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Ablation of interaction between IL-33 and ST2^{+} regulatory T cells increases immune cell-mediated hepatitis and activated NK cells liver infiltration

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

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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$^{-/-}$ 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$^{-/-}$
36 mice was associated with significantly higher levels of TNF-α and IL-1-β 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$^{-/-}$ mice following Con A-hepatitis. The
39 percentage of CD25^{+} NK cells was significantly higher in the livers of IL-33$^{-/-}$ 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$^{-/-}$ 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^{+} and CD8^{+} T cells, and the frequency of ST2^{+} Treg cells. In conclusion, IL-33 acts as a
45 potent immune modulator protecting the liver through activation of ST2^{+}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 $CD4^+CD25^+Foxp3^+$ 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 $ST2^+$ 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-1α (18). However, the effects of
80 endogenous IL-33 during liver inflammation remain obscure, and the $ST2^{+}$ 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 $ST2^{-/-}$ 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 $ST2^{+}$ immune cells, by using $IL\text{-}33^{-/-}$
93 and WT mice to determine the functional role of endogenous IL-33 in Con A-induced
94 hepatitis.
95 $IL\text{-}33^{-/-}$ 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 $CD25^{+}$ 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 $ST2^+$ 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 ($^{-/-}$) 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-Ol, 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); phalloïdin (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 SuperScriptTM 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-γ, TNF-α, IL-1β, 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™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 FACSAria™ 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 ($CD4^+$, $CD8^+$, 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 (10^5 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-$33^{-/-}$ 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-$33^{-/-}$ mice were

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

173

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$^{-/-}$ mice. Serum transaminase (AST/ALT) levels 12 or 24 h
179 after Con A administration were significantly higher in IL-33$^{-/-}$ and WT mice than in control
180 mice treated with PBS (Fig. 1A). A significant difference between IL-33$^{-/-}$ and WT mice was
181 observed 24 h after Con A administration, but not at 12 h, indicating that IL-33$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$
189 mice was associated with significantly higher levels of TNF-α and IL-1β in IL-33$^{-/-}$ mice than
190 in WT mice at 24 h, but not at 12 h (Fig. 1D). By contrast, similar high levels of IFN-γ
191 transcripts were observed in IL-33$^{-/-}$ 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$^{-/-}$ mice and WT mice following Con A-hepatitis but IL-13 transcript level
194 significantly decreased at 24 h in IL-33$^{-/-}$ mice (Fig. 1D). Increased but comparable mRNA
195 expression of cytokine IL-17A was found in IL-33$^{-/-}$ 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-$33^{-/-}$ 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-$33^{-/-}$ 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-$33^{-/-}$ 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-$33^{-/-}$ mice, whereas CD69 expression
215 remained stable in NKT cells (Fig. 3A and 3B). There were significantly more intrahepatic
216 NK cells in IL-$33^{-/-}$ 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-$33^{-/-}$
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, $CD4^{+}$ and $CD8^{+}$ T cells in WT mice, but only

222 for $CD8^+$ T cells in IL-$33^{-/-}$ mice. The numbers of NK cells and B cells increased between 12
223 h and 24 h after Con A administration in IL-$33^{-/-}$ 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-$33^{-/-}$ mice, but the number of neutrophils increased significantly by 24 h after Con A
226 administration in both WT and IL-$33^{-/-}$ 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-$33^{-/-}$ mice), whereas, a small population of $CD4^+$
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 $CD4^+$ T cells in liver. We found
241 that Treg cells ($CD3^+CD4^+Foxp3^+$ cells) expressed ST2 on their membranes, whereas,
242 conventional $CD4^+$ T cells ($CD3^+CD4^+Foxp3^-$ 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-$33^{-/-}$ mice (notably at 24
245 h), as did the frequency of $ST2^+$ Treg cells after 24 h of activation, in both WT and IL-$33^{-/-}$
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$^{-/-}$ mice. There was a significant
249 difference in the intensity of the signal obtained for ST2 between WT and IL-33$^{-/-}$ mice at 24
250 h (Fig. 4D, MFI: 42.24±3.3 in WT mice vs. 32.13±1.3 in IL-33$^{-/-}$ mice). Thus, the number of
251 Treg cells, and particularly of ST2^{+} 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^{+} Treg cells displayed higher levels of
257 surface CD25 expression than ST2^{-} Treg cells in the livers of WT mice, and CD25 expression
258 was stronger in the ST2^{+} Treg cells of WT mice than in those of IL-33$^{-/-}$ mice (Fig. 4E). The
259 percentage of NK cells expressing CD25 was significantly higher in the livers of IL-33$^{-/-}$ mice
260 than in WT mice (Fig. 4E). Similar results were obtained for NKT cells (frequency of CD25^{+}
261 cells among NKT cells: 23.3%±2 for WT mice *vs.* 41.63%±5.8 for IL-33$^{-/-}$ mice; data not
262 shown). The MFI for CD25 staining in CD25^{+} NK cells was similar in the livers of WT and
263 IL-33$^{-/-}$ 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^{+} Treg cells in WT mice (Fig. 4E).
266 Thus, in the liver, IL-33 modulated ST2^{+} 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 ($CD4^+$, $CD8^+$, B cells, NK and Treg cells)
273 in WT and IL-$33^{-/-}$ mice with Con A-induced hepatitis. The total numbers of splenocytes in
274 WT and IL-$33^{-/-}$ 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 $CD8^+$ T cells in both WT and IL-
276 $33^{-/-}$ 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-$33^{-/-}$ 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 $ST2^+$ cells, were observed at 24 h in both WT and IL-
280 $33^{-/-}$ 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-$33^{-/-}$ 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 $CD25^{high}ST2^+$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 $CD4^+$ T cells at 15 h without inducing proliferation, and a higher concentration (0.5 µg/ml)
291 that activated almost 40% of $CD4^+$ 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 $ST2^+$ cells in Treg cells and the MFI of ST2 staining for $ST2^+$ Treg cells (Fig.
295 6C). Moreover, $ST2^+$ 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, $CD4^+$ and $CD8^+$ T
298 cells (Fig. 6C). Thus, IL-33 had an effect on Treg cells, by selecting and overactivating the
299 $ST2^+$ 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$^{-/-}$ 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-1α/β, 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-γ 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-γ production was observed between WT and IL-33$^{-/-}$
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-γ 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$^{-/-}$ 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-α, IL-1β) 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$^{-/-}$ 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^{+} frequency higher in IL-33$^{-/-}$

