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1 **Ablation of interaction between IL-33 and ST2<sup>+</sup> regulatory T cells increases immune**  
2 **cell-mediated hepatitis and activated NK cells liver infiltration**

3

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18

19 **Keywords:** Con A-hepatitis, immune cells, liver, IL-33-deficient mice, ST2 receptor,  
20 regulatory T cells

21

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25

26 **List of abbreviations:** AST: aspartate aminotransferase, ALT: alanine aminotransferase, IL-  
27 1RAcP: interleukin-1 receptor accessory protein, knockout: (-/-), PI: post-injection.

28

29 \* These authors contributed equally to this work and shared first co-authorship.

30 **Abstract**

31 The IL-33/ST2 axis plays a protective role in T cell-mediated hepatitis, but little is known  
32 about the functional impact of endogenous IL-33 on liver immunopathology. We used IL33-  
33 deficient mice to investigate the functional effect of endogenous IL-33 in concanavalin A  
34 (Con A)-hepatitis. IL-33<sup>-/-</sup> mice displayed more severe Con A liver injury than WT mice,  
35 consistent with a hepatoprotective effect of IL-33. The more severe hepatic injury in IL-33<sup>-/-</sup>  
36 mice was associated with significantly higher levels of TNF- $\alpha$  and IL-1- $\beta$  and a larger number  
37 of NK cells infiltrating the liver. The expression of Th2 cytokines (IL-4, IL10) and IL-17 was  
38 not significantly varied between WT and IL-33<sup>-/-</sup> mice following Con A-hepatitis. The  
39 percentage of CD25<sup>+</sup> NK cells was significantly higher in the livers of IL-33<sup>-/-</sup> mice than in  
40 WT mice in association with up-regulated expression of CXCR3 in liver. Regulatory T cells  
41 (Treg cells) strongly infiltrated the liver in both WT and IL-33<sup>-/-</sup> mice, but Con A treatment  
42 increased their membrane expression of ST2 and CD25 only in WT mice. *In vitro*, IL-33 had  
43 a significant survival effect, increasing the total number of splenocytes, including B cells,  
44 CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and the frequency of ST2<sup>+</sup> Treg cells. In conclusion, IL-33 acts as a  
45 potent immune modulator protecting the liver through activation of ST2<sup>+</sup>Treg cells and  
46 control of NK cells.

47

48 **Introduction**

49 Interleukin-33 (IL-33), a member of the IL-1 family, drives immune responses by interacting  
50 with its specific receptor ST2, and IL-RAcP (1, 39). IL-33 is mostly produced in the nucleus  
51 of cells in barrier tissues, such as epithelial and endothelial cells (primarily in the lung, skin  
52 and brain) (29, 39), but it is also produced by hematopoietic cells such as macrophages or  
53 mast cells following stimulation *in vitro* (31). ST2 is produced by various immune cells,  
54 including mast cells, granulocytes, dendritic cells, NK/NKT cells and Th2 lymphocytes (28),  
55 and this receptor has also recently been detected on regulatory T cells (Treg cells) (27). Treg  
56 cells form a heterogeneous population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells. They are involved in both  
57 the normal physiological and pathological suppression of immune reactivity. Treg cells play  
58 an important role in many diseases, including autoimmune diseases, inflammatory disorders,  
59 transplant rejection, tumorigenesis and infections (41).

60 The role of the IL-33/ST2 axis remains unclear. IL-33 drives innate immune responses in  
61 various inflammatory conditions *in vivo* (30) and has been shown to induce anti-helminth and  
62 antiviral responses (10, 35). These findings indicate a role for the IL-33/ST2 axis in mediating  
63 adaptive immune responses. Indeed, IL-33 acts as a central mediator, driving Th2  
64 differentiation by inducing the production of IL-5 and IL-13 (32, 34). Moreover, IL-33  
65 suppress protective Th1 differentiation in protozoan infections (36) but promotes Th1  
66 differentiation in a mouse model of viral infection (7, 8). The IL-33/ST2 axis may therefore  
67 orchestrate both Th1 and Th2 immune responses, depending on the type of activated  
68 cell/tissue involved, and the microenvironment and cytokine network in damaged tissues. IL-  
69 33 has been implicated in many diseases. It has protective effects against obesity,  
70 atherosclerosis, and helminth infection, but exacerbates asthma, arthritis, experimental  
71 autoimmune encephalomyelitis and dermatitis (22). Finally, by interacting with ST2<sup>+</sup> Treg  
72 cells, IL-33 seems to control the immune system in various pathological conditions, including

73 heart transplantation (43), breast cancer growth or metastases (20) and experimental colitis  
74 (14, 38).

75 Liver endothelial cells constitutively express IL-33, and we have shown IL-33 to be  
76 overexpressed in hepatocytes during necrotic NKT-TRAIL-mediated hepatic cell death (4, 5)  
77 and viral hepatitis (2). IL-33 is also produced by the hepatic stellate cells in hepatic fibrosis  
78 (26). Thus, during liver inflammation, IL-33 is released after liver cell death (4) and it was  
79 considered to act as an “alarmin”, like HMGB1 and IL-1 $\alpha$  (18). However, the effects of  
80 endogenous IL-33 during liver inflammation remain obscure, and the ST2<sup>+</sup> liver immune cells  
81 and the effects of IL-33 on these target cells have yet to be characterized.

82 In this study, we aimed to decipher the role of endogenous IL-33 and its target immune cell  
83 populations during liver inflammation induced by concanavalin A (Con A) in mice. Liver  
84 inflammation rapidly leads to the production of copious amounts of IL-33 by hepatocytes and  
85 liver endothelial cells (5). The Con A-induced hepatitis model is representative of  
86 autoimmune, viral or immune cell-mediated hepatitis in humans and liver injury in this model  
87 is caused principally by NK, NKT cells and TRAIL (40, 42). Previous studies on Con A-  
88 induced hepatitis have generated conflicting results: Chen *et al.* demonstrated a protective role  
89 of an IL-33-blocking antibody in liver injury (11), whereas, Volarevic *et al.* showed IL-33 to  
90 have beneficial effects on hepatitis in studies on ST2<sup>-/-</sup> mice or in which recombinant IL-33  
91 was administered to mice (44). We investigated the mechanism of liver activation/protection  
92 by IL-33 and the interaction of this cytokine with target ST2<sup>+</sup> immune cells, by using IL-33<sup>-/-</sup>  
93 and WT mice to determine the functional role of endogenous IL-33 in Con A-induced  
94 hepatitis.

95 IL-33<sup>-/-</sup> mice displayed more severe liver injury than WT mice following Con A  
96 administration. This exacerbated liver injury was associated with of the infiltration of large  
97 number of immune cells, including CD25<sup>+</sup> NK cells in particular. Treg cells infiltrated the

98 livers of both IL-33-deficient and WT mice, but membrane levels of ST2 and CD25 were  
99 higher in Con A-treated WT mice. In summary, Con A-treated mice lacking the IL-33 gene  
100 displayed lower levels of infiltrating ST2<sup>+</sup> Treg cell activation and exacerbated liver injury.

101

102

### 103 **Materials and Methods**

#### 104 **Animals and treatment protocol**

105 Eight- to 10-week-old wild-type (WT) C57Bl/6 (Janvier, Le Genest-sur-isle, France) or IL-  
106 33 knockout (<sup>-/-</sup>) C57Bl/6 mice (matched for age and sex) (provided by Dr Jean-Philippe  
107 Girard) (33) each received an intravenous (i.v.) injection of Con A (Sigma-Aldrich), at a dose  
108 of 20 mg/kg body weight, to induce acute hepatitis. Mice were killed 12 or 24 hours post-  
109 injection (PI). All mice were reared in specific pathogen-free conditions at the local animal  
110 house facilities. The study was conducted in accordance with French law and institutional  
111 guidelines for animal welfare. All efforts were made to minimize suffering and the number of  
112 animals involved. The protocol was approved by the "Comité Rennais d'Ethique en matière  
113 d'Expérimentation Animale", the local ethics committee accredited by the French Ministry of  
114 Research and Higher Education (protocol agreement number: R-2012-CPP-OI, researcher  
115 agreement for M. Samson #35-96 and C. Piquet-Pellorce #35-82).

116

#### 117 **Histopathological, biochemical and immunohistochemical analyses**

118 The histopathological (H&E staining) and serum biochemical analyses (AST/ALT) were  
119 performed as described earlier (5). Briefly, immunolocalisation of IL-33 was performed using  
120 primary antibody goat IgG anti-mouse-IL-33 (R&D Systems) and secondary HRP-conjugated  
121 rabbit anti-goat antibody (Dako, USA) with hematoxylin counterstaining in Ventana machine  
122 (Ventana Medical Systems, Inc. USA). Immuno-localisation of ST2 was performed using 3

123 stainings: rat IgG2b anti-mouse-ST2 (clone RMST2-33, ebiosciences) coupled with a  
124 secondary Cy-3 anti-rat antibody (Dako, USA); phalloidin (F-actin staining) coupled with  
125 alexa 350 (Interchim, Montluçon, France) and Draq-5 (DNA dye) coupled with far red  
126 fluorescence (Biostatus, Loughborough, UK).

