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

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Noel G, Arshad MI, Filliol A, Genet V, Rauch M, Lucas-Clerc C, Lehuen A, Girard J-P, Piquet-Pellorce C, Samson M. Ablation of interaction between IL-33 and ST2$^+$ regulatory T cells increases immune cell-mediated hepatitis and activated NK cell liver infiltration. *Am J Physiol Gastrointest Liver Physiol* 311: G313–G323, 2016. First published June 23, 2016; doi:10.1152/ajpgi.00097.2016.—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 IL-33-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 wild-type (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-1β and a larger number of NK cells infiltrating the liver. The expression of Th2 cytokines (IL-4, IL-10) 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 upregulated expression of CXCR3 in the 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.

concanavalin A-hepatitis; immune cells; liver; interleukin-33-deficient mice; ST2 receptor; regulatory T cells

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 suppresses 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 high-motility group box protein 1 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 auto-

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immune, viral, or immune cell-mediated hepatitis in humans, and liver injury in this model is caused principally by NK and NKT cells and TRAIL (40, 42). Previous studies on Con A-induced hepatitis have generated conflicting results. Chen et al. (11) demonstrated a protective role of an IL-33-blocking antibody in liver injury, whereas, Volarevic et al. (44) showed IL-33 to have beneficial effects on hepatitis in studies on $ST2^{-/-}$ mice or in which recombinant IL-33 was administered to mice. 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-$33^{-/-}$ and wild-type (WT) mice to determine the functional role of endogenous IL-33 in Con A-induced hepatitis.

IL-$33^{-/-}$ mice displayed more severe liver injury than WT mice following Con A administration. This exacerbated liver injury was associated with the infiltration of a 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-wk-old 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 injection of Con A (Sigma-Aldrich), at a dose of 20 mg/kg body wt, to induce acute hepatitis. Mice were killed 12 or 24 h postinjection (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 (hematoxylin and eosin staining) and serum biochemical analyses (alanine aminotransferase/aspartate aminotransferase, AST/ALT) were performed as described earlier (5). Briefly, immunolocalization of IL-33 was performed using primary antibody goat IgG anti-mouse-IL-33 (R&D Systems) and secondary horseradish peroxidase-conjugated rabbit anti-goat antibody (Dako) with hematoxylin counterstaining in Ventana machine (Ventana Medical Systems). Immunolocalization of ST2 was performed using three stainings: rat IgG2b anti-mouse-ST2 (clone RMST2-33, eBiosciences) coupled with a secondary Cy-3 anti-rat antibody (Dako), phalloïdin (F-actin staining) coupled with Alexa 350 (Interchim), and Draq-5 (DNA dye) coupled with far red fluorescence (Biostatus).

RNA isolation and RT-qPCR. Total RNA was extracted from mouse liver pieces using TRIzol Reagent (Invitrogen). First-strand cDNA was produced using the SuperScriptTM II Reverse Transcriptase (Invitrogen). Real-time qPCR was performed using the fluorescent dye SYBR green with the double-strand specific dye SYBRs green system (Applied Biosystems) and the 7300 sequence detection system ABI Prism sequence detector (Applied Biosystems). Total cDNA (30 ng) was used as a template for amplification with the specific primer pair (Table 1) used at a 300-nM final concentration. Each measurement was performed in duplicate. The mRNA levels of mouse IFN-γ, TNF-α, IL-1β, IL-4, IL-10, IL-13, IL-17A, and CXCR3 were normalized to the mRNA expression of ubiquitous housekeeping gene *18S*.

