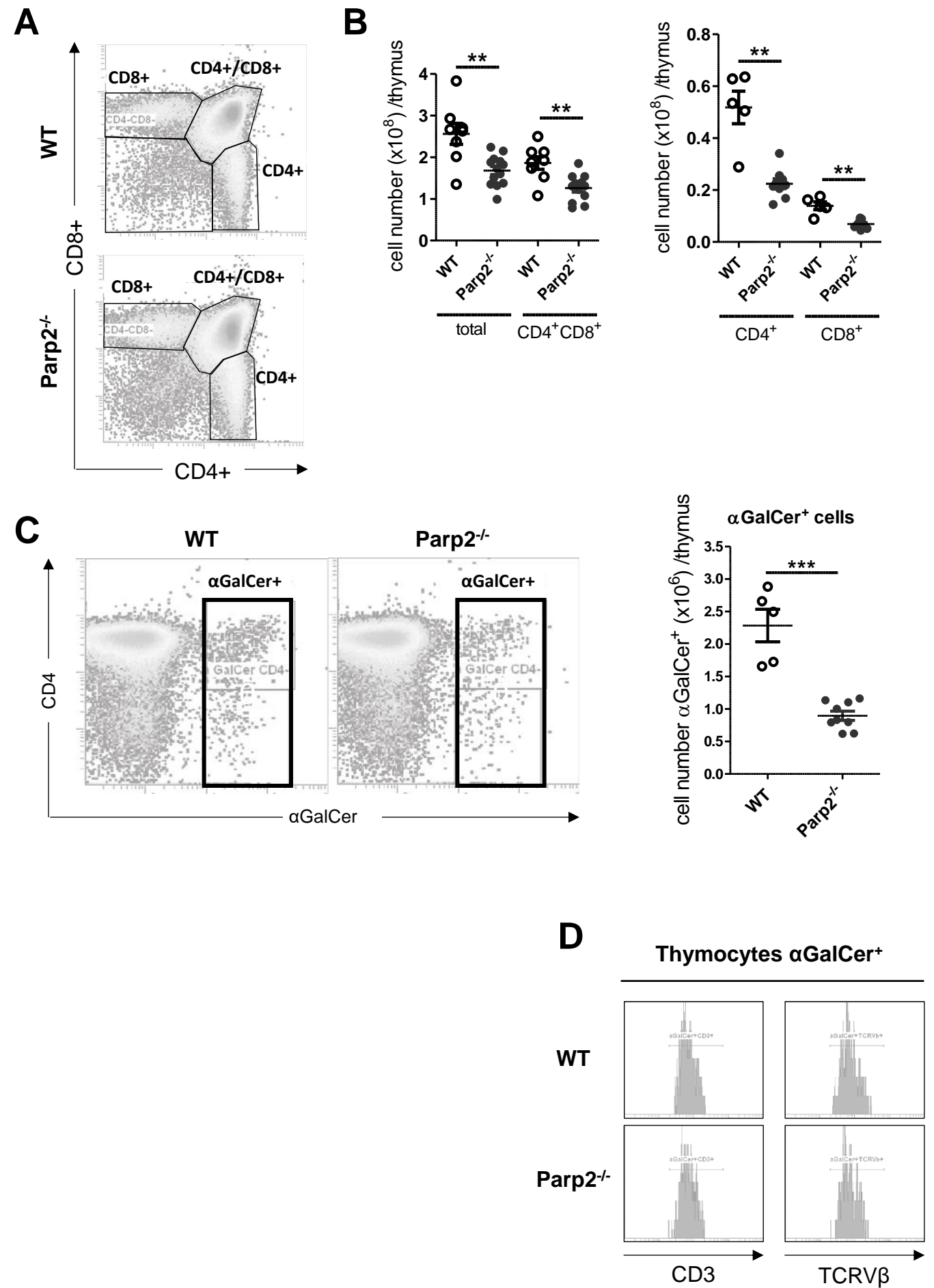
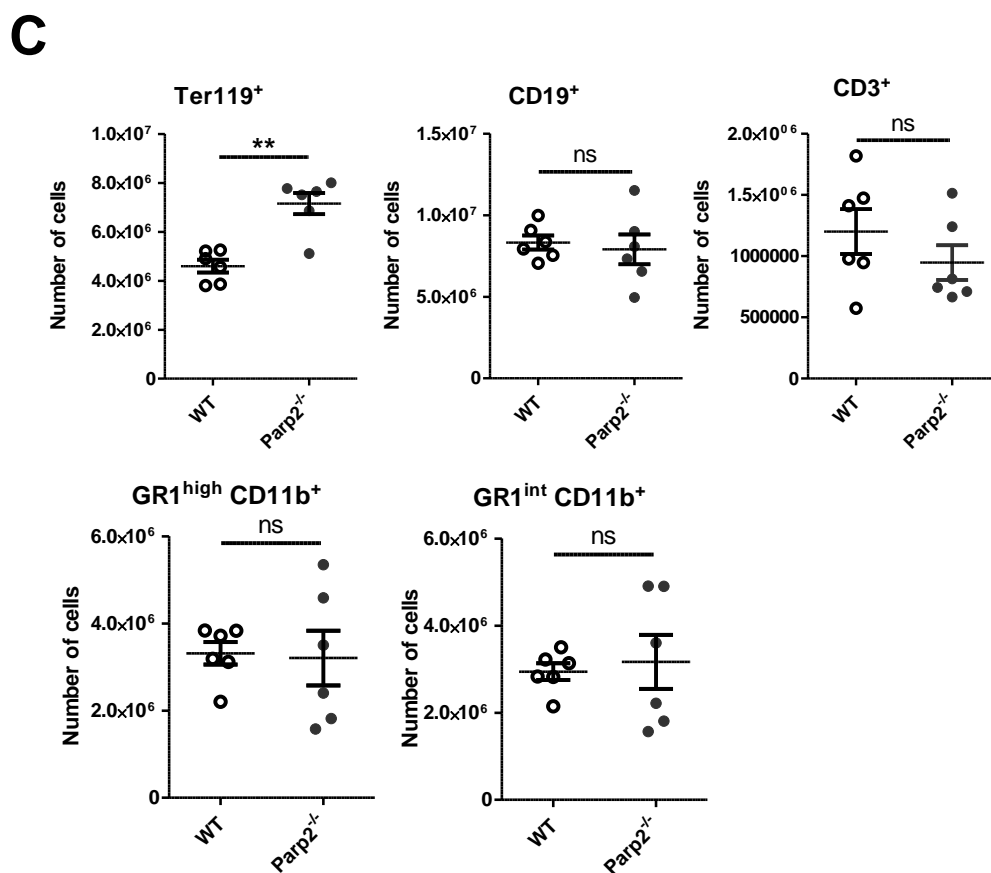