127

### 128 **RNA isolation and RT-qPCR**

129 Total RNA was extracted from mouse liver pieces using TRIzol Reagent (Invitrogen,  
130 Carlsbad, CA). First-strand cDNA was produced using the SuperScript™ II Reverse  
131 Transcriptase (Invitrogen). Real-time qPCR was performed using the fluorescent dye SYBR  
132 Green with the double-strand specific dye SYBRs Green system (Applied Biosystems) and  
133 the 7300 sequence detection system ABI Prism sequence detector (Applied Biosystems).  
134 Total cDNA (30 ng) was used as a template for amplification with the specific primer pair  
135 (Table 1) used at a 300nM final concentration. Each measurement was performed in  
136 duplicate. The mRNA level of mouse IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-4, IL-10, IL-13, IL-17A,  
137 CXCR3 were normalized to the mRNA expression of ubiquitous house-keeping gene 18S.

138

### 139 **Isolation of liver and spleen immune cells and flow cytometry**

140 The liver immune cells were isolated as previously described (13, 23), with a viability > 95%.  
141 Splenocytes were obtained by passing spleens through a 70-mm cell strainer to dissociate the  
142 cells before red blood cells lysis. Cells were resuspended in staining buffer (10% FCS in PBS)  
143 and incubated with anti-CD16/32 antibody (BD Pharmingen) to block non-specific binding.  
144 The cells were then labeled with the appropriate fluorochrome-conjugated antibodies/reagents  
145 (BD Pharmingen and eBioscience): orange live dead, anti-CD3-V500 (clone 500-A2), anti-  
146 CD4-PE-Cy7 (clone RM4-5), anti-CD8-APC-Cy7 (clone 53-6.7), anti-CD25-PEeFluor610  
147 (clone PC61.5 or clone 3C7), anti-NK1.1-PerCP-Cy-5.5 (clone PK136), anti-ST2-PE (clone

148 RMST2-33), anti-CD11b-PE Cy<sup>TM</sup>7 (clone M1/70), CD11c-APC (clone HL3), anti-Gr1-  
149 V450-Ly.6G/C (clone RB6-8C5) and CD19-APC (clone 1D3). After membrane staining,  
150 intranuclear staining was carried out with the Foxp3-Alexa488 antibody (clone MF23),  
151 according to the manufacturer's instructions. The stained cells were analyzed on a  
152 FACS Aria<sup>TM</sup> II flow cytometer with BD FACSDiva software (BD Bioscience) and the data  
153 were analyzed with CXP software (Beckman Coulter). Doublet cells were excluded on the  
154 basis of forward and side scatter.

155

#### 156 ***In vitro* stimulation of mouse primary splenocytes (immune cells) with Con A**

157 Splenocytes (CD4<sup>+</sup>, CD8<sup>+</sup>, B cells and Treg cells) from WT mouse spleen were purified as  
158 described above. The splenocytes were then dispensed into a round-bottomed 96-well culture  
159 plate (10<sup>5</sup> cells/well) and activated with two concentrations of Con A (0.1 and 0.5 µg/ml) in  
160 the presence or absence of 10 ng/ml IL-33 (Peprotech). Cells were counted and analyzed by  
161 flow cytometry at 4 and 7 days of Con A stimulation. Proliferation was quantified by staining  
162 a sample of splenocytes with CFSE (1 µg/ml, 10 min at 37°C) before activation. The dilution  
163 of the CFSE signal in the wells was then observed, by flow cytometry, on days 4 and 7 of  
164 stimulation.

165

#### 166 **Statistical analysis**

167 The results shown are representative of at least three independent experiments and are  
168 expressed as means ± SEM. We used Mann-Whitney *U* tests, as implemented in GraphPad  
169 Prism5 software, to compare parameters between WT and IL-33<sup>-/-</sup> mice in *in vivo* studies (#  
170 *p*<0.05, ## *p*<0.01 and ### *p*<0.001) and in comparisons between groups in *in vitro* studies (\*  
171 *p*<0.05, \*\* *p*<0.01 and \*\*\* *p*<0.001). Time-course analyses of WT and IL-33<sup>-/-</sup> mice were



172 carried out independently, by one-way ANOVA with Dunn's multiple comparison test (\*  
173  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ).

174

## 175 **Results**

### 176 **IL-33 deficiency sensitizes mice to Con A-induced liver injury**

177 We investigated the role of IL-33 in liver disease, by comparing the time course of Con A  
178 hepatic injury in WT and IL-33<sup>-/-</sup> mice. Serum transaminase (AST/ALT) levels 12 or 24 h  
179 after Con A administration were significantly higher in IL-33<sup>-/-</sup> and WT mice than in control  
180 mice treated with PBS (Fig. 1A). A significant difference between IL-33<sup>-/-</sup> and WT mice was  
181 observed 24 h after Con A administration, but not at 12 h, indicating that IL-33<sup>-/-</sup> mice were  
182 more sensitive to Con A liver injury than WT mice, but with a time lag to the manifestation of  
183 this effect (Fig. 1A). Massive parenchymal and perivascular zones of hepatic injury were  
184 observed in IL-33<sup>-/-</sup> and WT mice following Con A-hepatitis (Fig. 1B). Immunostaining for  
185 IL-33 in liver tissues demonstrated constitutive IL-33 production by the vascular endothelial  
186 and sinusoidal endothelial cells of control mouse livers (Fig. 1C). We observed an induction  
187 of IL-33 production by the hepatocytes of WT mouse livers following Con A-hepatitis (as  
188 previously described (3); Fig. 1C). The more severe Con A-induced liver injury in IL-33<sup>-/-</sup>  
189 mice was associated with significantly higher levels of TNF- $\alpha$  and IL-1 $\beta$  in IL-33<sup>-/-</sup> mice than  
190 in WT mice at 24 h, but not at 12 h (Fig. 1D). By contrast, similar high levels of IFN- $\gamma$   
191 transcripts were observed in IL-33<sup>-/-</sup> mice and WT mice, 12 h after Con A administration (Fig.  
192 1D). The signature of Th2 cytokines such as IL-4 and IL-10 showed comparable liver mRNA  
193 expression in IL-33<sup>-/-</sup> mice and WT mice following Con A-hepatitis but IL-13 transcript level  
194 significantly decreased at 24 h in IL-33<sup>-/-</sup> mice (Fig. 1D). Increased but comparable mRNA  
195 expression of cytokine IL-17A was found in IL-33<sup>-/-</sup> mice and WT mice following Con A-  
196 hepatitis (Fig. 1D). Thus, IL-33 deficiency led to more severe liver injury 24 h after Con A

197 administration, together with the establishment of a more pro-inflammatory cytokine micro-  
198 environment.

199

200 **Deletion of the IL-33 gene leads to higher levels of intrahepatic leukocytes and**  
201 **significant NK-cell infiltration of the liver during Con A-induced hepatitis**

202 We determined the signature of immune cell infiltration during liver injury, by quantifying the  
203 leukocyte population in the livers of mice challenged with or without Con A, by flow  
204 cytometry with a live-dead marker (Fig. 2A). The increase in the total number of liver  
205 leukocytes was significantly larger in WT mice than in PBS-treated controls at 12 h, and in  
206 IL-33<sup>-/-</sup> mice than in PBS-treated controls at 24 h. Thus, 24 h after Con A administration,  
207 greater leukocyte infiltration had occurred in the IL-33<sup>-/-</sup> mice than in WT mice (Fig. 2B). A  
208 strong decrease in the number of NKT cells and a significant increase in the number of NK  
209 cells was evident 12 h after Con A administration, in both WT and IL-33<sup>-/-</sup> mice (Fig. 2B),  
210 suggesting that NKT cells are involved in the early stages of liver damage, with NK cells  
211 playing a role later on. This hypothesis was confirmed by quantifying liver CD69  
212 expression/MFI (a lymphoid cell activation marker) between 12 h and 24 h after Con A  
213 administration. Indeed, a marked increase in CD69 expression on NK cells was observed  
214 following Con A administration, in both WT and IL-33<sup>-/-</sup> mice, whereas CD69 expression  
215 remained stable in NKT cells (Fig. 3A and 3B). There were significantly more intrahepatic  
216 NK cells in IL-33<sup>-/-</sup> mice than in WT mice 24 h after Con A administration (Fig. 2B).  
217 Accordingly, the liver mRNA expression of CXCR3 (chemokine receptor involved in  
218 recruitment of immune cells) was significantly raised at 24 h of Con A liver injury in IL-33<sup>-/-</sup>  
219 mice than in WT mice (Fig. 3C).

220 This tendency for the number of infiltrating cells to increase by 12 h and then return to the  
221 basal state was observed for NK cells, B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in WT mice, but only

222 for CD8<sup>+</sup> T cells in IL-33<sup>-/-</sup> mice. The numbers of NK cells and B cells increased between 12  
223 h and 24 h after Con A administration in IL-33<sup>-/-</sup> mice (Fig. 2B). A non-significant difference  
224 (PBS vs. Con A) in the numbers of macrophages and dendritic cells was observed in both WT  
225 and IL-33<sup>-/-</sup> mice, but the number of neutrophils increased significantly by 24 h after Con A  
226 administration in both WT and IL-33<sup>-/-</sup> mice (Fig. 2C). In conclusion, IL-33 deficiency  
227 resulted in a late increase in immune cell infiltration into the liver, with NK cells particularly  
228 abundant in the infiltrate, predisposing the affected mice to more severe Con A liver injury.