Table 1. *Primers used for qPCR*

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′

Isolation of liver and spleen immune cells and flow cytometry. The liver immune cells were isolated as previously described (13, 23), with a viability >95%. Splenocytes were obtained by passing spleens through a 70-mm cell strainer to dissociate the cells before red blood cell lysis. Cells were resuspended in staining buffer (10% FCS in PBS) and incubated with anti-CD16/32 antibody (BD Pharmingen) to block nonspecific binding. The cells were then labeled with the appropriate fluorochrome-conjugated antibodies/reagents (BD Pharmingen and eBioscience): orange live dead, anti-CD3-V500 (clone 500-A2), anti-CD4-PE-Cy7 (clone RM4-5), anti-CD8-APC-Cy7 (clone 53-6.7), anti-CD25-PEeFluor610 (clone PC61.5 or clone 3C7), anti-NK1.1-PerCP-Cy-5.5 (clone PK136), anti-ST2-PE (clone RMST2-33), anti-CD11b-PE Cy7 (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 carboxyfluorescein succinimidyl ester (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 ± SE. 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. 1*A*). 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. 1*A*). Massive parenchymal and perivascular zones of hepatic injury were observed in IL-33$^{-/-}$ and WT mice following Con A-hepatitis (Fig. 1*B*). 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. 1*C*). We observed an induction of IL-33 production by the hepatocytes of WT mouse livers following Con A-hepatitis (as previously described, Ref. 3) (Fig. 1*C*). 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. 1*D*). 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. 1*D*). 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. 1*D*). Increased but comparable mRNA expression of cytokine IL-17A was found in IL-33$^{-/-}$ mice and WT mice following Con A-hepatitis (Fig. 1*D*). Thus IL-33 deficiency led to more severe liver injury 24 h after Con A administration, together with the establishment of a more proinflammatory cytokine microenvironment.

Deletion of the IL-33 gene leads to higher levels of intrahepatic leukocytes and significant NK-cell infiltration of the liver during Con A-induced hepatitis. We determined the signature of immune cell infiltration during liver injury by quantifying the leukocyte population in the livers of mice challenged with or without Con A, by flow cytometry with a live-dead marker (Fig. 2*A*). The increase in the total number of liver leukocytes was significantly larger in WT mice than in PBS-treated controls at 12 h and in IL-33$^{-/-}$ mice than in PBS-treated controls at 24 h. Thus, 24 h after Con A administration, greater leukocyte infiltration had occurred in the IL-33$^{-/-}$ mice than in WT mice (Fig. 2*B*). A strong decrease in the number of NKT cells and a significant increase in the number of NK cells were evident 12 h after Con A administration, in both WT and IL-33$^{-/-}$ mice (Fig. 2*B*), suggesting that NKT cells are involved in the early stages of liver damage, with NK cells playing a role later on. This hypothesis was confirmed by quantifying liver CD69 expression/MFI (a lymphoid cell activation marker) between 12 and 24 h after Con A administration. Indeed, a marked increase in CD69 expression on NK cells was observed following Con A administration, in both WT and IL-33$^{-/-}$ mice, whereas CD69 expression remained stable in NKT cells (Fig. 3, *A* and *B*). There were significantly more intrahepatic NK cells in IL-33$^{-/-}$ mice than in WT mice 24 h after Con A administration (Fig. 2*B*). Accordingly, the liver mRNA expression of CXCR3 (chemokine receptor involved in recruitment of immune cells) was significantly raised at 24 h of Con A liver injury in IL-33$^{-/-}$ mice compared with in WT mice (Fig. 3*C*).

This tendency for the number of infiltrating cells to increase by 12 h and then return to the basal state was observed for NK cells, B cells, and 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 and 24 h after Con A administration in IL-33$^{-/-}$ mice (Fig. 2*B*). A nonsignificant 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. 2*C*). 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. 4*A*). Furthermore, flow cytometry showed that NKT cells displayed weak membrane ST2 expression (MFI 24 h after Con A treatment: 1.793 ± −0.100 in WT mice vs. 1.778 ± 0.050 in IL-33$^{-/-}$ mice), whereas a small population of CD4$^+$ T cells displayed strong membrane ST2 expression in Con A-treated WT mice (Fig. 4*B*). 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 intranuclear 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. 4*C*). 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. 4*D*). 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. 4*D*, MFI: 42.24 ± 3.30 in WT mice vs. 32.13 ± 1.30 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. 4*E*). The percentage of NK cells expressing CD25 was significantly higher in the livers of IL-33$^{-/-}$ mice than in WT mice (Fig. 4*E*). Similar results were obtained for NKT cells (frequency of CD25$^+$ cells among NKT cells: 23.3 ± 2.0% for WT mice vs. 41.63 ± 5.80% 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$^{-/-}$