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 $CD4^+$ and $CD8^+$ 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 $CD4^+$ and $CD8^+$ T cells.
341 During later stages of Con A-induced inflammation, a regulatory mechanism dependent on
342 IL-33 may control $CD25^+$ 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 $ST2^+$ 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 $ST2^+$ 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-$33^{-/-}$ mice (with a MFI six times higher than that in NK
351 cells in WT mice and twice that in IL-$33^{-/-}$ mice). In IL-$33^{-/-}$ 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 $ST2^+$ 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-γ) (44); *ii)* an accumulation of $ST2^+$ 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 $ST2^+$ 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 $ST2^+$ Treg cells and the control of NK cell activation, through the direct regulation of NK
369 cells by $ST2^+$ Treg cells or through enhanced $ST2^+$ 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 $CD25^{high}ST2^+$ Treg cells use a mechanism
373 based on IL-2 deprivation to control activated $CD25^+$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-β 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 $ST2^+$ 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 $ST2^+$
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 $ST2^+$ Treg cells, control of NK cells
393 activation/cytotoxicity and promoting cell survival.

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396

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404

405 **Conflict of Interest:** The authors have no financial or commercial conflict of interest to
406 declare.

407

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552 heterogeneity in maintaining self-tolerance. *Immunol Rev* 259: 103-114, 2014.
553
554

555 **Figure legends**

556

557 **Figure 1: Liver injury and inflammatory cytokine expression in WT and IL-33$^{-/-}$ mice**
558 **treated with Con A.** (**A**) Serum AST/ALT levels (IU/l) in WT mice and IL-33$^{-/-}$ 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^{-}
564 $^{/-}$ mice. Arrows indicate IL-33-positive hepatocytes. The scale bar indicates 200 or 500 µm.
565 (**D**) Relative fold change in mRNA expression of TNF-α, IL-1β, IFN-γ, IL-4, IL-10, IL-13
566 and IL-17A in livers of WT and IL-33-/- 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$^{-/-}$ mice during**
571 **Con A-hepatitis.** Gating strategy (**A**) for quantification (**B**) of total liver leukocytes, NKT
572 cells (CD3^{med}NK1.1^{med}), NK cells (CD3^{-}NK1.1^{+}), CD4 and CD8 T cells (CD3^{high}), B cells
573 (CD19^{+}) and (**C**) neutrophils (NK1.1^{-}CD11b^{high}Gr1^{high}), macrophages (NK1.1^{-}
574 CD11b^{high}Gr1^{med}) and dendritic cells (CD11c^{+}), in the livers of WT and IL-33$^{-/-}$ 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-/- (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-/- 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$^{-/-}$ 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 $CD3^+CD4^+$ T-cell population of the liver infiltrate in Con A-
596 treated mice (WT and IL-33$^{-/-}$). (**D**) Quantification of Treg cells ($CD4^+Foxp3^+$) in the liver,
597 expression and fluorescence intensity (MFI) for ST2 on Treg cells in the livers of WT and IL-
598 33$^{-/-}$ 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 ($n\geq4$
600 for PBS control and $n\geq6$ 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$^{-/-}$ mice 24 h after Con A administration (α,
602 $p\leq0.05$ *vs.* PBS, Mann-Whitney *U* test) (n=5 for all groups).
603

604 **Figure 5: Quantification of splenocytes in WT and IL-33$^{-/-}$ mice during Con A hepatitis.**
605 Quantification and characterization of the splenocytes (CD4^{+}, CD8^{+}, B cells, NK and Treg
606 cells) of WT and IL-33$^{-/-}$ mice following Con A-induced (12 or 24 h post-injection) hepatitis;
607 comparison with PBS control mice (*n*≥5 for all groups).
608
609 **Figure 6: Effect of IL-33 in Con A-activated splenocytes *in vitro*.** (**A**) Splenocytes (105
610 cells/well) were activated in vitro with 2 concentrations of Con A (0.1 or 0.5µg/ml) with or
611 without IL-33 (10ng/ml). (**B**) Expression of membrane CD69 on CD4+ and CD8+ T cells
612 were quantified at 15h and frequency of CD69 positive cells were calculated. (**C**) Dilution of
613 CFSE in CD4+ and CD8+ T cells during Con A activation at 0.5µg/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^{+} and CD8^{+} T cells and
616 characterization of surface ST2 and CD25 expression in Treg cells (CD4^{+}Foxp3^{+}) after the
617 Con A stimulation (0.1 or 0.5 µg/ml) of splenocytes (10^{5} cells/well) *in vitro,* with or without
618 IL-33 (10 ng/ml) (*n*=5 for all conditions).
619

