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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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Abstract

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

Introduction

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

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

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

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

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

$IL\text{-}33^{-/-}$ mice displayed more severe liver injury than WT mice following Con A administration. This exacerbated liver injury was associated with of the infiltration of large number of immune cells, including $CD25^+$ NK cells in particular. Treg cells infiltrated the

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

Materials and Methods

Animals and treatment protocol

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

Histopathological, biochemical and immunohistochemical analyses

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

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

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

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

Statistical analysis

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

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

Results

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

We investigated the role of IL-33 in liver disease, by comparing the time course of Con A hepatic injury in WT and IL-33$^{-/-}$ mice. Serum transaminase (AST/ALT) levels 12 or 24 h after Con A administration were significantly higher in IL-33$^{-/-}$ and WT mice than in control mice treated with PBS (Fig. 1A). A significant difference between IL-33$^{-/-}$ and WT mice was observed 24 h after Con A administration, but not at 12 h, indicating that IL-33$^{-/-}$ mice were more sensitive to Con A liver injury than WT mice, but with a time lag to the manifestation of this effect (Fig. 1A). Massive parenchymal and perivascular zones of hepatic injury were observed in IL-33$^{-/-}$ and WT mice following Con A-hepatitis (Fig. 1B). Immunostaining for IL-33 in liver tissues demonstrated constitutive IL-33 production by the vascular endothelial and sinusoidal endothelial cells of control mouse livers (Fig. 1C). We observed an induction of IL-33 production by the hepatocytes of WT mouse livers following Con A-hepatitis (as previously described (3); Fig. 1C). The more severe Con A-induced liver injury in IL-33$^{-/-}$ mice was associated with significantly higher levels of TNF-α and IL-1β in IL-33$^{-/-}$ mice than in WT mice at 24 h, but not at 12 h (Fig. 1D). By contrast, similar high levels of IFN-γ transcripts were observed in IL-33$^{-/-}$ mice and WT mice, 12 h after Con A administration (Fig. 1D). The signature of Th2 cytokines such as IL-4 and IL-10 showed comparable liver mRNA expression in IL-33$^{-/-}$ mice and WT mice following Con A-hepatitis but IL-13 transcript level significantly decreased at 24 h in IL-33$^{-/-}$ mice (Fig. 1D). Increased but comparable mRNA expression of cytokine IL-17A was found in IL-33$^{-/-}$ mice and WT mice following Con A-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

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

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

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

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

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

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

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

IL-33 promotes immune cell survival and selects $CD25^{high}ST2^+$Treg target cells *in vitro*

Finally, the direct effect of IL-33 on target Treg cells was determined by stimulating splenocytes *in vitro* with Con A, with and without IL-33 (10 ng/ml), for four or seven days. Two concentrations of Con A were used: a low concentration (0.1 μg/ml) activating 5 to 10% of $CD4^+$ T cells at 15 h without inducing proliferation, and a higher concentration (0.5 μg/ml) that activated almost 40% of $CD4^+$ T cells at 15 h and induced cell proliferation by day 7 (Fig. 6A). IL-33 had no effect on T-cell proliferation on days 4 and 7 (Fig. 6B). It had no significant effect on Treg cell number following Con A stimulation *in vitro,* but increased the frequency of $ST2^+$ cells in Treg cells and the MFI of ST2 staining for $ST2^+$ Treg cells (Fig. 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

Discussion

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

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

In contrast, IL-33 deficiency resulted in more severe injury (higher transaminase levels) 24 h after Con A administration, with significant increase in the levels of inflammatory cytokines (TNF-α, IL-1β) and total immune cell number, with NK cells particularly abundant, infiltrating the liver from the spleen. The frequency of CD25 expression on liver NK cells was higher in IL-33$^{-/-}$ mice than in WT mice, resulting in the presence of twice as many activated NK cells in the livers of IL-33-deficient mice 24 h after Con A injection. Similar results were 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

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

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

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

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

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

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

Acknowledgments

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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≥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≥6 for PBS control and
577 n≥10 for Con A challenge. (**C**) n≥4 for PBS control and n≥6 for Con A challenge.

578

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

Figure 4: Expression of ST2 on cells infiltrating the liver in WT and IL-33$^{-/-}$ mice during Con A-hepatitis. (**A**) Sections of WT mice liver treated with Con A (24h) were stained with anti-mouse ST2 (green), phalloidin (red) and Draq-5 (blue) for immuno-fluorescence analysis. (**B**) Surface ST2 expression in various cell populations from the liver infiltrate in WT mice following Con A administration (solid gray graphs correspond to the isotypic control). The gating strategy was identical to that used in Fig. 2B. (**C**) Surface ST2 expression and intracellular Foxp3 expression in CD3^{+}CD4^{+} T-cell population of the liver infiltrate in Con A-treated mice (WT and IL-33$^{-/-}$). (**D**) Quantification of Treg cells (CD4^{+}Foxp3^{+}) in the liver, expression and fluorescence intensity (MFI) for ST2 on Treg cells in the livers of WT and IL-33$^{-/-}$ mice following Con A administration (12 or 24 h post-injection). MFI is the ratio of the fluorescence intensities obtained with specific anti-ST2 and isotype control antibodies ($n\geq4$ for PBS control and $n\geq6$ for Con A challenge). (**E**) Surface CD25 expression in the Treg and NK cells of the liver and spleen of WT and IL-33$^{-/-}$ mice 24 h after Con A administration (α, $p\leq0.05$ *vs.* PBS, Mann-Whitney *U* test) ($n=5$ for all groups).