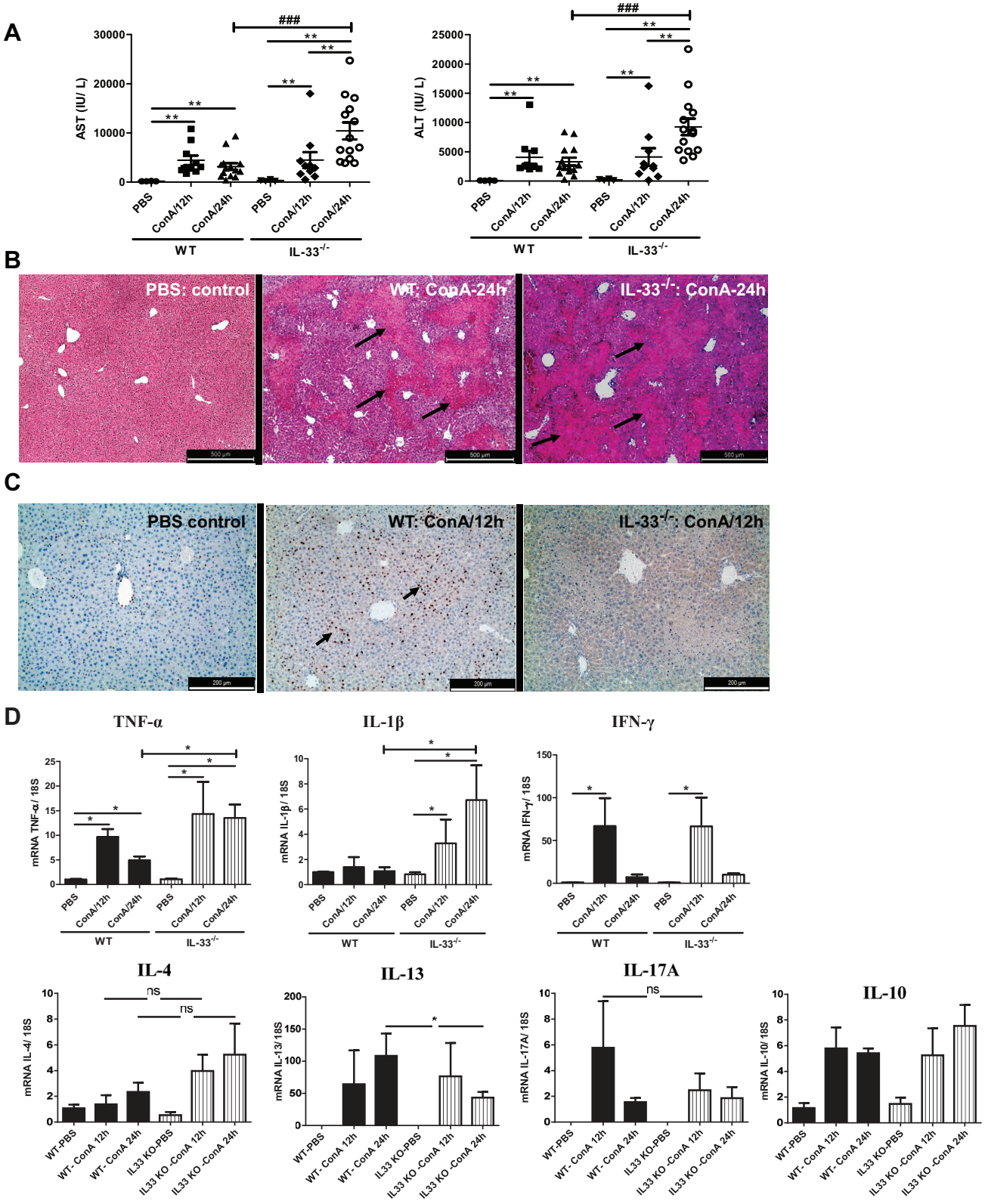

Fig. 1. Liver injury and inflammatory cytokine expression in wild-type (WT) and IL-33$^{-/-}$ mice treated with concanavalin A (Con A). *A*: serum alanine aminotransferase/aspartate aminotransferase (AST/ALT) levels (IU/l) in WT mice and IL-33$^{-/-}$ mice treated with Con A (20 mg/kg iv), 12 or 24 h postinjection ($n = 4$ for PBS control and $n \geq 10$ for Con A challenge). See *Statistical analysis* in text for definition of significance symbols. *B*: sections of liver from untreated mice and from mice treated with Con A were stained with hematoxylin and eosin. Arrows indicate the zones of liver injury. *C*: immunolocalization of IL-33 with goat anti-mouse-IL-33 primary antibody and a horseradish peroxidase-conjugated rabbit anti-goat antibody, with hematoxylin counterstaining on sections of livers from control, WT, and IL-33$^{-/-}$ mice. Arrows indicate IL-33-positive hepatocytes. The scale bar indicates 200 or 500 μm. *D*: relative fold change in mRNA expression of TNF-α, IL-1β, IFN-γ, IL-4, IL-10, IL-13, and IL-17A in livers of WT and IL-33$^{-/-}$ mice treated with Con A at 12 and 24 h postinjection. 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.