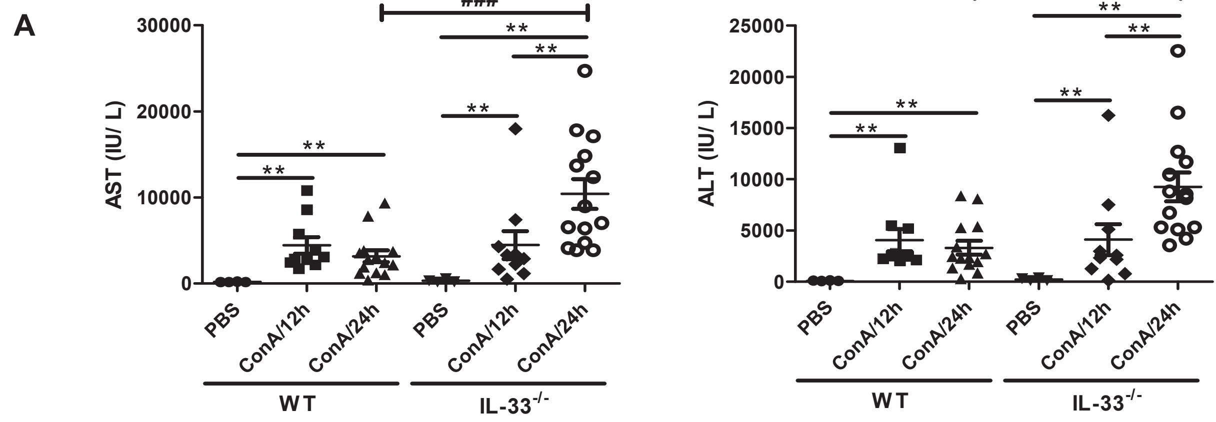
620 **Table 1**. Primers used for qPCR

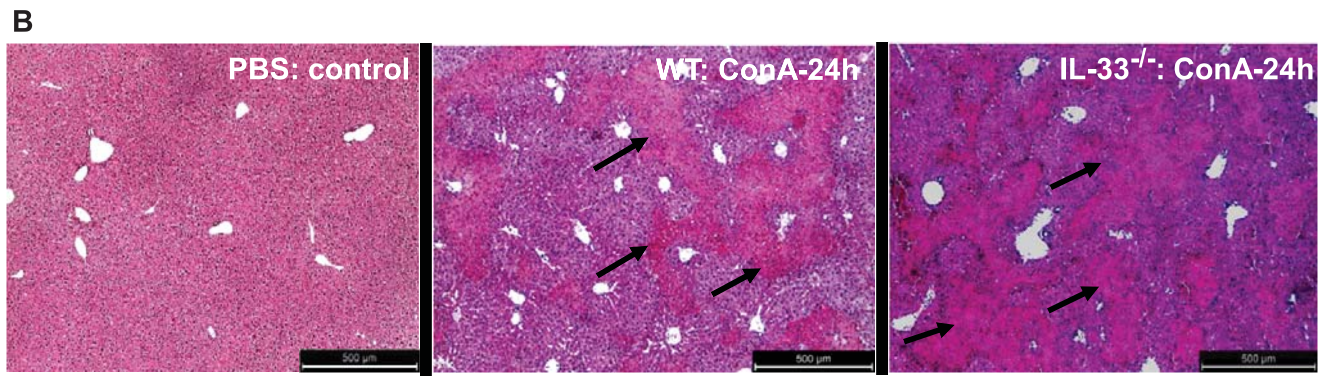
621

Gene	Forward	Reverse
m18S	5'-TTGGCAAATGCTTTCGCTC-3'	5'-CGCCGCTAGAGGTGAAATTC-3'
mIFN-γ	5'-AGGTCAACAACCCACAGGTC-3'	5'-ATCAGCAGCGACTCCTTTTC-3'
mTNF-α	5'-TAGCTCCCAGAAAAGCAAGC-3'	5'-TTTTCTGGAGGGAGATGTGG-3'
mIL-1β	5'-GAAGAAGTGCCCATCCTCTG-3'	5'-AGCTCATATGGGTCCGACAG-3'
IL-4	5'-GGCTTCCAAGGTGCTTGG-3'	5'-GGACTTGGACTCATTCATGG-3'
IL-10	5'-GAATTCCCTGGGTGAGAAGC-3'	5'-TTCATGGCCTTGTAGACACC-3'
IL-17A	5'-GCTCCAGAAGGCCCTCAGA-3'	5'AGCTTTCCCTCCGCATTGA-3'
IL-13	5'-CTGAGCAACATCACACAAGAC-3'	5'-ACAGAGGCCATGCAATATCC-3'
CXCR3	5'-GTTCTGGTCTCCAGAGG-3'	5'CTCCCACAAAGGCATAGAGC-3'