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

Figure 6: Effect of IL-33 in Con A-activated splenocytes *in vitro*. (**A**) Splenocytes (105 cells/well) were activated in vitro with 2 concentrations of Con A (0.1 or 0.5µg/ml) with or without IL-33 (10ng/ml). (**B**) Expression of membrane CD69 on CD4+ and CD8+ T cells were quantified at 15h and frequency of CD69 positive cells were calculated. (**C**) Dilution of CFSE in CD4+ and CD8+ T cells during Con A activation at 0.5µg/ml. Grey filled graphs represented Con A activation without IL-33 and black lines graphs represented activation in the presence of IL-33. Quantification of total cells, B cells, $CD4^+$ and $CD8^+$ T cells and characterization of surface ST2 and CD25 expression in Treg cells ($CD4^+Foxp3^+$) after the Con A stimulation (0.1 or 0.5 µg/ml) of splenocytes (10^5 cells/well) *in vitro,* with or without IL-33 (10 ng/ml) (n=5 for all conditions).

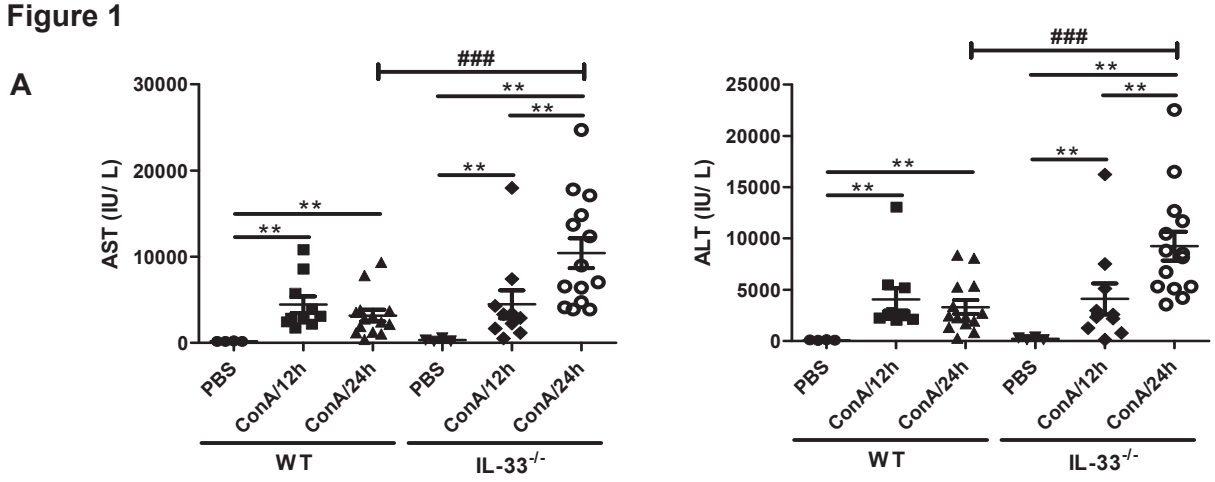
620 **Table 1**. Primers used for qPCR

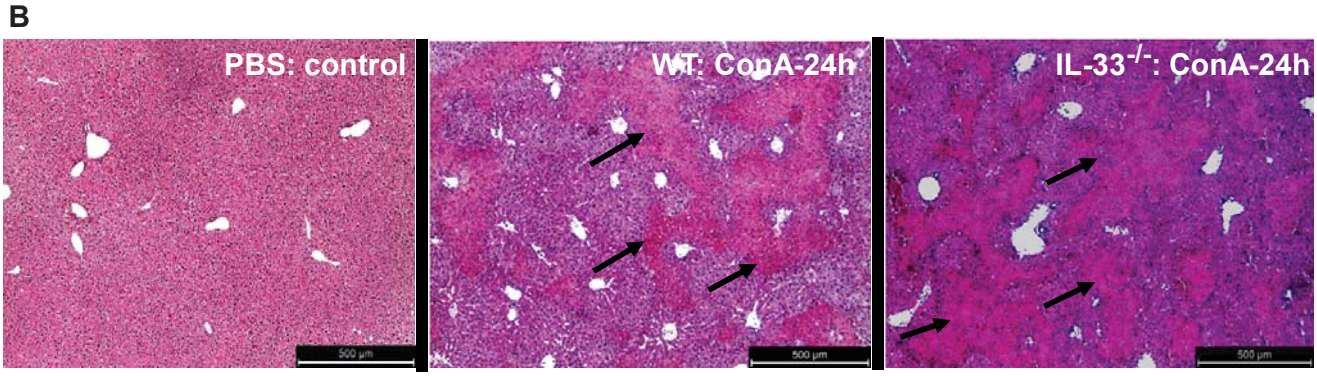
621

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


A
AST (IU/ L)
30000
20000
10000
0
###
**
**
**
**
**
PBS
ConA/12h
ConA/24h
PBS
ConA/12h
ConA/24h
WT
IL-33-/-
ALT (IU/ L)
25000
20000
15000
10000
5000
0
###
**
**
**
**
**
PBS
ConA/12h
ConA/24h
PBS
ConA/12h
ConA/24h
WT
IL-33-/-