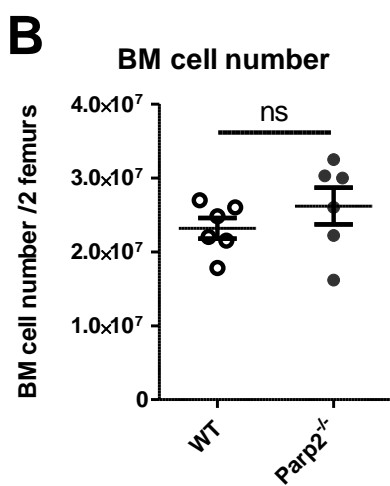
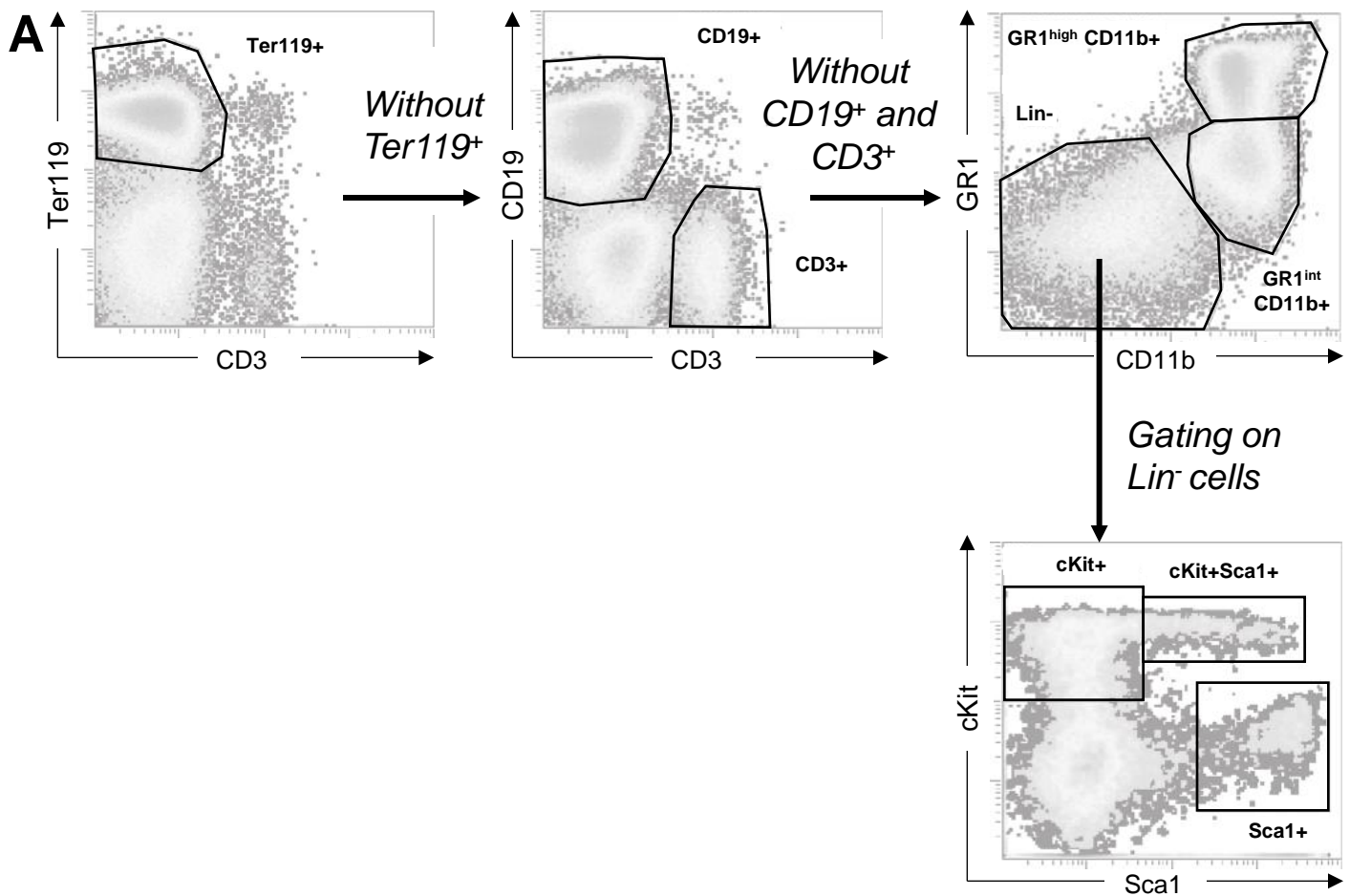