622

Figure 1





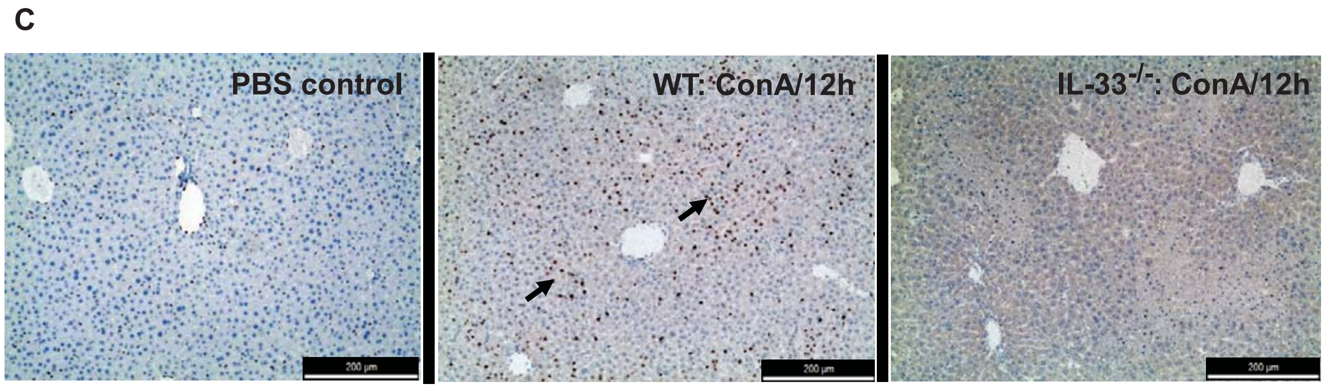


Figure 1

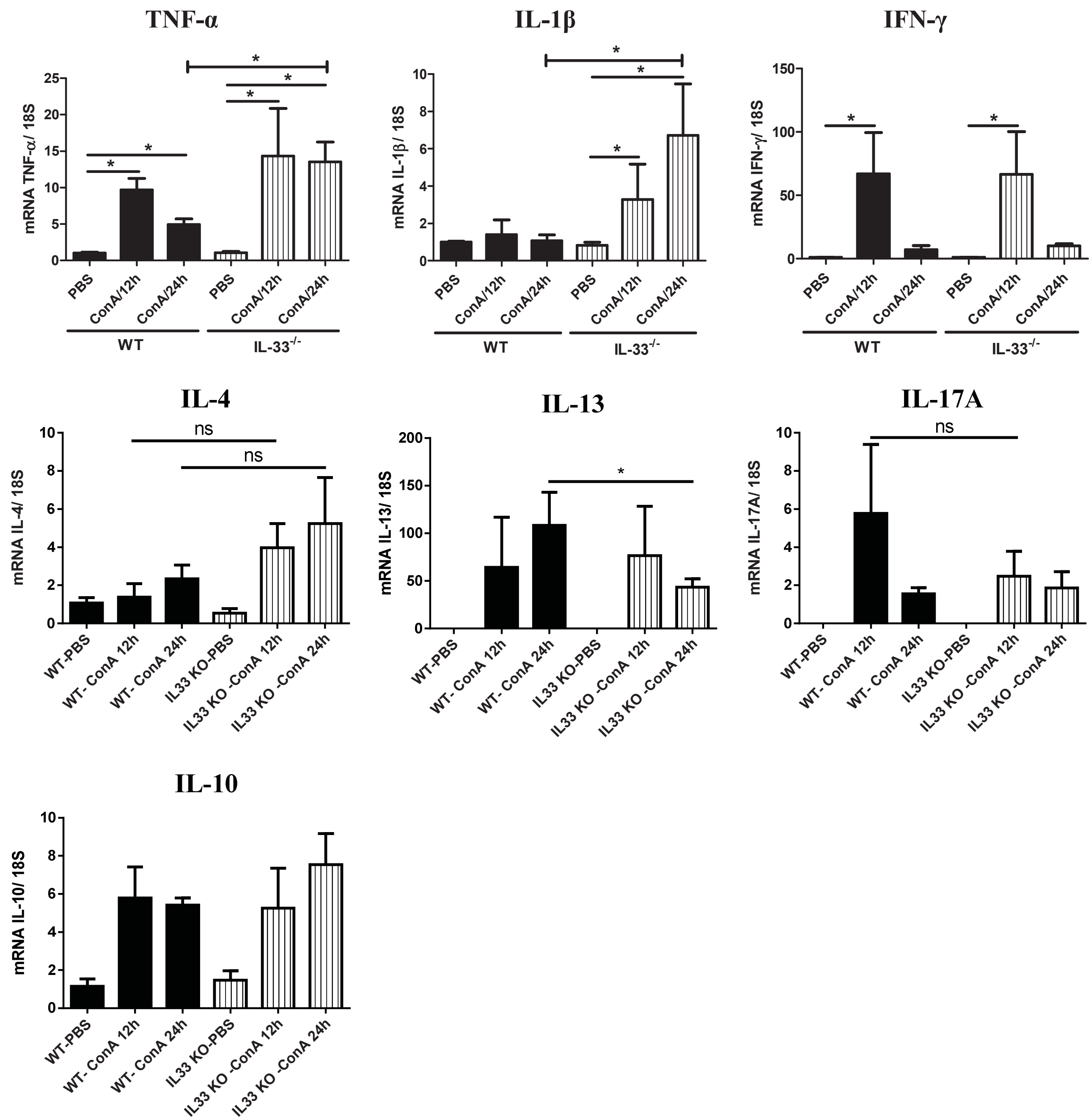