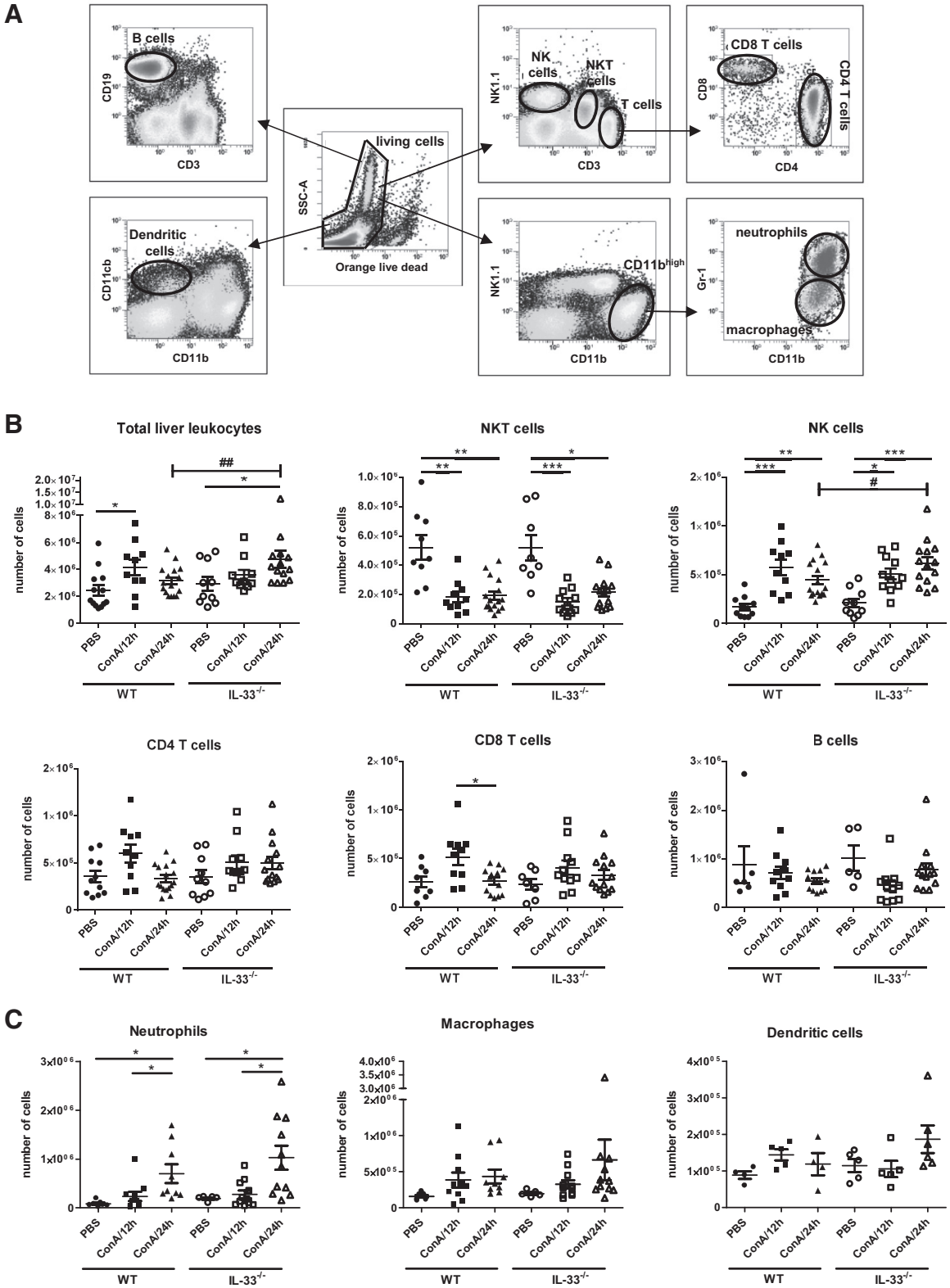

Fig. 2. Quantification of the cells infiltrating the liver in WT and IL-33$^{-/-}$ mice during Con A-hepatitis. Gating strategy (*A*) for quantification of total liver leukocytes, NKT cells (CD3^{med}NK1.1^{med}), NK cells (CD3^{-}NK1.1^{+}), CD4 and CD8 T cells (CD3^{high}), and B cells (CD19^{+}) (*B*) and neutrophils (NK1.1^{-}CD11b^{high}Gr1^{high}), macrophages (NK1.1^{-}CD11b^{high}Gr1^{med}), and dendritic cells (CD11c^{+}) (*C*), in the livers of WT and IL-33$^{-/-}$ mice following Con A administration (12 or 24 h postinjection). A viability dye (orange live dead) was used to exclude dead cells at the beginning of the analysis. *B*: $n \geq 6$ for PBS control and $n \geq 10$ for Con A challenge. *C*: $n \geq 4$ for PBS control and $n \geq 6$ for Con A challenge. See *Statistical analysis* in text for definition of significance symbols.