B
PBS: control
WT: ConA-24h
IL-33-/-: ConA-24h

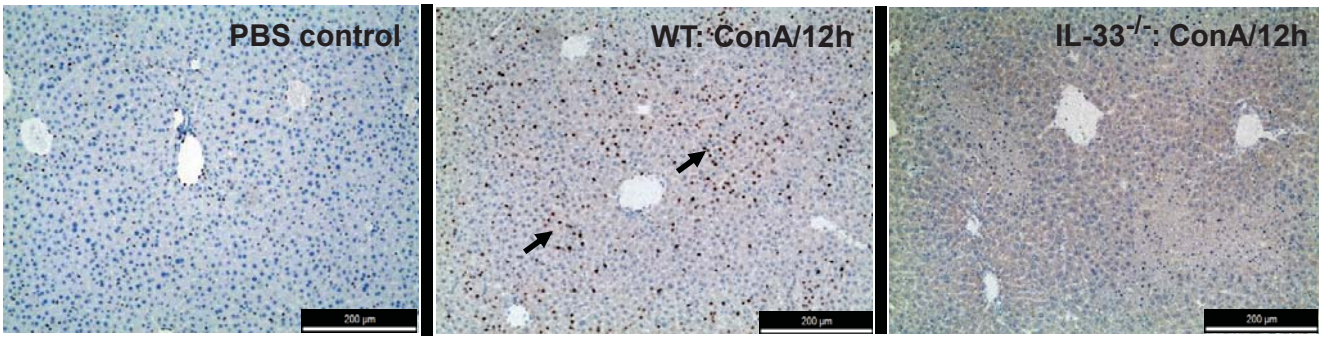

C
PBS control
WT: ConA/12h
IL-33-/-: ConA/12h

Figure 1

D

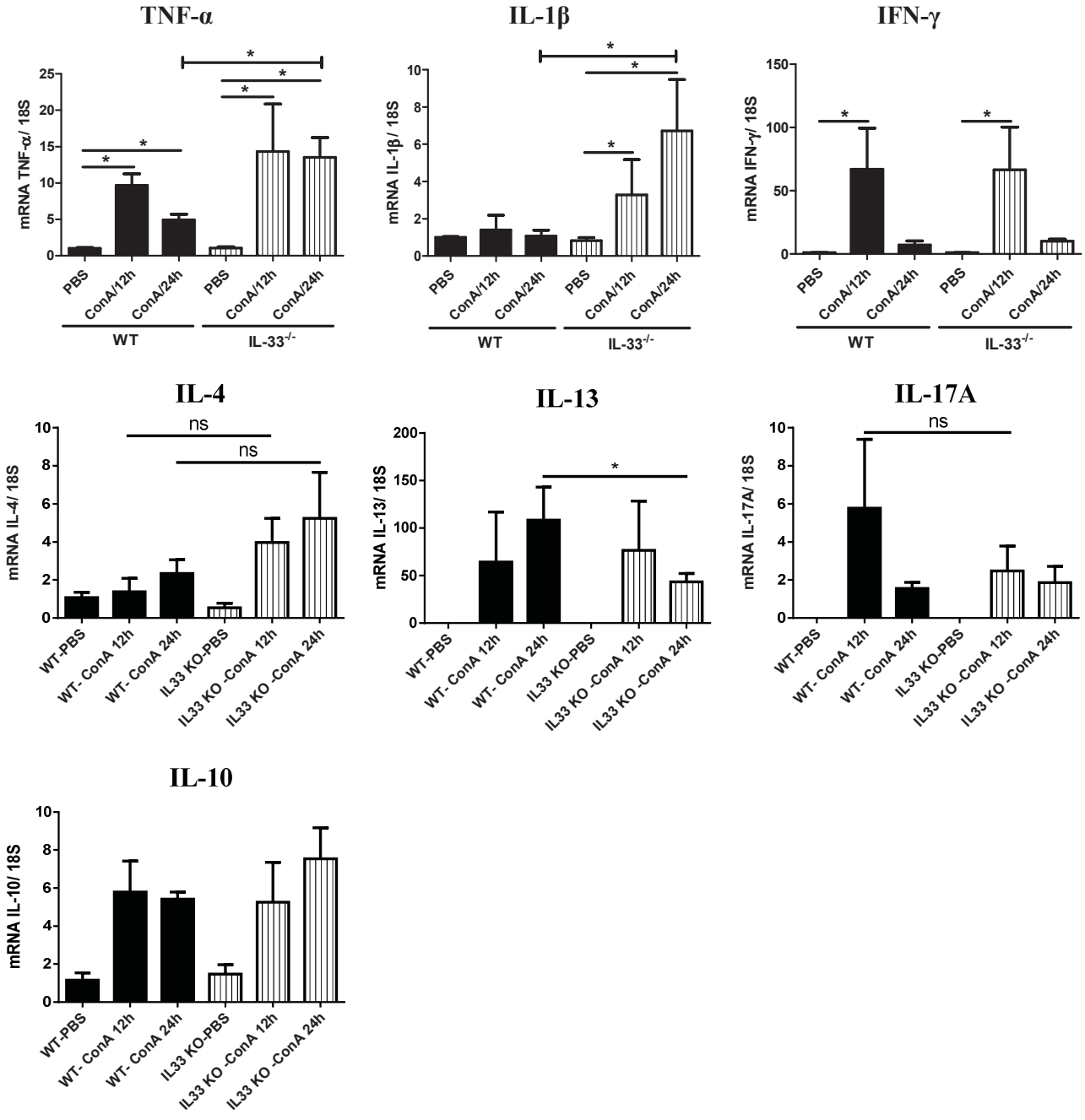

TNF-α
mRNA TNF-α/ 18S
PBS
ConA/12h
ConA/24h
WT
IL-33-/-
IL-1β
mRNA IL-1β/ 18S
IFN-γ
mRNA IFN-γ/ 18S
IL-4
mRNA IL-4/ 18S
ns
WT-PBS
WT- ConA 12h
WT- ConA 24h
IL33 KO-PBS
IL33 KO -ConA 12h
IL33 KO -ConA 24h
IL-13
mRNA IL-13/ 18S
IL-17A
mRNA IL-17A/ 18S
IL-10
mRNA IL-10/ 18S