229

### 230 **Treg cells infiltrate the liver during Con A hepatitis and display stronger ST2 and CD25** 231 **expression in the presence of IL-33**

232 We then investigated ST2 expression in the liver during hepatitis. Immunolocalization studies  
233 on liver tissues indicated that ST2 was present on liver sinusoidal endothelial cells but not on  
234 hepatocyte membranes in Con A-treated mice (Fig. 4A). Furthermore, flow cytometry showed  
235 that NKT cells displayed weak membrane ST2 expression (MFI 24 h after Con A treatment:  
236 1.793±0.1 in WT mice vs. 1.778±0.05 in IL-33<sup>-/-</sup> mice), whereas, a small population of CD4<sup>+</sup>  
237 T cells displayed strong membrane ST2 expression in Con A-treated WT mice (Fig. 4B). No  
238 ST2 expression was evident on the other immune cells infiltrating the liver characterized in  
239 Fig. 2. Treg cells are recognized target cells of IL-33. We therefore analyzed the intra-nuclear  
240 expression of Foxp3 and the membrane expression of ST2 in CD4<sup>+</sup> T cells in liver. We found  
241 that Treg cells (CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells) expressed ST2 on their membranes, whereas,  
242 conventional CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> cells) did not, this expression being stronger  
243 after Con A treatment than after the control PBS treatment (Fig. 4C). The number of Treg  
244 cells infiltrating the liver significantly increased in both WT and IL-33<sup>-/-</sup> mice (notably at 24  
245 h), as did the frequency of ST2<sup>+</sup> Treg cells after 24 h of activation, in both WT and IL-33<sup>-/-</sup>  
246 mice (Fig. 4D). The intensity of the fluorescent signals (MFI) for ST2 in Treg cells infiltrating

247 the liver increased after Con A challenge in WT mice (at both 12 and 24 h), whereas, a similar  
248 increase in intensity was observed only at 12 h in IL-33<sup>-/-</sup> mice. There was a significant  
249 difference in the intensity of the signal obtained for ST2 between WT and IL-33<sup>-/-</sup> mice at 24  
250 h (Fig. 4D, MFI: 42.24±3.3 in WT mice vs. 32.13±1.3 in IL-33<sup>-/-</sup> mice). Thus, the number of  
251 Treg cells, and particularly of ST2<sup>+</sup> Treg cells, infiltrating the liver increased during Con A-  
252 induced hepatitis, regardless of the presence or absence of IL-33. ST2 was strongly expressed  
253 on Treg cells in the presence of endogenous IL-33.

254 IL-2 is important for NK-cell function (37). We therefore investigated the effect of IL-33 on  
255 CD25 (IL-2 receptor) expression in the liver. We analyzed the expression of CD25 by Treg  
256 and NK cells 24 h after Con A administration. ST2<sup>+</sup> Treg cells displayed higher levels of  
257 surface CD25 expression than ST2<sup>-</sup> Treg cells in the livers of WT mice, and CD25 expression  
258 was stronger in the ST2<sup>+</sup> Treg cells of WT mice than in those of IL-33<sup>-/-</sup> mice (Fig. 4E).—The  
259 percentage of NK cells expressing CD25 was significantly higher in the livers of IL-33<sup>-/-</sup> mice  
260 than in WT mice (Fig. 4E). Similar results were obtained for NKT cells (frequency of CD25<sup>+</sup>  
261 cells among NKT cells: 23.3%±2 for WT mice vs. 41.63%±5.8 for IL-33<sup>-/-</sup> mice; data not  
262 shown). The MFI for CD25 staining in CD25<sup>+</sup> NK cells was similar in the livers of WT and  
263 IL-33<sup>-/-</sup> mice (31±3.3 and 34±2.2, respectively; data not shown). As IL-33 was produced in  
264 large amounts in the liver following Con A injection, these results were not reproduced in the  
265 spleen, except for the stronger expression of CD25 by ST2<sup>+</sup> Treg cells in WT mice (Fig. 4E).  
266 Thus, in the liver, IL-33 modulated ST2<sup>+</sup> Treg cells activation and control NK cells, thereby  
267 limiting liver injury.

268

269 **NK and Treg cells migrate from the spleen to the liver during Con A-hepatitis and IL-33**  
270 **regulates late NK-cell migration to the liver**

271 We investigated the migration of immune cells from the spleen to the liver during hepatitis  
272 and the impact of IL-33, by analyzing splenocytes (CD4<sup>+</sup>, CD8<sup>+</sup>, B cells, NK and Treg cells)  
273 in WT and IL-33<sup>-/-</sup> mice with Con A-induced hepatitis. The total numbers of splenocytes in  
274 WT and IL-33<sup>-/-</sup> mice were similar, 12 and 24 h after Con A injection (Fig. 5). Con A  
275 treatment lead to a significant decrease in the frequency of CD8<sup>+</sup> T cells in both WT and IL-  
276 33<sup>-/-</sup> mice (Fig. 5). A significant decrease in the number of Treg and NK cells was evident at  
277 12 h in the spleen of both WT and IL-33<sup>-/-</sup> mice, consistent with the migration of these two  
278 types of immune cells from the spleen to the liver, as shown in Figs. 2B and 4E. High rates of  
279 recolonization by Treg cells, especially ST2<sup>+</sup> cells, were observed at 24 h in both WT and IL-  
280 33<sup>-/-</sup> mice, but only low levels of NK-cell recolonization were observed in WT mice (Fig. 5).  
281 The number of NK cells was significantly higher in WT than in IL-33<sup>-/-</sup> mice 24 h after Con A  
282 administration (Fig. 5). Thus, Treg and NK cells were activated in the spleen and migrated to  
283 the liver within 12 hours of treatment and endogenous IL-33 impact the recruitment of NK  
284 cells from the spleen to the liver.

285

#### 286 **IL-33 promotes immune cell survival and selects CD25<sup>high</sup>ST2<sup>+</sup>Treg target cells *in vitro***

287 Finally, the direct effect of IL-33 on target Treg cells was determined by stimulating  
288 splenocytes *in vitro* with Con A, with and without IL-33 (10 ng/ml), for four or seven days.  
289 Two concentrations of Con A were used: a low concentration (0.1 µg/ml) activating 5 to 10%  
290 of CD4<sup>+</sup> T cells at 15 h without inducing proliferation, and a higher concentration (0.5 µg/ml)  
291 that activated almost 40% of CD4<sup>+</sup> T cells at 15 h and induced cell proliferation by day 7 (Fig.  
292 6A). IL-33 had no effect on T-cell proliferation on days 4 and 7 (Fig. 6B). It had no  
293 significant effect on Treg cell number following Con A stimulation *in vitro*, but increased the  
294 frequency of ST2<sup>+</sup> cells in Treg cells and the MFI of ST2 staining for ST2<sup>+</sup> Treg cells (Fig.  
295 6C). Moreover, ST2<sup>+</sup> Treg cells displayed stronger surface CD25 expression than the other

296 Treg cells after 4 and 7 days of Con A stimulation (Fig. 6C). IL-33 had a significant effect on  
297 cell survival, resulting in a higher total number of splenocytes, B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T  
298 cells (Fig. 6C). Thus, IL-33 had an effect on Treg cells, by selecting and overactivating the  
299 ST2<sup>+</sup> Treg cell population and favoring lymphoid cell survival during Con A activation *in*  
300 *vitro*.

301

302

303 **Discussion**

304 The IL-33/ST2 axis plays a key role in several diseases, including hepatitis. In this study, we  
305 investigated the functional role of endogenous IL-33 during immune cell-mediated Con A  
306 hepatitis in WT and IL-33<sup>-/-</sup> mice and its impact on the target cells infiltrating the liver. The  
307 direct effect of IL-33 on immune cells survival and activation was also accessed *in vitro*.

308 Like other members of the IL-1 family (IL-1 $\alpha/\beta$ , IL-18), IL-33 has a pro-inflammatory effect  
309 on innate immunity, by activating ST2, a receptor present in several types of immune cell  
310 (24). It also promotes the production of IFN- $\gamma$  and IL-12 by NKT and NK cells in the liver  
311 (9). In the Con A-induced hepatitis model, activated NKT cells are responsible for most of the  
312 damage to the liver (3, 40). ST2 is expressed on the NKT cell membrane, but no difference in  
313 liver injury, NKT cell number or IFN- $\gamma$  production was observed between WT and IL-33<sup>-/-</sup>  
314 mice 12 h after Con A administration. Significant immune cell infiltration was observed only  
315 in WT mice at this time point, suggesting that IL-33 was involved in liver immune cells  
316 recruitment. By contrast, Chen *et al.* (11) showed that IL-33 blockade greatly reduced liver  
317 damage, by inhibiting the activation and IFN- $\gamma$  production by NKT cells. Thus, it can be  
318 inferred that *i*) a high dose of Con A used in our model minimized IL-33 activation, *ii*) a  
319 compensatory mechanism is activated in IL-33<sup>-/-</sup> mice, making it possible for NKT cell  
320 interactions to occur in the absence of IL-33.