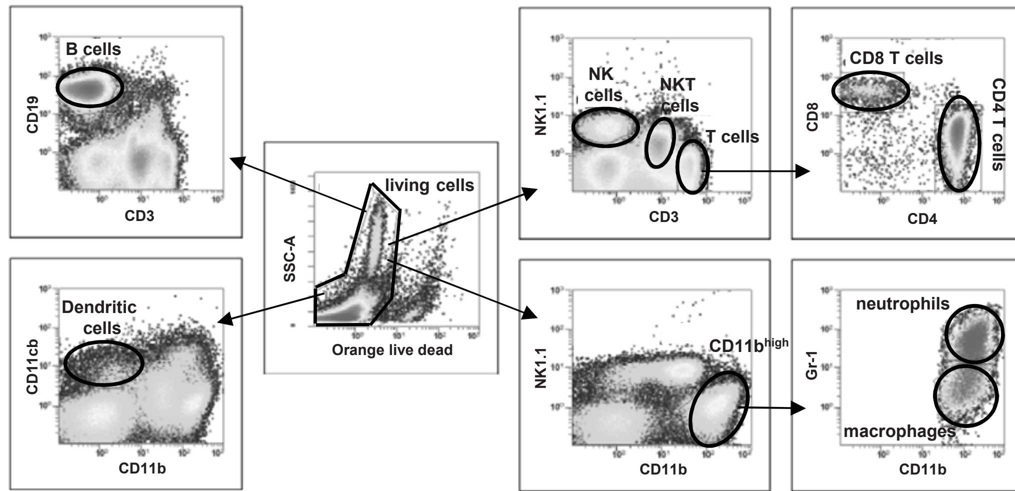
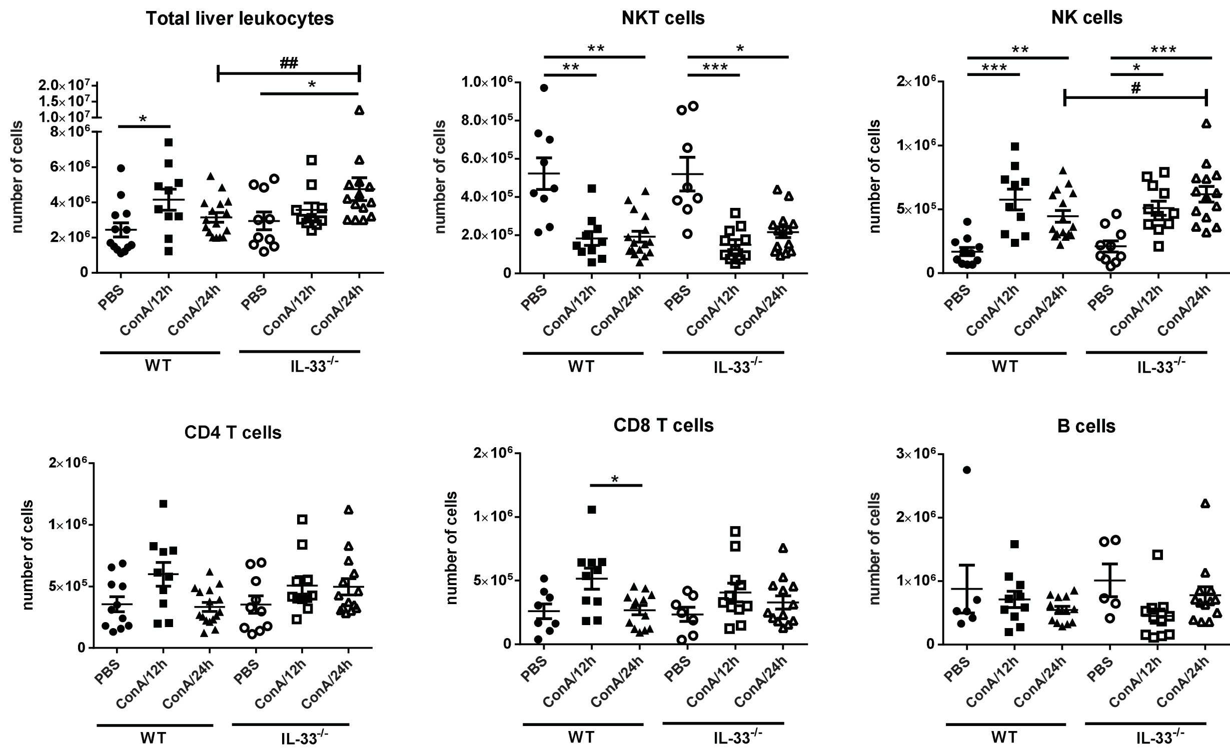