Figure 2

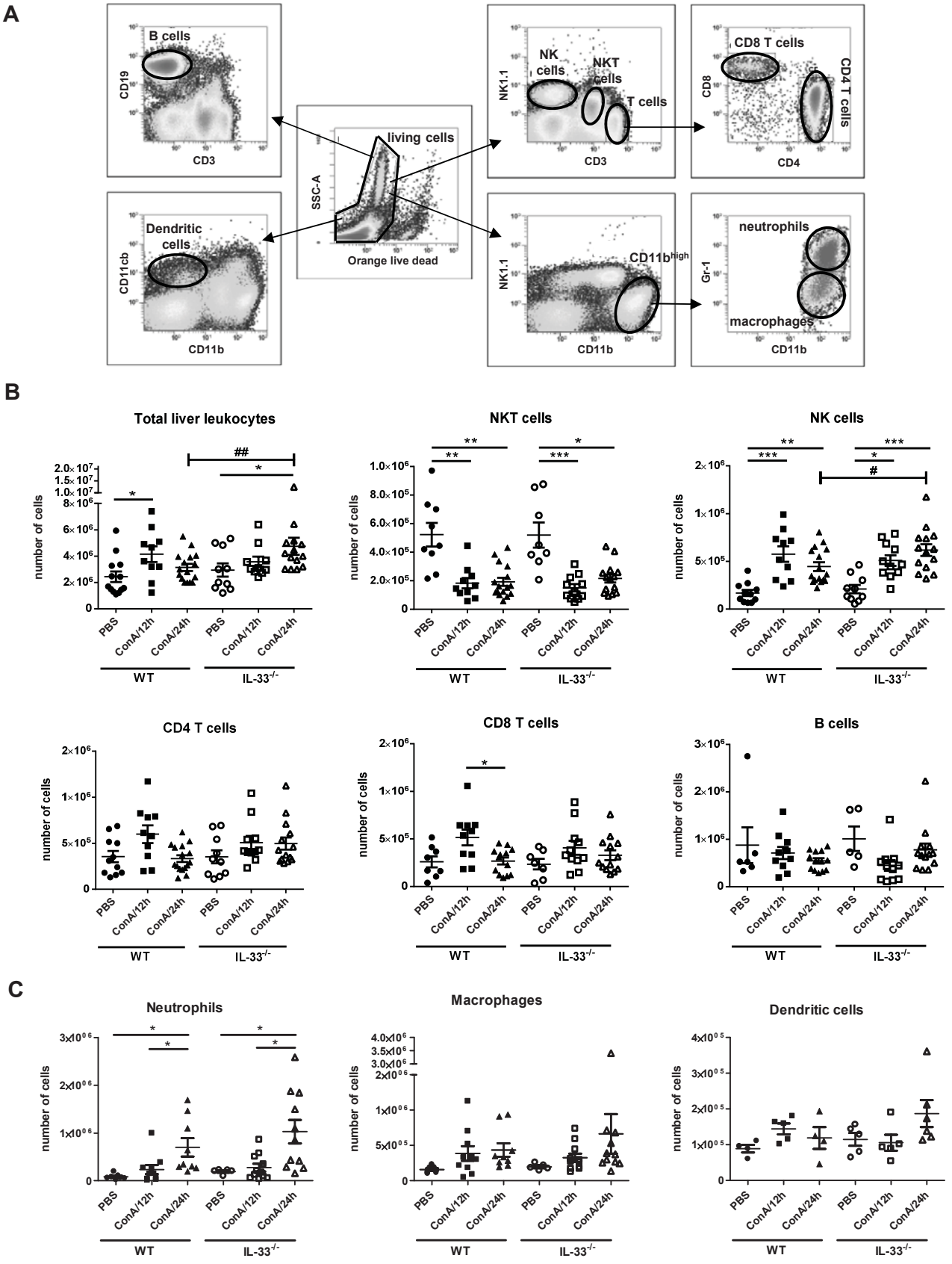

A
B cells
CD19
CD3
living cells
SSC-A
Orange live dead
NK cells
NKT cells
T cells
NK1.1
CD3
CD8 T cells
CD8
CD4 T cells
CD4
Dendritic cells
CD11cb
CD11b
CD11b^high
NK1.1
CD11b
neutrophils
Gr-1
macrophages
CD11b
B
Total liver leukocytes
NKT cells
NK cells
CD4 T cells
CD8 T cells
B cells
number of cells
PBS
ConA/12h
ConA/24h
WT