321 In contrast, IL-33 deficiency resulted in more severe injury (higher transaminase levels) 24 h  
322 after Con A administration, with significant increase in the levels of inflammatory cytokines  
323 (TNF- $\alpha$ , IL-1 $\beta$ ) and total immune cell number, with NK cells particularly abundant,  
324 infiltrating the liver from the spleen. The frequency of CD25 expression on liver NK cells was  
325 higher in IL-33<sup>-/-</sup> mice than in WT mice, resulting in the presence of twice as many activated  
326 NK cells in the livers of IL-33-deficient mice 24 h after Con A injection. Similar results were  
327 obtained for activated NKT cells (similar numbers, but CD25<sup>+</sup> frequency higher in IL-33<sup>-/-</sup>

328 mice). The significantly raised expression of CXCR3 in liver of IL-33-deficient mice than  
329 WT mice at 24 h of Con A administration reinforced the recruitment of NK cells. The NK,  
330 NKT cells and lymphocytes express the CXCR3 in mouse liver (6) and play a role in  
331 recruitment of these cells during liver inflammation (15). These results are consistent with the  
332 smaller number of cells infiltrating the liver 24 h after Con A administration reported by  
333 Volarevic *et al.* in mice treated with IL-33 (1 µg/mouse), and the smaller number of activated  
334 NK and NKT cells and less severe liver damage in these IL-33-treated Balb/c mice (44). By  
335 contrast to our findings, these authors showed that prior treatment with IL-33 decreased the  
336 numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In another study, the pre-treatment with rIL-33 (10  
337 µg/mouse) did not protect C57Bl/6 mice against ConA-hepatitis (11). The differences in the  
338 results obtained may be due to the low dose of Con A or the mice used by Volarevic *et al.*  
339 (44) and the prior activation of Treg cells by IL-33, both of which may decrease the activation  
340 and recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

341 During later stages of Con A-induced inflammation, a regulatory mechanism dependent on  
342 IL-33 may control CD25<sup>+</sup> NKT and NK cells, as these activated cells were found to be  
343 essential to trigger liver injury. The IL-33/ST2 axis promotes Treg cell function in cancer  
344 growth and metastasis (20) and in colon inflammation (38). Treg cells were reported to be  
345 protective (protective effect induced by prior treatment with galectin-9 or GP96) against Con  
346 A-induced hepatitis in previous studies (21, 25). We identified the ST2<sup>+</sup> Treg cell population  
347 in the liver and spleen and showed that these cells strongly infiltrated the liver within 24 h of  
348 Con A administration, independently of IL-33. In WT mice, these ST2<sup>+</sup> Treg cells displayed  
349 high levels of ST2 expression (with a MFI 25 times higher than that for NKT cells) and higher  
350 levels of CD25 expression than in IL-33<sup>-/-</sup> mice (with a MFI six times higher than that in NK  
351 cells in WT mice and twice that in IL-33<sup>-/-</sup> mice). In IL-33<sup>-/-</sup> mice, 24 h after Con A injection,  
352 activated effector cells (NK and NKT cells) were more numerous and Treg cells were less



353 activated than in WT mice, consistent with strong, protective activation of ST2<sup>+</sup> Treg cells by  
354 IL-33 to probably control effector cell activation. These results are consistent with *i*) the  
355 larger numbers of Treg cells infiltrating the liver 24 h after Con A administration (due to the  
356 exogenous injection of IL-33) reported by Volarevic *et al.* for mice subjected to prior  
357 treatment with IL-33, associated with the presence of smaller numbers of activated NK and  
358 NKT cells (producing IFN- $\gamma$ ) (44); *ii*) an accumulation of ST2<sup>+</sup> Treg cells within tumors,  
359 associated with lower levels of NK-cell activation and cytotoxicity (20); *iii*) the direct  
360 suppression of NK-mediated hepatocytotoxicity by Treg cells in HBV-associated liver disease  
361 (12).

362 Thus, our results, consistent with the findings of Chen *et al.* (11) and Volarevic *et al.* (44),  
363 strongly suggest that the IL-33/ST2 axis plays a dual role in Con A-induced hepatitis. IL-33  
364 may first activate NKT cells, thereby aggravating liver injury, as previously reported (11).  
365 Later on after the infiltration of Treg cells into the liver, IL-33 strongly activates ST2<sup>+</sup> Treg  
366 cells, with a protective effect on the liver, probably due to the limitation of NK cell activation.  
367 Our results provide a possible explanation of link between IL-33-mediated overactivation of  
368 ST2<sup>+</sup> Treg cells and the control of NK cell activation, through the direct regulation of NK  
369 cells by ST2<sup>+</sup> Treg cells or through enhanced ST2<sup>+</sup> Treg-cell survival.

370 The survival and cytotoxicity of NK cells were dependent on IL-2, as previously described  
371 (37). Treg cells can limit NK cell cytotoxicity by decreasing the availability of IL-2 (16, 17).  
372 We can therefore speculate that the overactivated CD25<sup>high</sup>ST2<sup>+</sup> Treg cells use a mechanism  
373 based on IL-2 deprivation to control activated CD25<sup>+</sup>NK cells in the liver. This regulation  
374 mechanism may also operate in the spleen, because NK cells started to recolonize the spleen  
375 after 24 h of Con A-induced inflammation only in WT mice, suggesting that IL-33 inhibited  
376 NK cell activation and/or migration.

377 Nevertheless, Treg cells constitute a heterogeneous immune cell population capable of using  
378 multiple mechanisms to modulate immune responses in various inflammatory conditions (45).  
379 Other molecules, including membrane-bound TGF- $\beta$  and OX40 may be involved in this  
380 control mechanism and upregulated on hepatic Treg cells (12). A regulatory role for MDSCs  
381 (myeloid-derived suppressor cells) in the Con A hepatic model cannot be excluded, because  
382 Huang *et al.* demonstrated that IL-33-stimulated macrophages produce G-CSF, in turn  
383 boosting MDSC levels (19).

384 IL-33-mediated cell survival mechanisms may also be involved, as the injection of  
385 recombinant IL-33 had a protective effect on hepatocytes, by repressing the expression of pro-  
386 apoptotic genes and increasing the expression of anti-apoptotic genes during Con A hepatitis  
387 (28). We found that, during splenocyte activation with Con A *in vitro*, rIL-33 stimulation  
388 overactivated and selected ST2<sup>+</sup> Treg cells and favored the survival of other immune cell  
389 populations (T and B cells). This provides a proof-of-concept that the stimulation of ST2<sup>+</sup>  
390 Treg cells with IL-33 favored survival of liver cells.

391 In conclusion, we evidenced that the IL-33/ST2 axis induced a hepatoprotective effect during  
392 Con A-induced hepatitis, by modulation of ST2<sup>+</sup> Treg cells, control of NK cells  
393 activation/cytotoxicity and promoting cell survival.

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404

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406 declare.

407

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553  
554

555 **Figure legends**

556

557 **Figure 1: Liver injury and inflammatory cytokine expression in WT and IL-33<sup>-/-</sup> mice**  
558 **treated with Con A.** (A) Serum AST/ALT levels (IU/l) in WT mice and IL-33<sup>-/-</sup> mice treated  
559 with Con A (20 mg/kg i.v.), 12 or 24 h post injection ( $n=4$  for PBS control and  $n\geq 10$  for Con  
560 A challenge). (B) Sections of liver from untreated mice and from mice treated with Con A  
561 were stained with H&E. Arrows indicate the zones of liver injury. (C) Immunolocalization of  
562 IL-33 with goat anti-mouse-IL-33 primary antibody and an HRP-conjugated rabbit anti-goat  
563 antibody, with hematoxylin counterstaining on sections of livers from control, WT and IL-33<sup>-/-</sup>  
564 mice. Arrows indicate IL-33-positive hepatocytes. The scale bar indicates 200 or 500  $\mu\text{m}$ .  
565 (D) Relative fold change in mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , IL-4, IL-10, IL-13  
566 and IL-17A in livers of WT and IL-33<sup>-/-</sup> mice treated with Con A at 12 and 24h of post  
567 injection. The PBS-treated mice serve as a reference for mRNA expression.  $n=5$  for all  
568 conditions. \*  $p<0.05$  and with a Mann-Whitney U test.

569

570 **Figure 2: Quantification of the cells infiltrating the liver in WT and IL-33<sup>-/-</sup> mice during**  
571 **Con A-hepatitis.** Gating strategy (A) for quantification (B) of total liver leukocytes, NKT  
572 cells (CD3<sup>med</sup>NK1.1<sup>med</sup>), NK cells (CD3<sup>-</sup>NK1.1<sup>+</sup>), CD4 and CD8 T cells (CD3<sup>high</sup>), B cells  
573 (CD19<sup>+</sup>) and (C) neutrophils (NK1.1<sup>-</sup>CD11b<sup>high</sup>Gr1<sup>high</sup>), macrophages (NK1.1<sup>-</sup>  
574 CD11b<sup>high</sup>Gr1<sup>med</sup>) and dendritic cells (CD11c<sup>+</sup>), in the livers of WT and IL-33<sup>-/-</sup> mice  
575 following Con A administration (12 or 24 h post-injection). A viability dye (orange live dead)  
576 was used to exclude dead cells at the beginning of the analysis. (B)  $n\geq 6$  for PBS control and  
577  $n\geq 10$  for Con A challenge. (C)  $n\geq 4$  for PBS control and  $n\geq 6$  for Con A challenge.