Figure 2





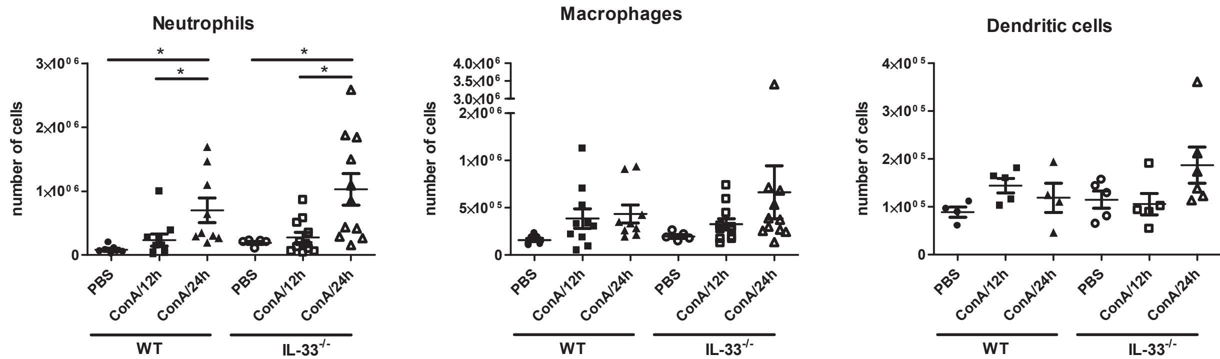


Figure 3

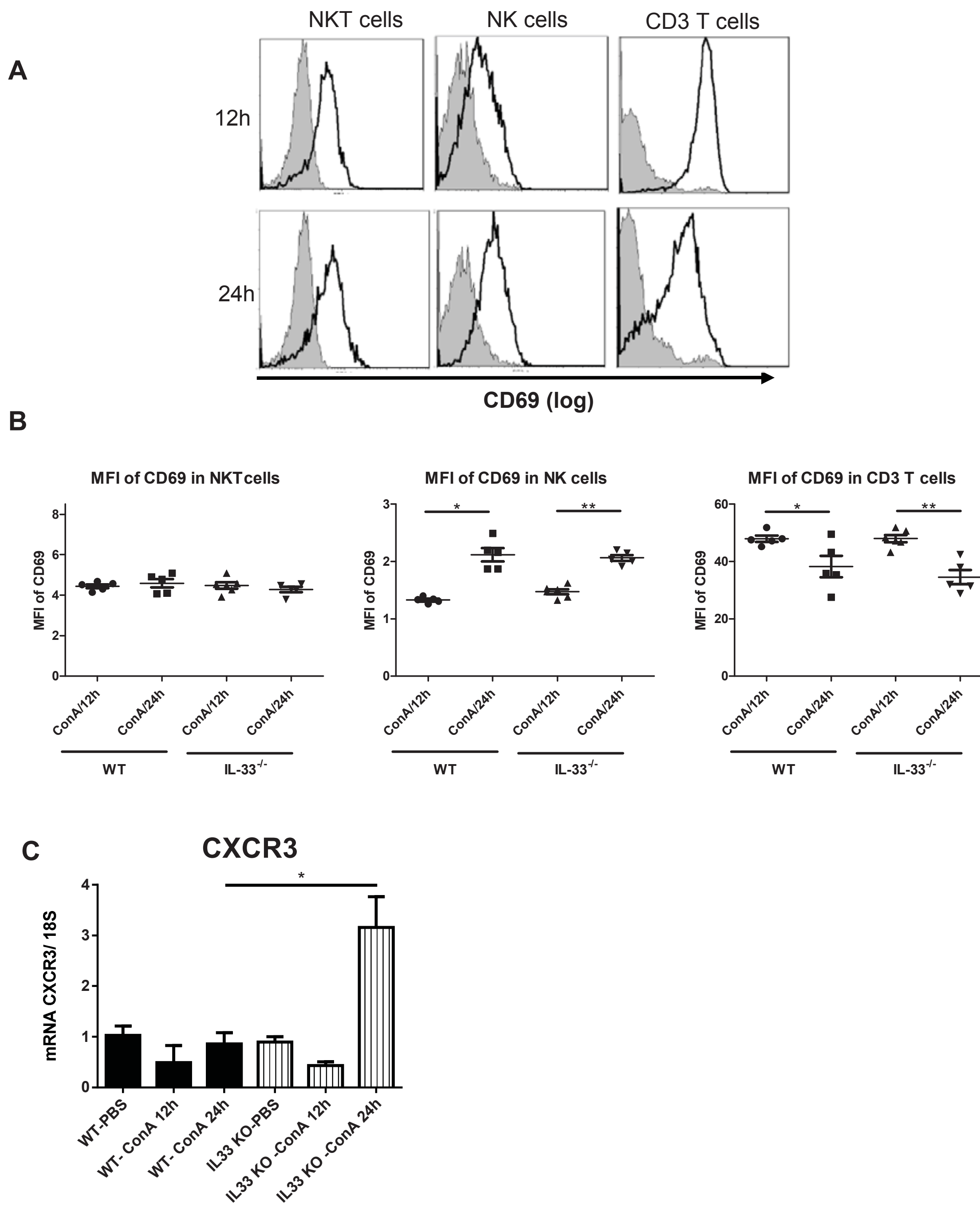
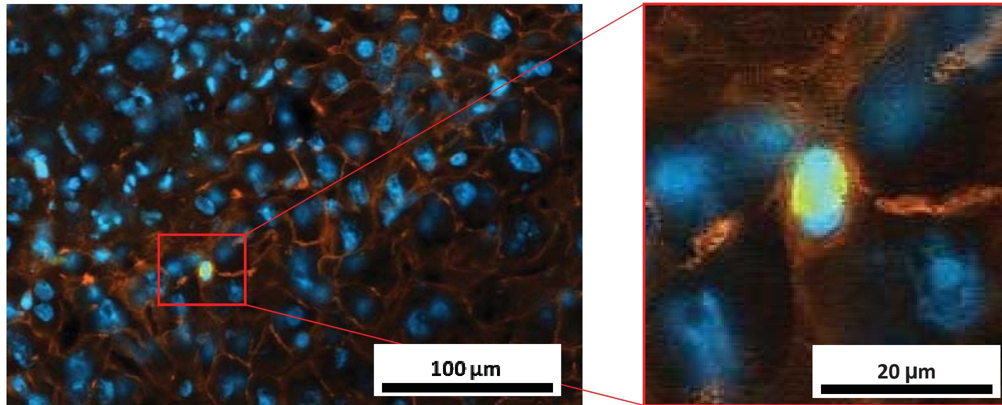
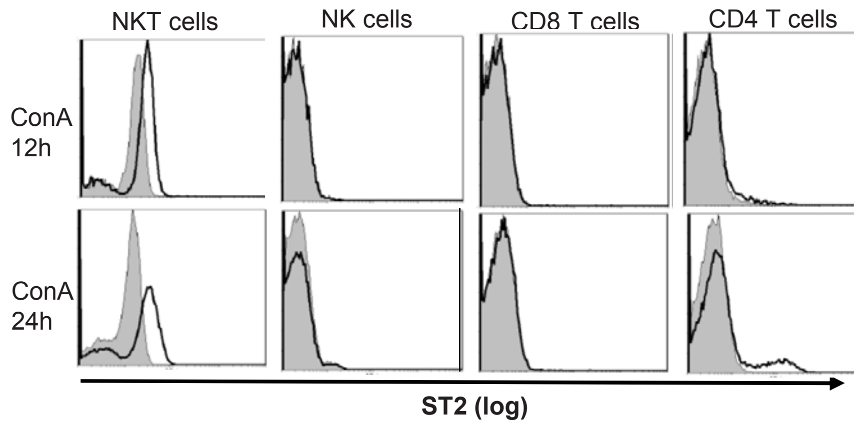