IL-33-/-
C
Neutrophils
Macrophages
Dendritic cells

Figure 3

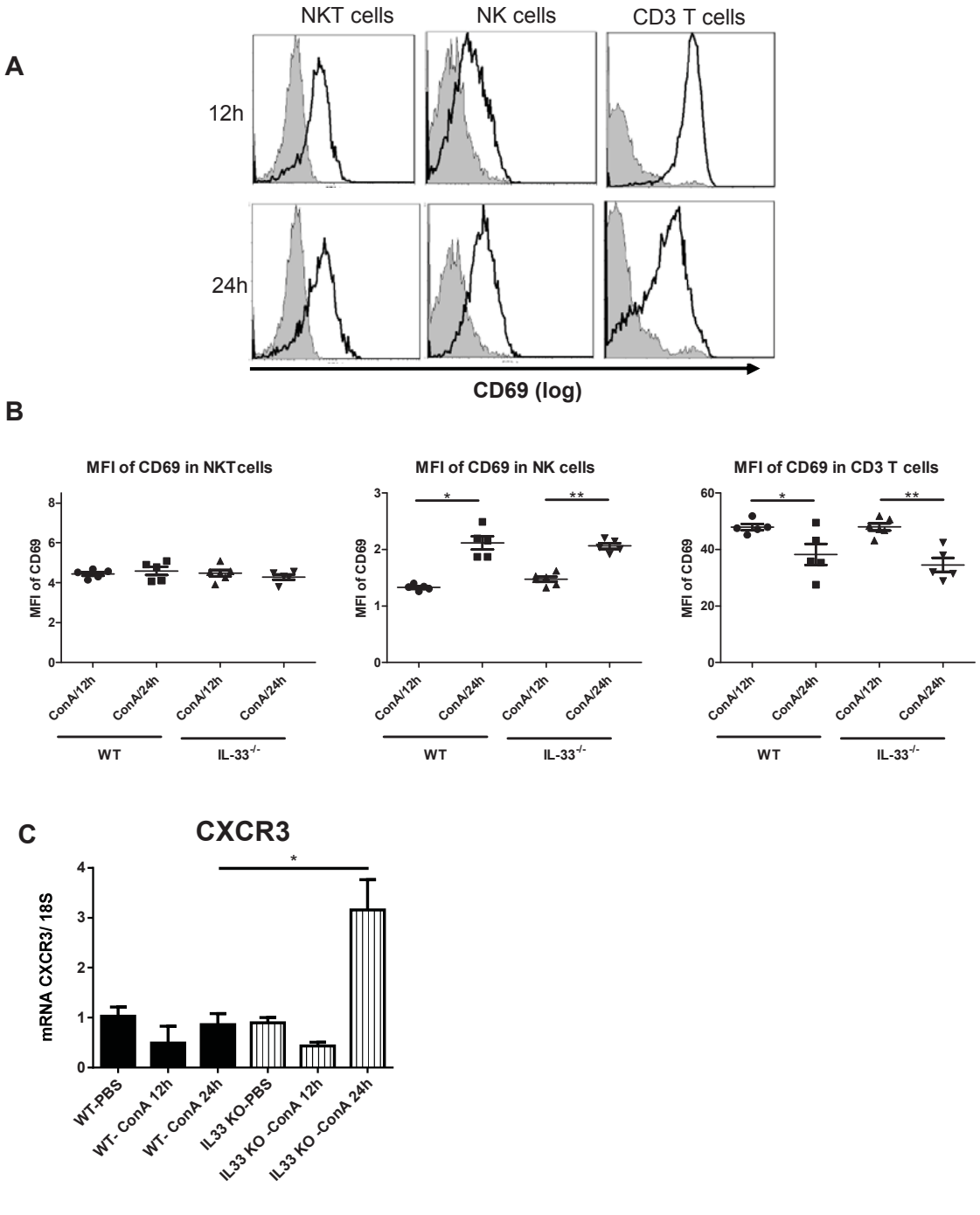

A
NKT cells
NK cells
CD3 T cells
12h
24h
CD69 (log)
B
MFI of CD69 in NKTcells
MFI of CD69 in NK cells
MFI of CD69 in CD3 T cells
MFI of CD69
ConA/12h
ConA/24h
WT
IL-33-/-
C
CXCR3
mRNA CXCR3/ 18S
WT-PBS
WT- ConA 12h
WT- ConA 24h
IL33 KO-PBS
IL33 KO -ConA 12h
IL33 KO -ConA 24h