578



579 **Figure 3: Quantification of liver CD69 expression/MFI during Con A-hepatitis.**  
580 Expression (A) and MFI (B) of surface CD69 on membrane of different infiltrate cell  
581 populations in liver of WT (A, B) and IL-33<sup>-/-</sup> (B) mice following Con A administration. n=5  
582 for all conditions. \* p<0.05 and \*\* p<0.01 with a Mann-Whitney U test. Grey filled graphs in  
583 A represent CD69 staining of each population after PBS treatment. These staining are used as  
584 reference for MFI calcul in B, thus MFI of non-treated population (PBS) is always 1. (C)  
585 Relative fold change in mRNA expression of CXCR3 in livers of WT and IL-33<sup>-/-</sup> mice  
586 treated with Con A at 12 and 24h of post injection. The PBS-treated mice serve as a reference  
587 for mRNA expression. n=5 for all conditions. \* p<0.05 and with a Mann-Whitney U test.

588

589 **Figure 4: Expression of ST2 on cells infiltrating the liver in WT and IL-33<sup>-/-</sup> mice during**  
590 **Con A-hepatitis.** (A) Sections of WT mice liver treated with Con A (24h) were stained with  
591 anti-mouse ST2 (green), phalloidin (red) and Draq-5 (blue) for immuno-fluorescence analysis.  
592 (B) Surface ST2 expression in various cell populations from the liver infiltrate in WT mice  
593 following Con A administration (solid gray graphs correspond to the isotypic control). The  
594 gating strategy was identical to that used in Fig. 2B. (C) Surface ST2 expression and  
595 intracellular Foxp3 expression in CD3<sup>+</sup>CD4<sup>+</sup> T-cell population of the liver infiltrate in Con A-  
596 treated mice (WT and IL-33<sup>-/-</sup>). (D) Quantification of Treg cells (CD4<sup>+</sup>Foxp3<sup>+</sup>) in the liver,  
597 expression and fluorescence intensity (MFI) for ST2 on Treg cells in the livers of WT and IL-  
598 33<sup>-/-</sup> mice following Con A administration (12 or 24 h post-injection). MFI is the ratio of the  
599 fluorescence intensities obtained with specific anti-ST2 and isotype control antibodies ( $n \geq 4$   
600 for PBS control and  $n \geq 6$  for Con A challenge). (E) Surface CD25 expression in the Treg and  
601 NK cells of the liver and spleen of WT and IL-33<sup>-/-</sup> mice 24 h after Con A administration ( $\alpha$ ,  
602  $p \leq 0.05$  vs. PBS, Mann-Whitney U test) ( $n=5$  for all groups).

603

604 **Figure 5: Quantification of splenocytes in WT and IL-33<sup>-/-</sup> mice during Con A hepatitis.**  
605 Quantification and characterization of the splenocytes (CD4<sup>+</sup>, CD8<sup>+</sup>, B cells, NK and Treg  
606 cells) of WT and IL-33<sup>-/-</sup> mice following Con A-induced (12 or 24 h post-injection) hepatitis;  
607 comparison with PBS control mice ( $n \geq 5$  for all groups).

608

609 **Figure 6: Effect of IL-33 in Con A-activated splenocytes *in vitro*.** (A) Splenocytes (10<sup>5</sup>  
610 cells/well) were activated *in vitro* with 2 concentrations of Con A (0.1 or 0.5 μg/ml) with or  
611 without IL-33 (10 ng/ml). (B) Expression of membrane CD69 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells  
612 were quantified at 15h and frequency of CD69 positive cells were calculated. (C) Dilution of  
613 CFSE in CD4<sup>+</sup> and CD8<sup>+</sup> T cells during Con A activation at 0.5 μg/ml. Grey filled graphs  
614 represented Con A activation without IL-33 and black lines graphs represented activation in  
615 the presence of IL-33. Quantification of total cells, B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and  
616 characterization of surface ST2 and CD25 expression in Treg cells (CD4<sup>+</sup>Foxp3<sup>+</sup>) after the  
617 Con A stimulation (0.1 or 0.5 μg/ml) of splenocytes (10<sup>5</sup> cells/well) *in vitro*, with or without  
618 IL-33 (10 ng/ml) ( $n = 5$  for all conditions).

619

620 **Table 1.** Primers used for qPCR

621

Gene	Forward	Reverse
m18S	5'-TTGGCAAATGCTTTCGCTC-3'	5'-CGCCGCTAGAGGTGAAATTC-3'
mIFN- $\gamma$	5'-AGGTCAACAACCCACAGGTC-3'	5'-ATCAGCAGCGACTCCTTTTC-3'
mTNF- $\alpha$	5'-TAGCTCCCAGAAAAGCAAGC-3'	5'-TTTTCTGGAGGGAGATGTGG-3'
mIL-1 $\beta$	5'-GAAGAAGTGCCCATCCTCTG-3'	5'-AGCTCATATGGGTCCGACAG-3'
IL-4	5'-GGCTCCAAGGTGCTTGG-3'	5'-GGACTTGGACTCATTTCATGG-3'
IL-10	5'-GAATCCCTGGGTGAGAAGC-3'	5'-TTCATGGCCTGTAGACACC-3'
IL-17A	5'-GCTCCAGAAGCCCTCAGA-3'	5'-AGCTTTCCTCCGCATTGA-3'
IL-13	5'-CTGAGCAACATCACACAAGAC-3'	5'-ACAGAGGCCATGCAATATCC-3'
CXCR3	5'-GTTCTGGTCTCCAGAGG-3'	5'-CTCCCACAAAGGCATAGAGC-3'