Figure 4

A



B



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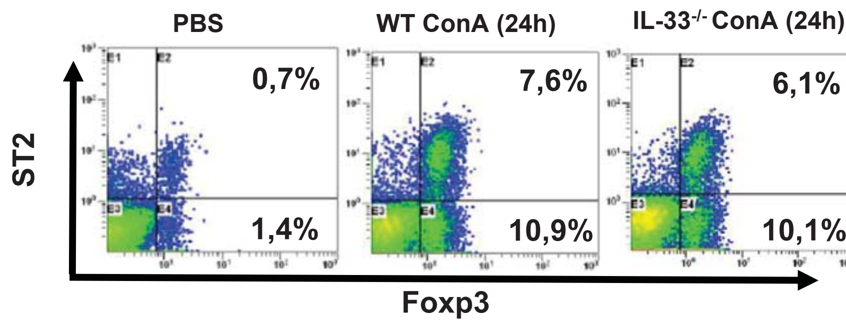

D

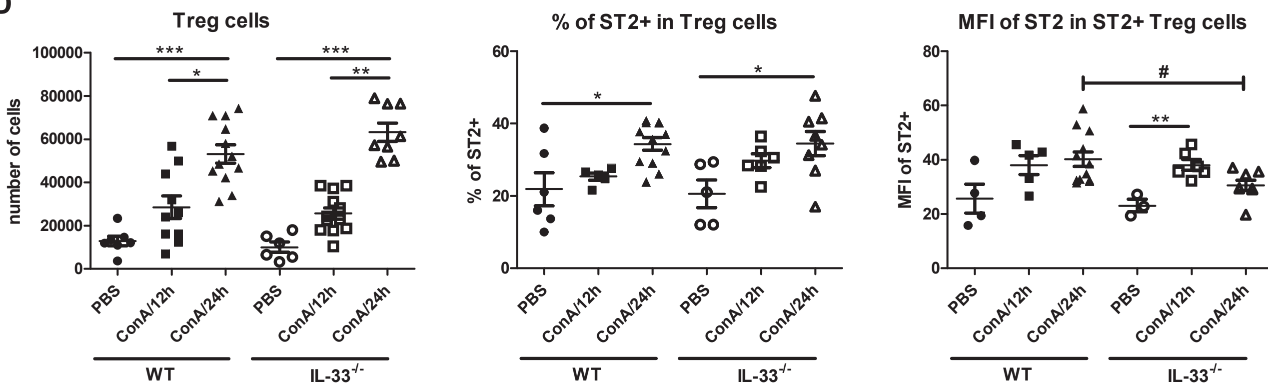

Figure 4

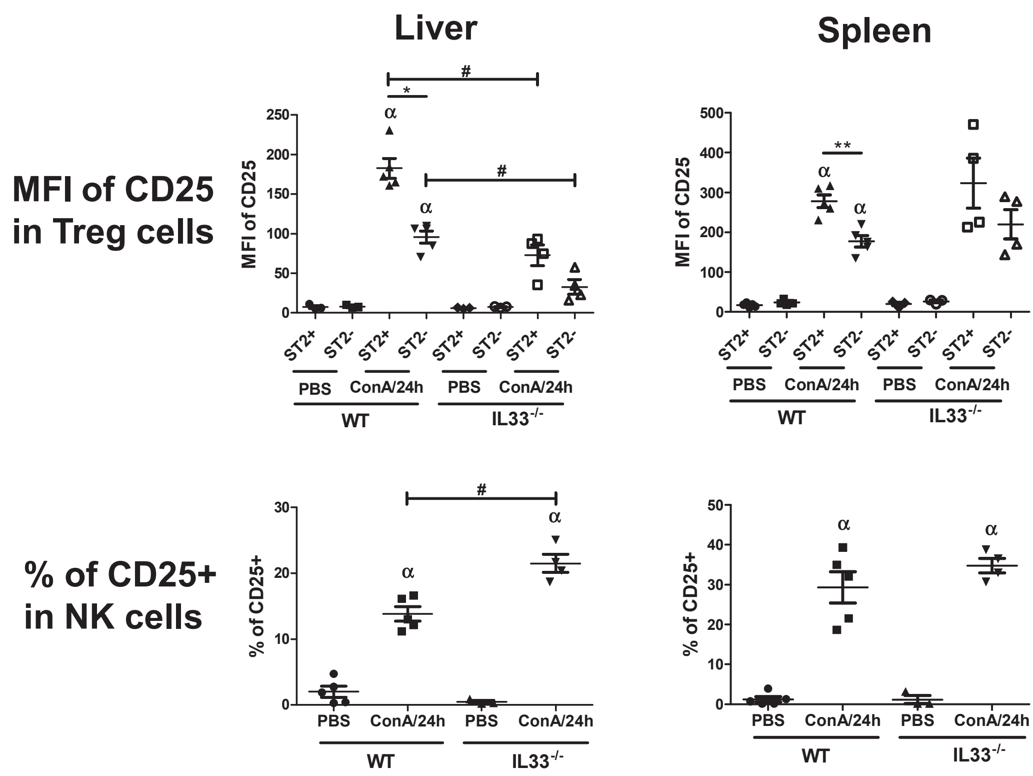

Figure 5

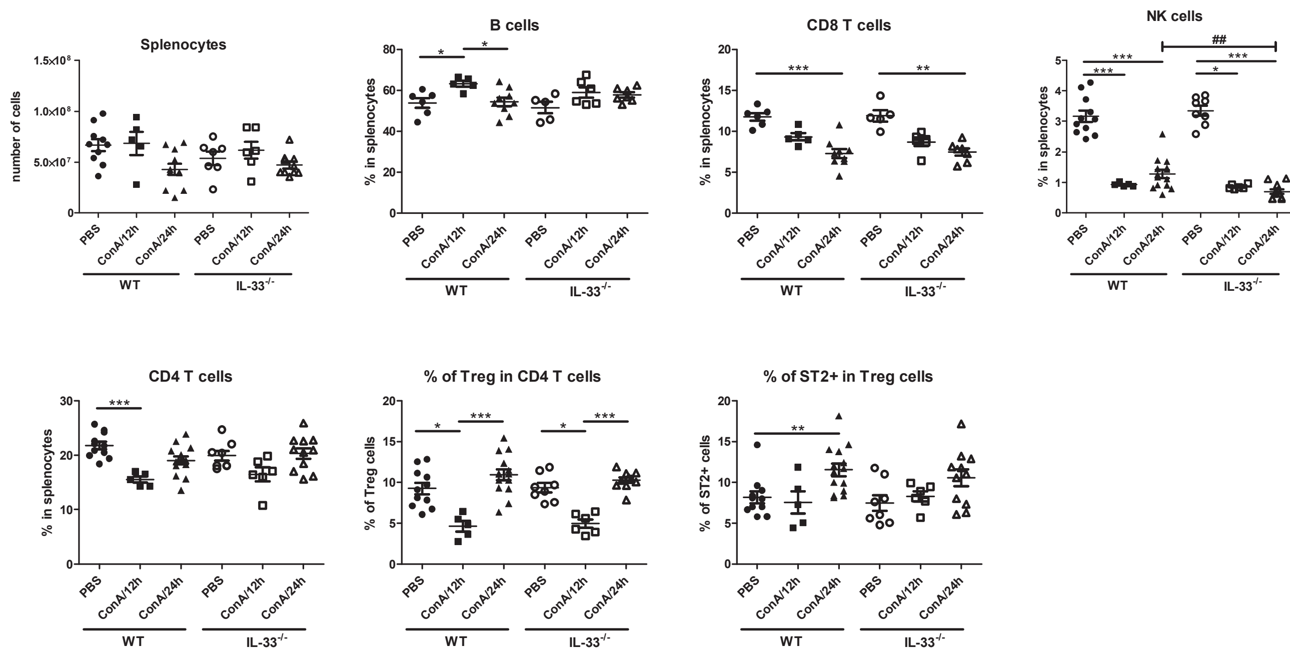

Figure 6