Figure 4

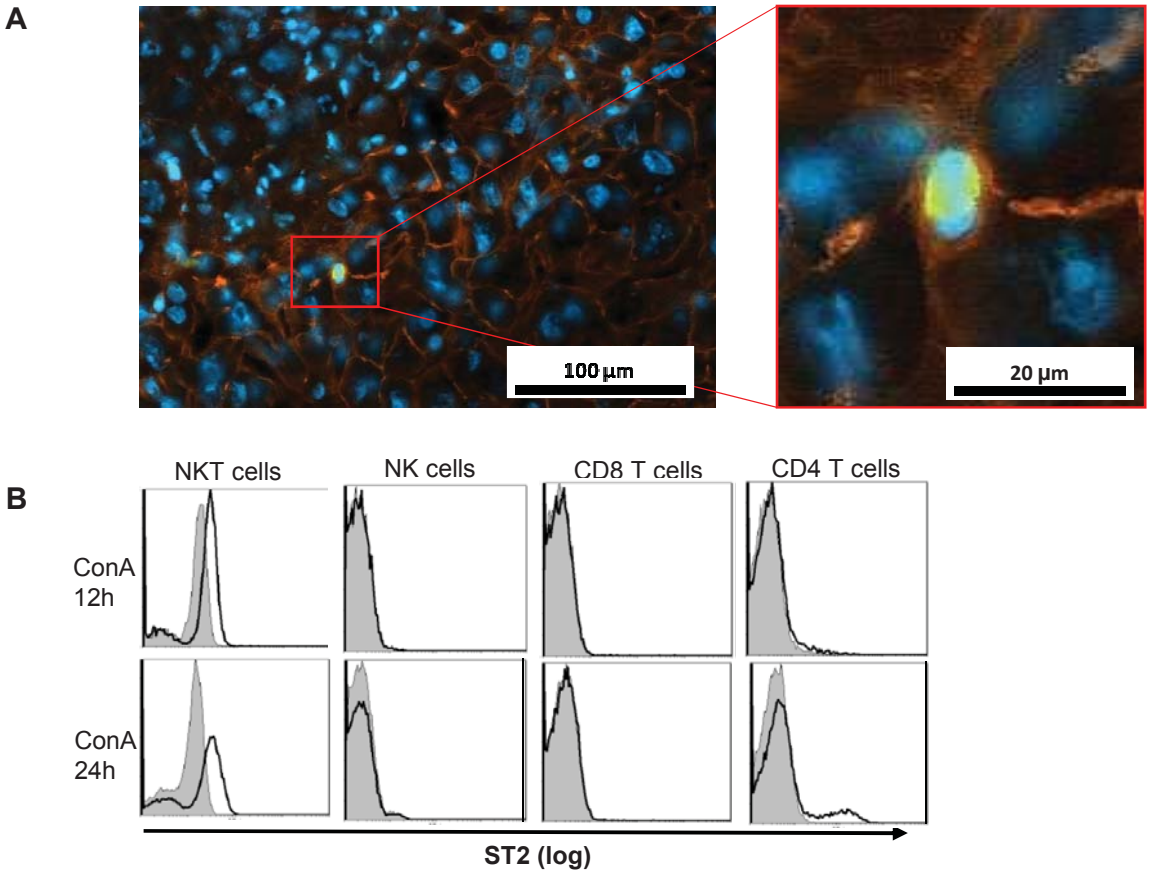

A
100 µm
20 µm
B
NKT cells
NK cells
CD8 T cells
CD4 T cells
ConA 12h
ConA 24h
ST2 (log)
C
PBS
WT ConA (24h)
IL-33-/- ConA (24h)
0,7%
1,4%
7,6%
10,9%
6,1%
10,1%
ST2
Foxp3
D
Treg cells
number of cells
% of ST2+ in Treg cells
% of ST2+
MFI of ST2 in ST2+ Treg cells
MFI of ST2+
PBS
ConA/12h
ConA/24h
WT
IL-33-/-