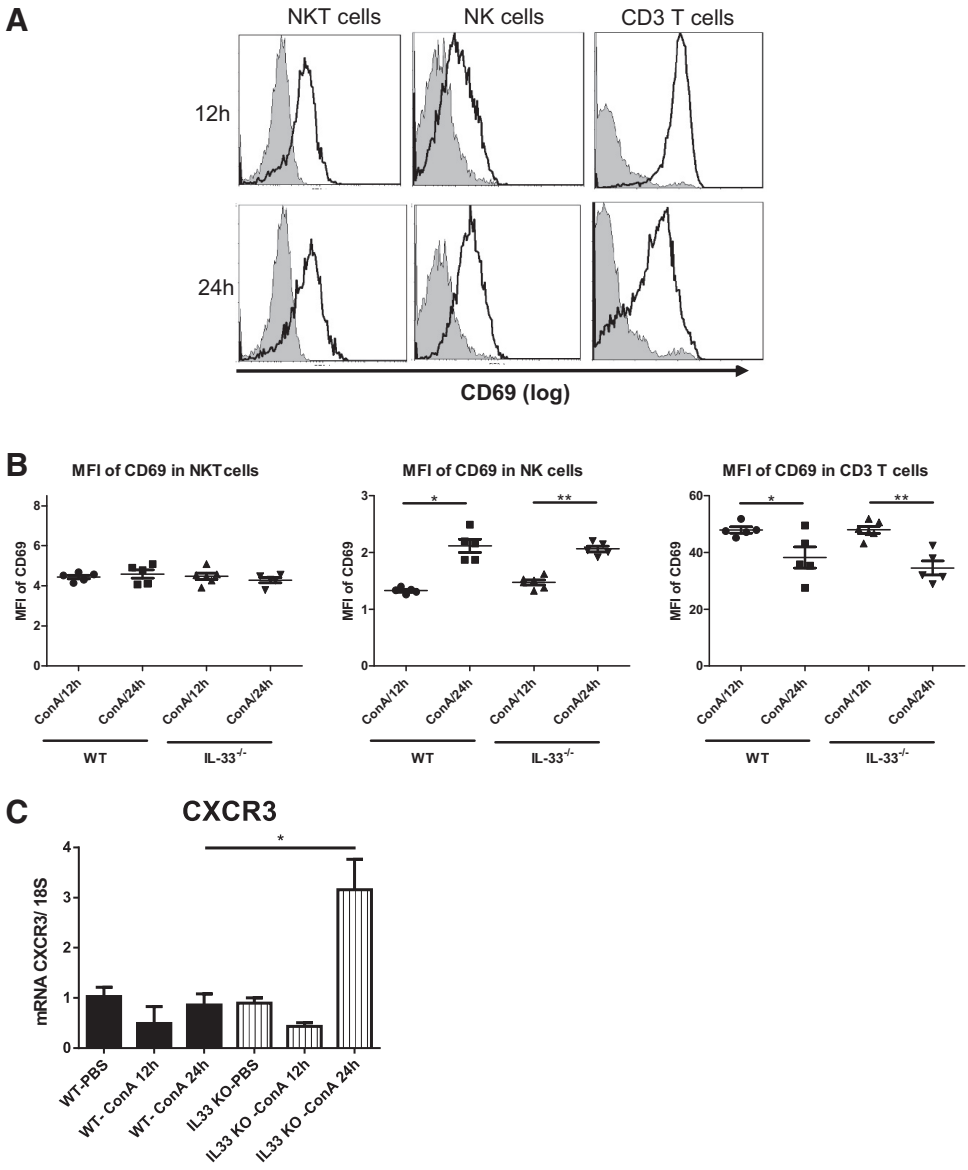

Fig. 3. Quantification of liver CD69 expression/MFI during Con A-hepatitis. Expression (*A*) and MFI (*B*) of surface CD69 on membrane of different infiltrate cell populations in liver of WT (*A* and *B*) and IL-33$^{-/-}$ (*B*) mice following Con A administration; $n = 5$ for all conditions. *$P < 0.05$ and **$P < 0.01$ with a Mann-Whitney *U*-test. Gray shaded graphs in *A* represent CD69 staining of each population after PBS treatment. These stainings are used as reference for MFI calculations in *B*; thus MFI of nontreated 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 24 h postinjection. 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.

mice (31.0 ± 3.3 and 34.0 ± 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. 4*E*). Thus, in the liver, IL-33 modulated ST2$^+$ Treg cell 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 cells, 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 led 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. 2*B* and 4*E*. 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 h of treatment, and endogenous IL-33 impacted 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 4 or 7 days. Two concentrations of Con A were used: a low concentration (0.1 μg/ml) activating 5–10% of CD4$^+$ T cells at 15 h without inducing proliferation, and a higher concentration (0.5 μg/ml) that activated almost 40% of