Figure 4

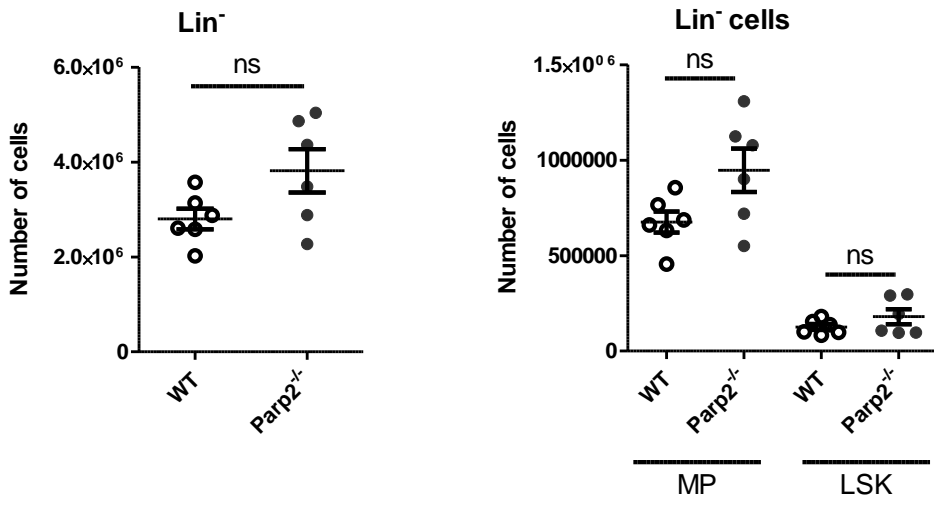


**Figure 5**



**Figure 6**



**D**

**Table 1. Sequence of primers used for qPCR**

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
Mouse 18S	5'-CGCCGCTAGAGGTGAAATTC-3'	5'-TTGGCAAATGCTTTCGCTC-3'
Mouse TNF $\alpha$	5'-TAGCTCCCAGAAAAGCAAGC-3'	5'-TTTTCTGGAGGGAGATGTGG-3'
Mouse IFN- $\gamma$	5'AGGTCAACAACCCACAGGTC3'	5'ATCAGCAGCGACTCCTTTTC3'
Mouse IL-1 $\beta$	5'-GAAGAAGTGCCCATCCTCTG-3'	5'-AGCTCATATGGGTCCGACAG-3'
Mouse IL-4	5'-GGCTTCCAAGGTGCTTGG-3'	5'-GGACTTGGACTCATTGATGG-3'