Figure 4

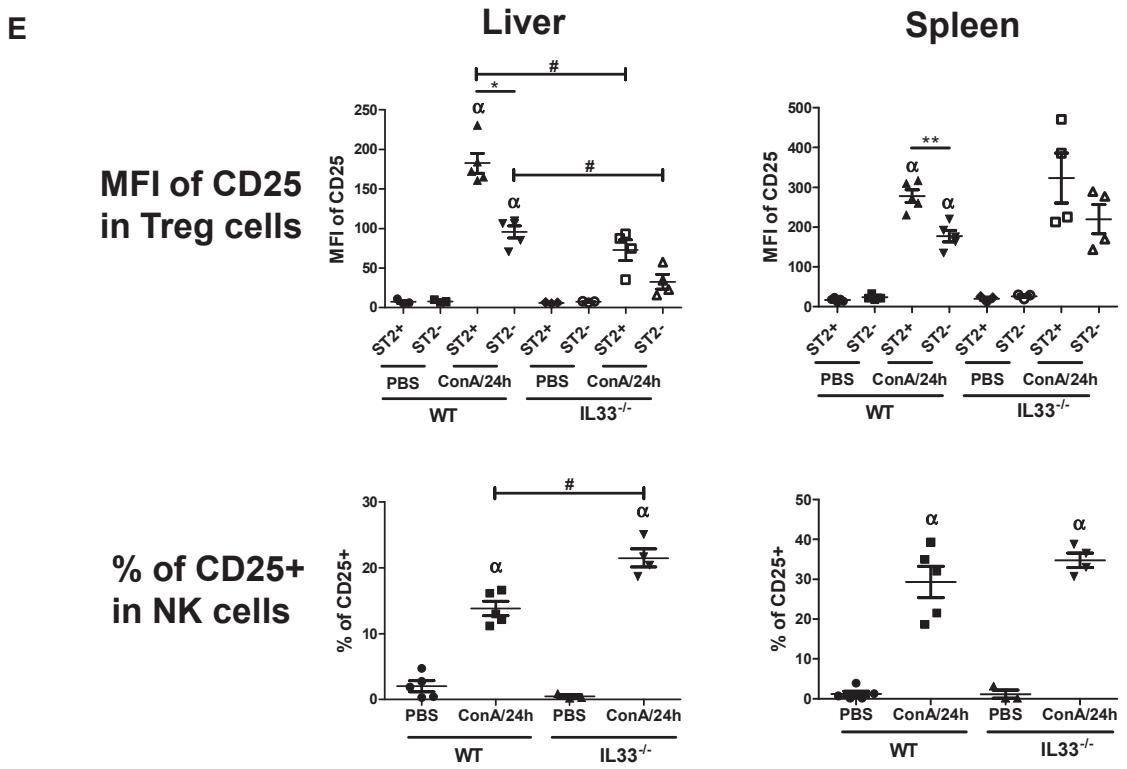

E
Liver
Spleen
MFI of CD25 in Treg cells
% of CD25+ in NK cells
MFI of CD25
% of CD25+
ST2+
ST2-
PBS
ConA/24h
WT
IL33-/-
#
*
**
α