622

Figure 1

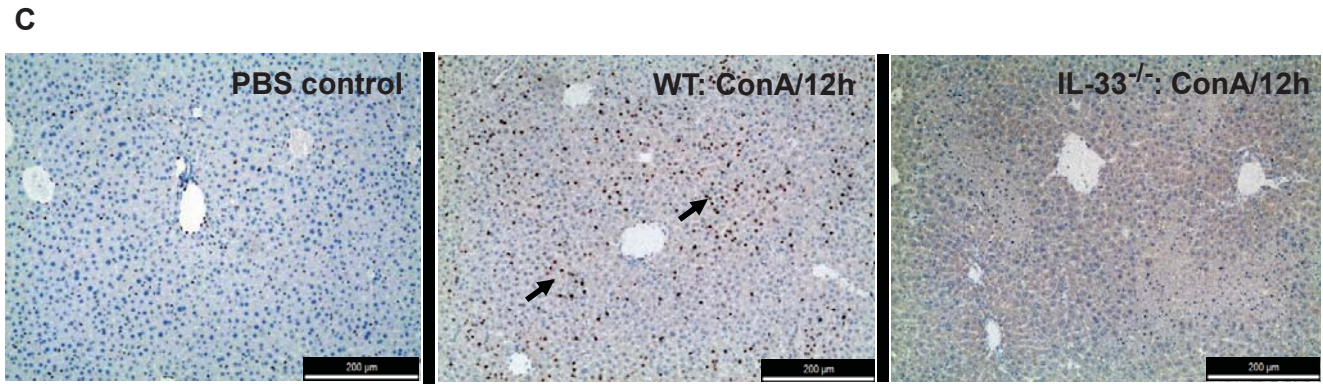
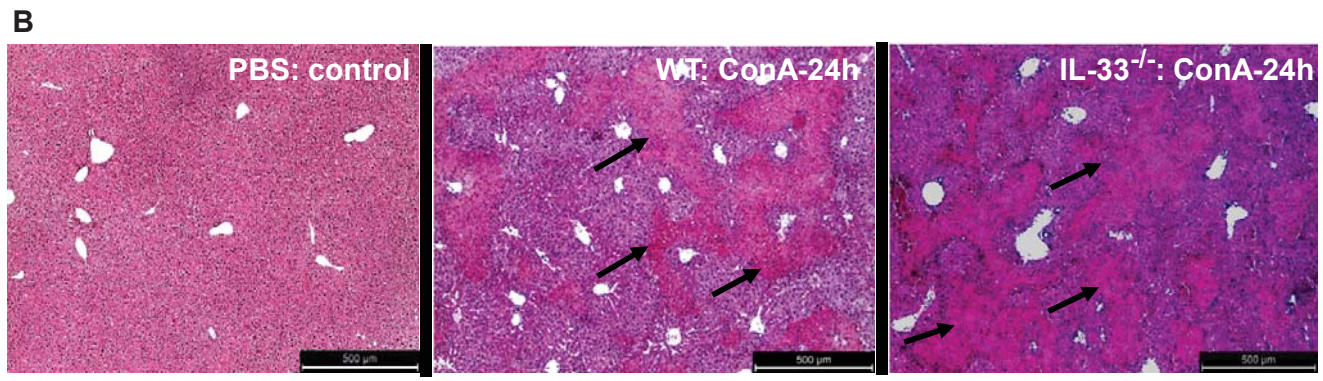
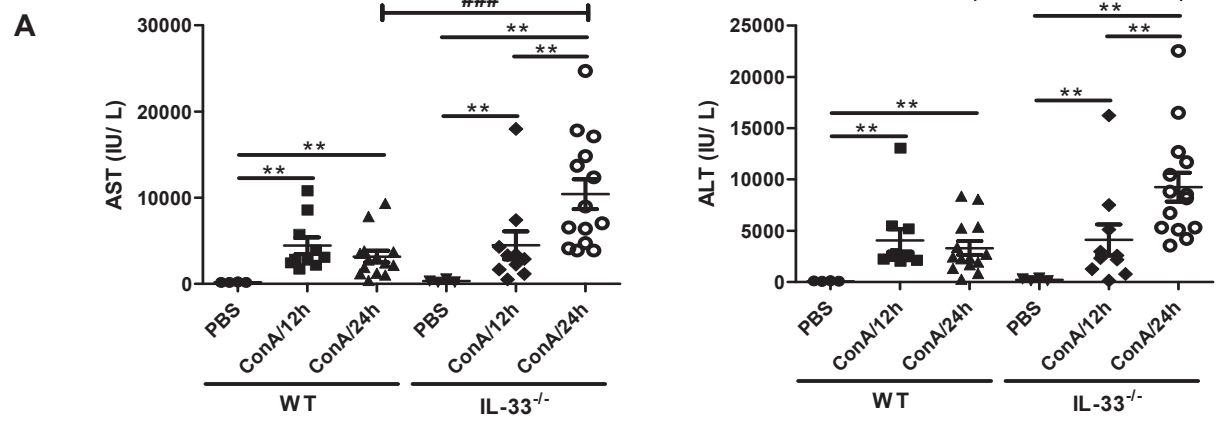
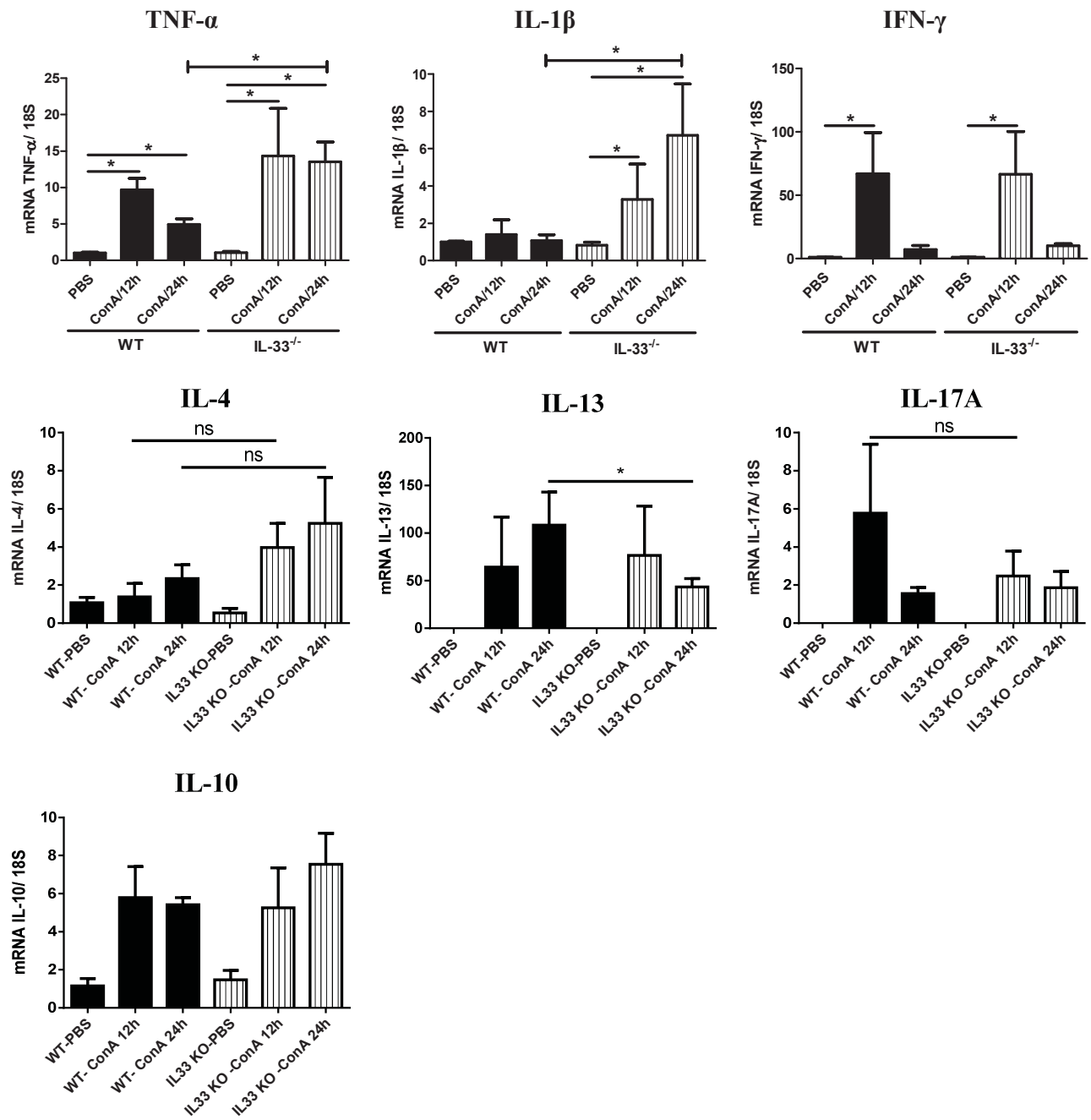