Figure 5

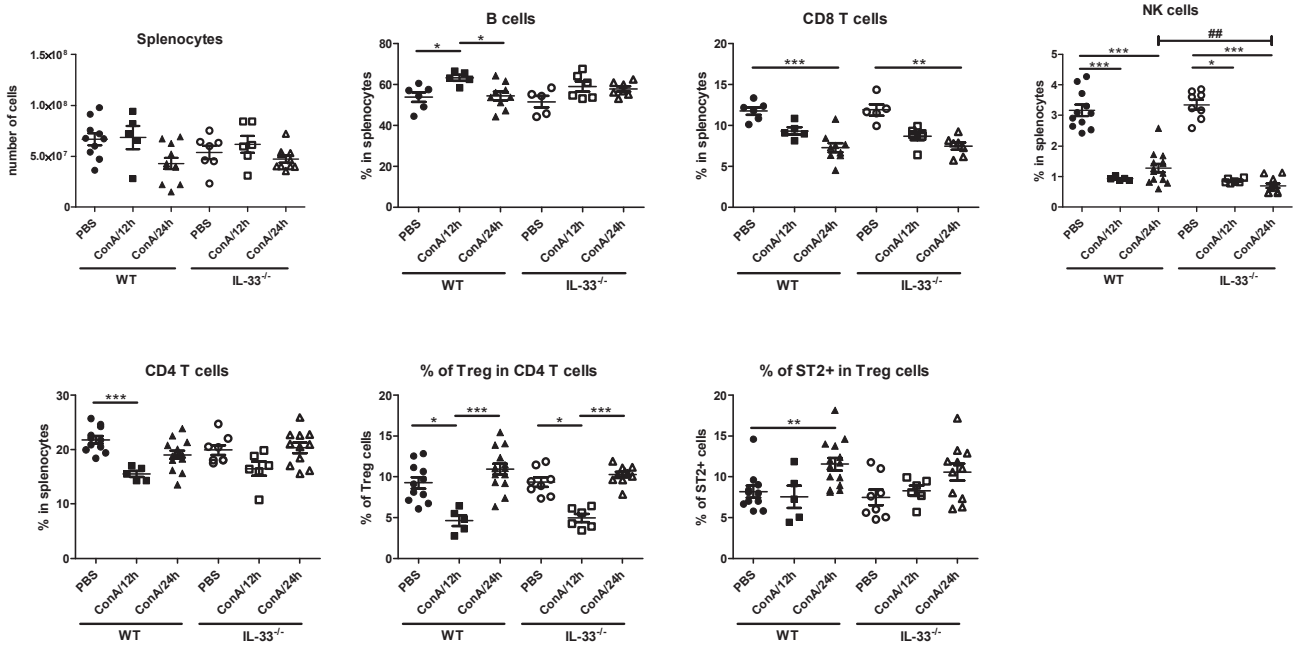
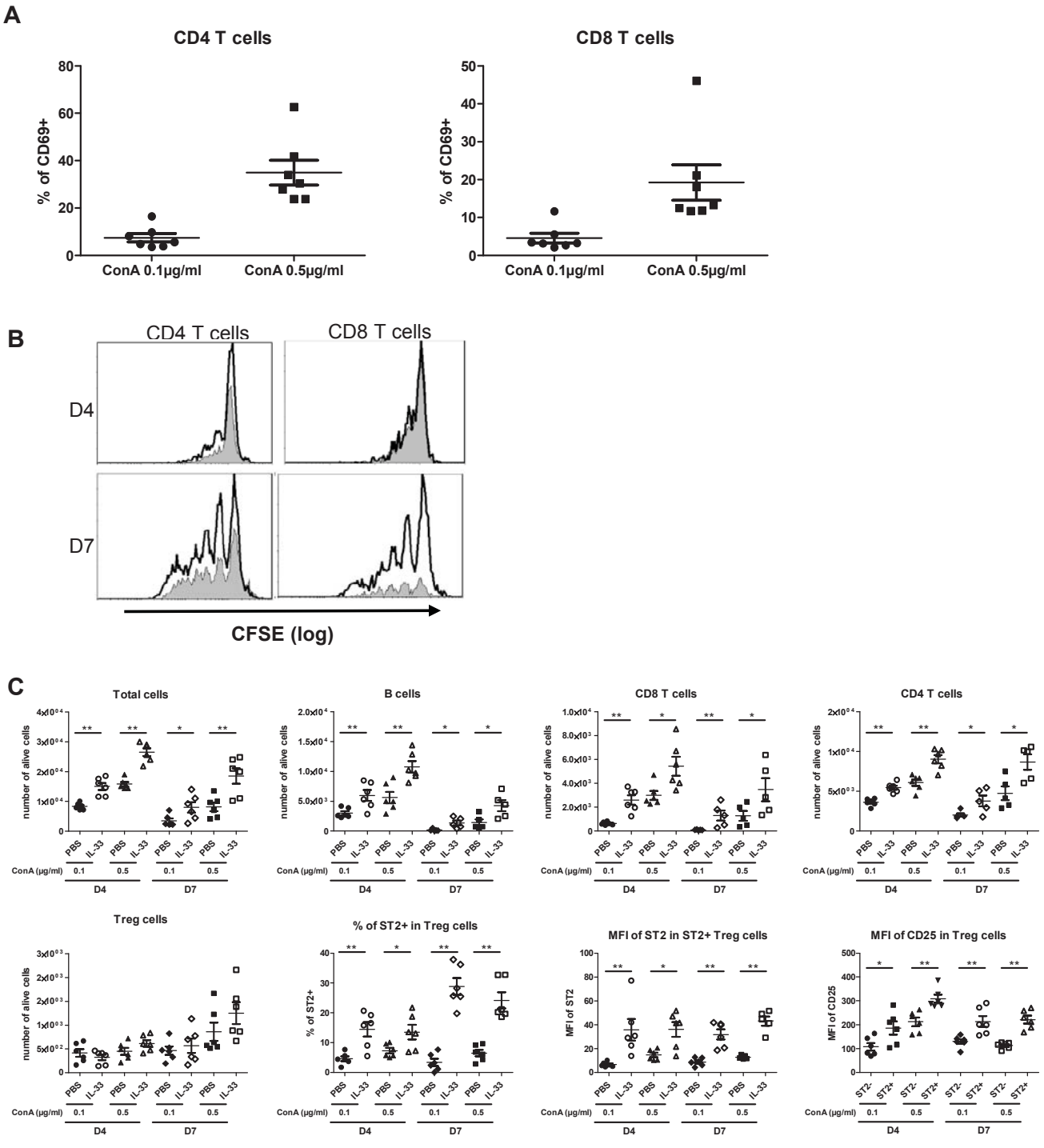

Splenocytes
number of cells
1.5x10^8
1.0x10^8
5.0x10^7
0
B cells
% in splenocytes
80
60
40
20
0
CD8 T cells
% in splenocytes
20
15
10
5
0
NK cells
% in splenocytes
5
4
3
2
1
0
##
***
*
**
PBS
ConA/12h
ConA/24h
WT
IL-33-/-
CD4 T cells
% in splenocytes
30
20
10
0
% of Treg in CD4 T cells
% of Treg cells
20
15
10
5
0
% of ST2+ in Treg cells
% of ST2+ cells
20
15
10
5
0

Figure 6


A
CD4 T cells
CD8 T cells
% of CD69+
ConA 0.1μg/ml
ConA 0.5μg/ml
B
CD4 T cells
CD8 T cells
D4
D7
CFSE (log)
C
Total cells
B cells
CD8 T cells
CD4 T cells
Treg cells
% of ST2+ in Treg cells
MFI of ST2 in ST2+ Treg cells
MFI of CD25 in Treg cells
number of alive cells
% of ST2+
MFI of ST2
MFI of CD25
PBS
IL-33
ConA (μg/ml)
0.1
0.5
D4
D7
ST2-
ST2+