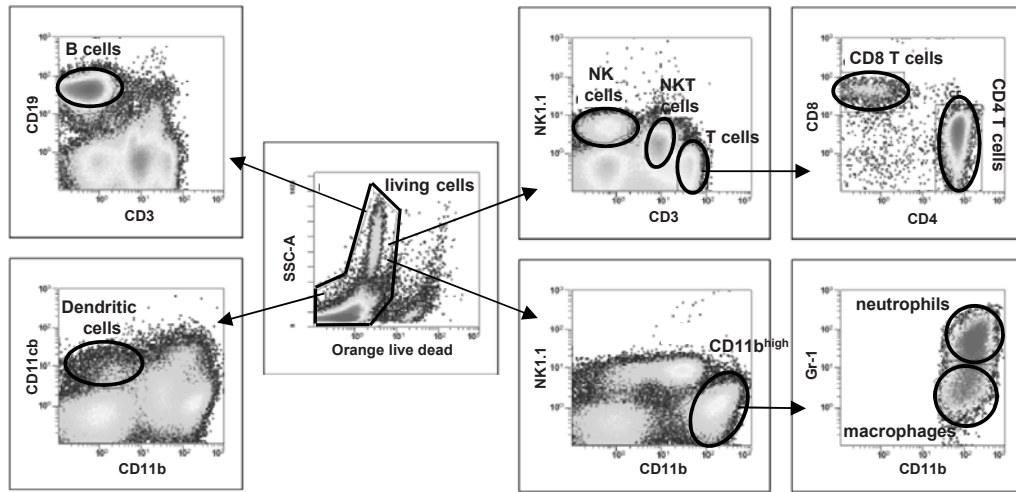
Figure 1

D

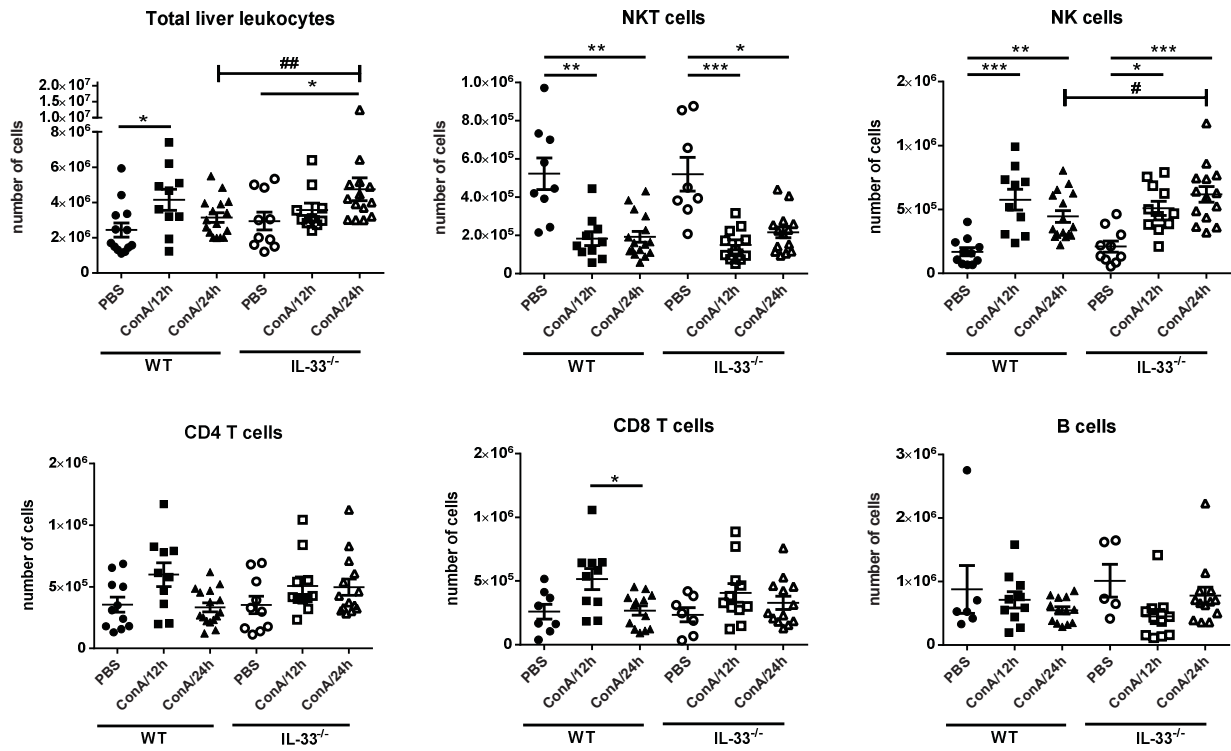


**Figure 2**

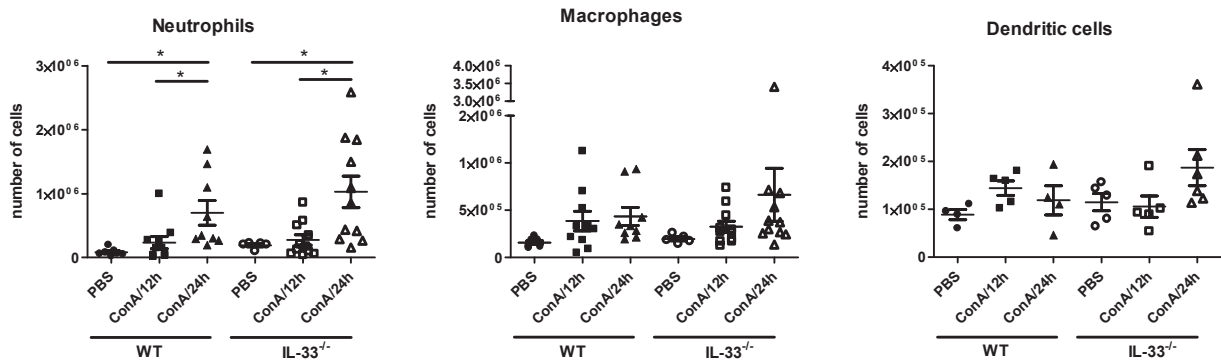
**A**



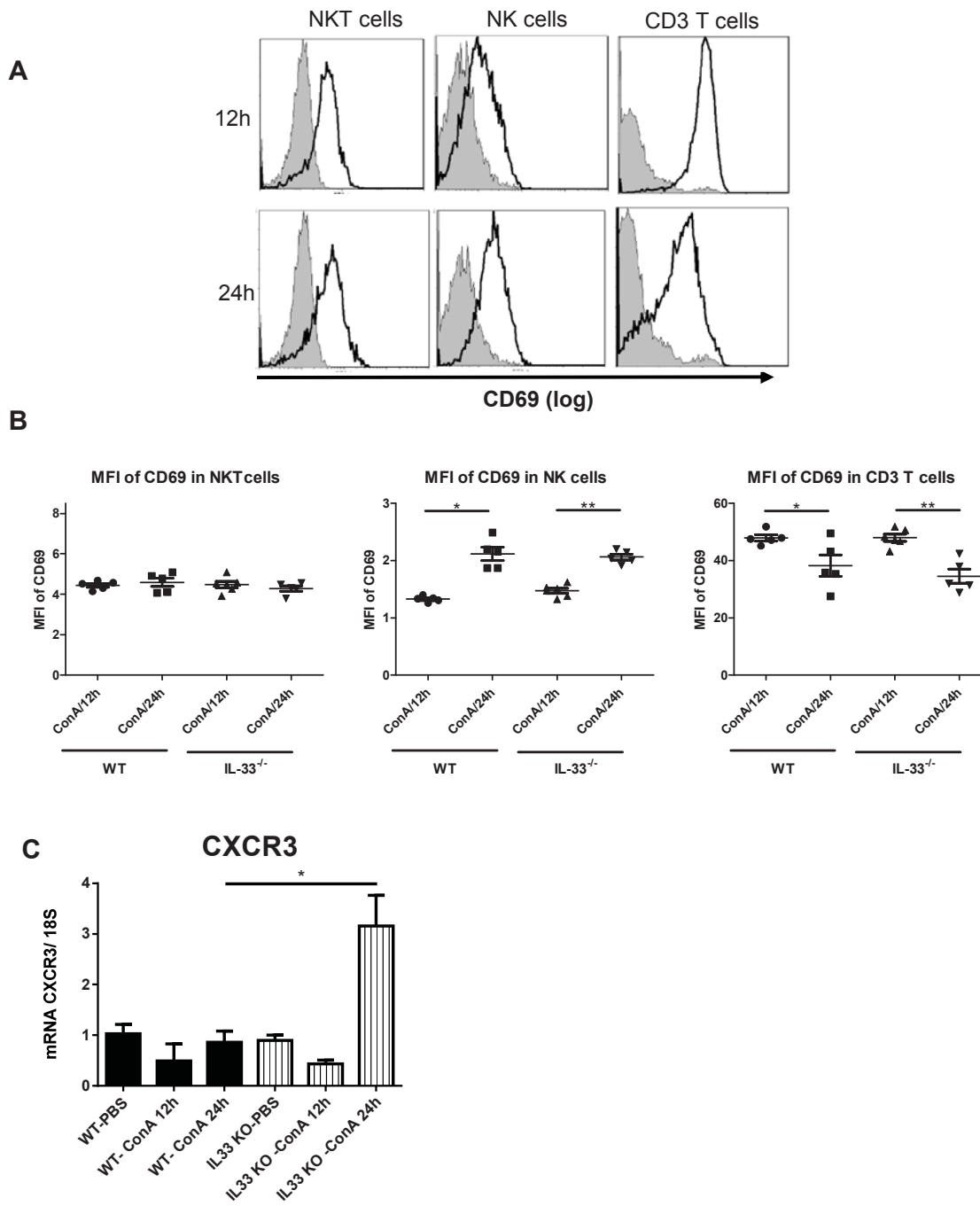
**B**



**C**

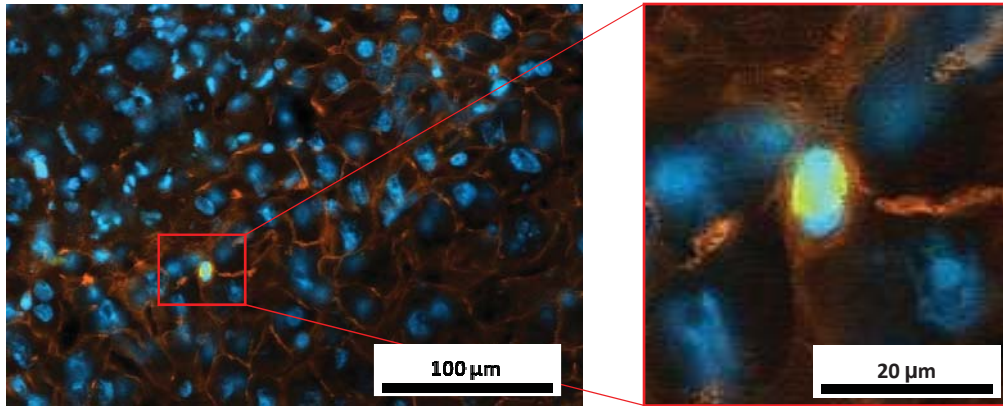


**Figure 3**

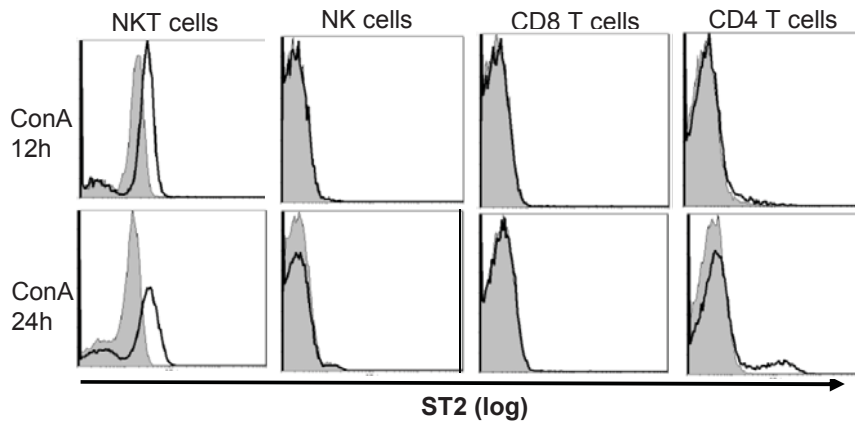


**Figure 4**

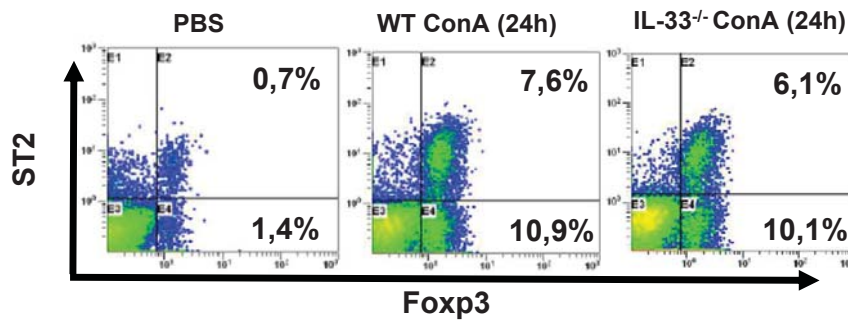
**A**



**B**



**C**



**D**

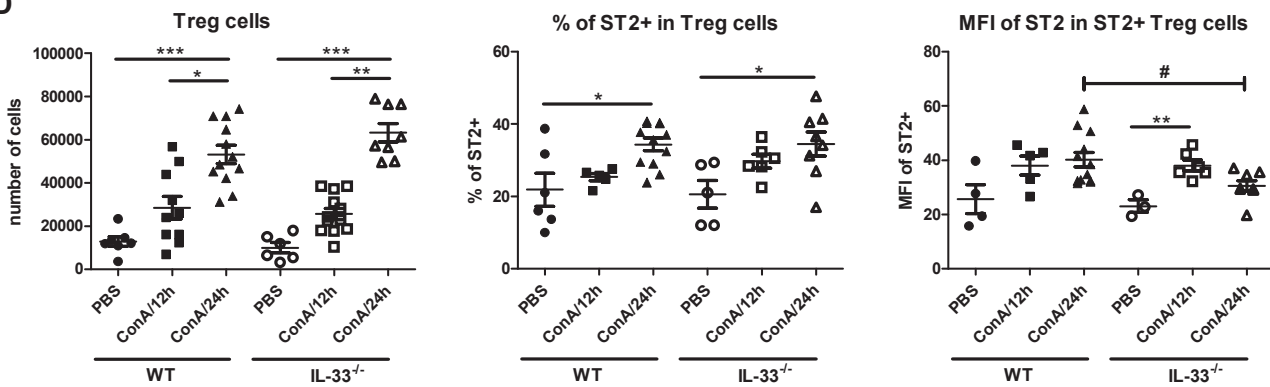




Figure 4

E

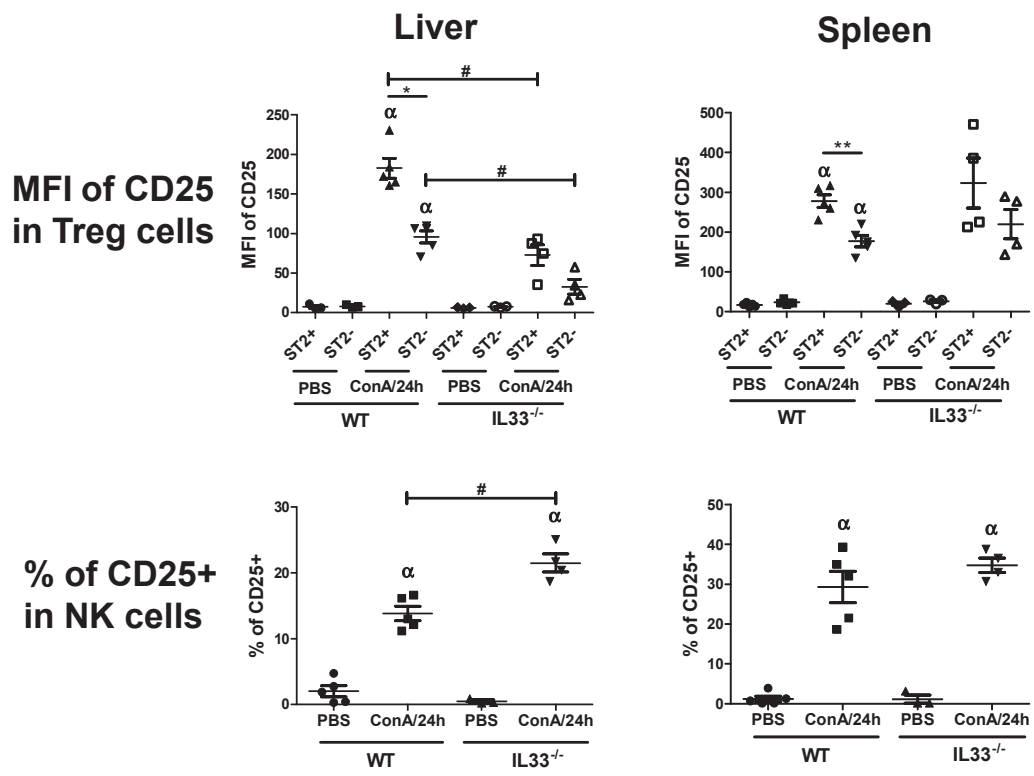
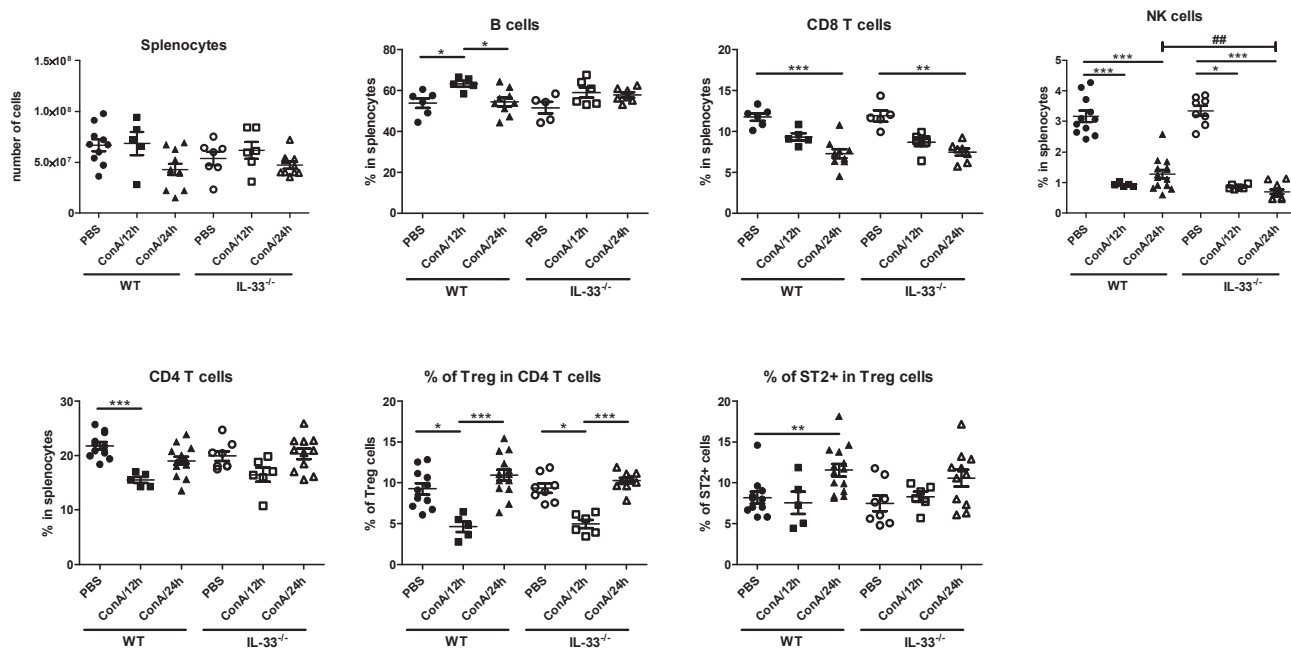
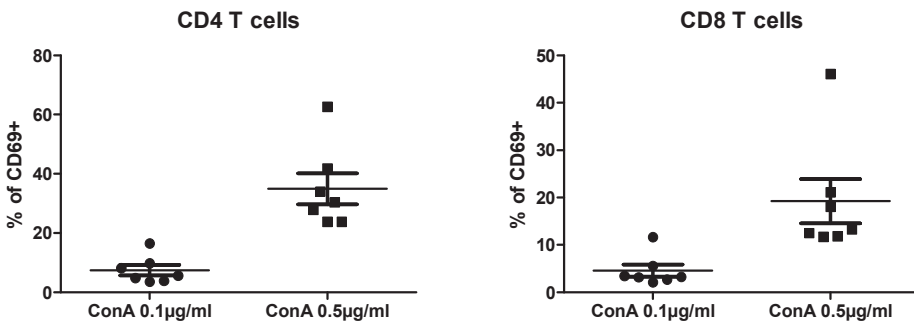


Figure 5

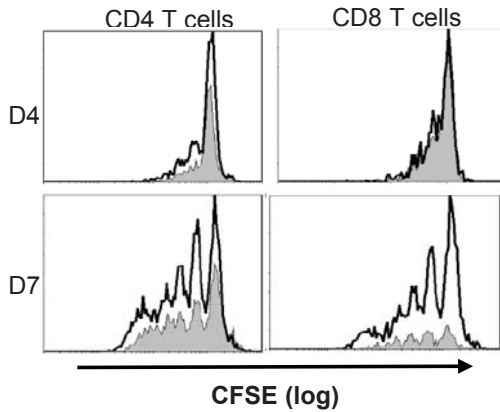


**Figure 6**

**A**



**B**



**C**