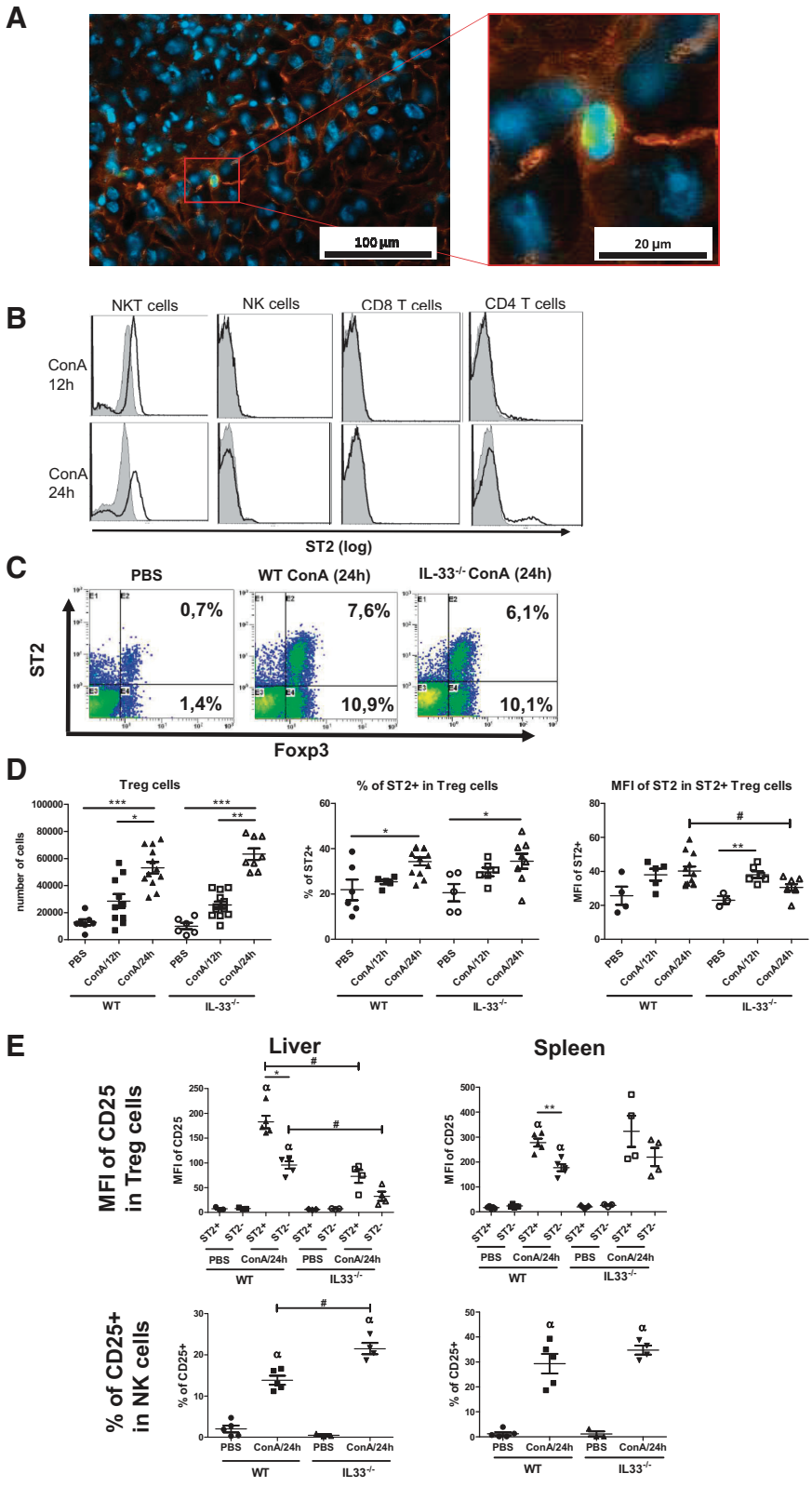

Fig. 4. Expression of ST2 on cells infiltrating the liver in WT and IL-33$^{-/-}$ mice during Con A-hepatitis. *A*: sections of WT mice liver treated with Con A (24 h) were stained with anti-mouse ST2 (green), phalloidin (red), and Draq-5 (blue) for immunofluorescence analysis. *B*: surface ST2 expression in various cell populations from the liver infiltrate in WT mice following Con A administration (gray shaded graphs correspond to the isotypic control). The gating strategy was identical to that used in Fig. 2*B*. *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 regulatory T (Treg) cells (CD4^{+}Foxp3^{+}) in the liver and 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 postinjection). MFI is the ratio of the fluorescence intensities obtained with specific anti-ST2 and isotype control antibodies ($n \geq 4$ for PBS control and $n \geq 6$ 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 ($\alpha P \leq 0.05$ vs. PBS, Mann-Whitney *U*-test) ($n = 5$ for all groups). See *Statistical analysis* in text for definition of significance symbols.

CD4^{+} T cells at 15 h and induced cell proliferation by *day 7* (Fig. 6*A*). IL-33 had no effect on T-cell proliferation on *days 4* and *7* (Fig. 6*B*). 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. 6*C*). Moreover, ST2^{+} Treg cells displayed stronger surface CD25 expression than the other Treg cells after 4 and 7 days of Con A stimulation (Fig. 6*C*). IL-33 had a significant effect on cell survival, resulting in a higher total number of splenocytes, B cells, and CD4^{+} and

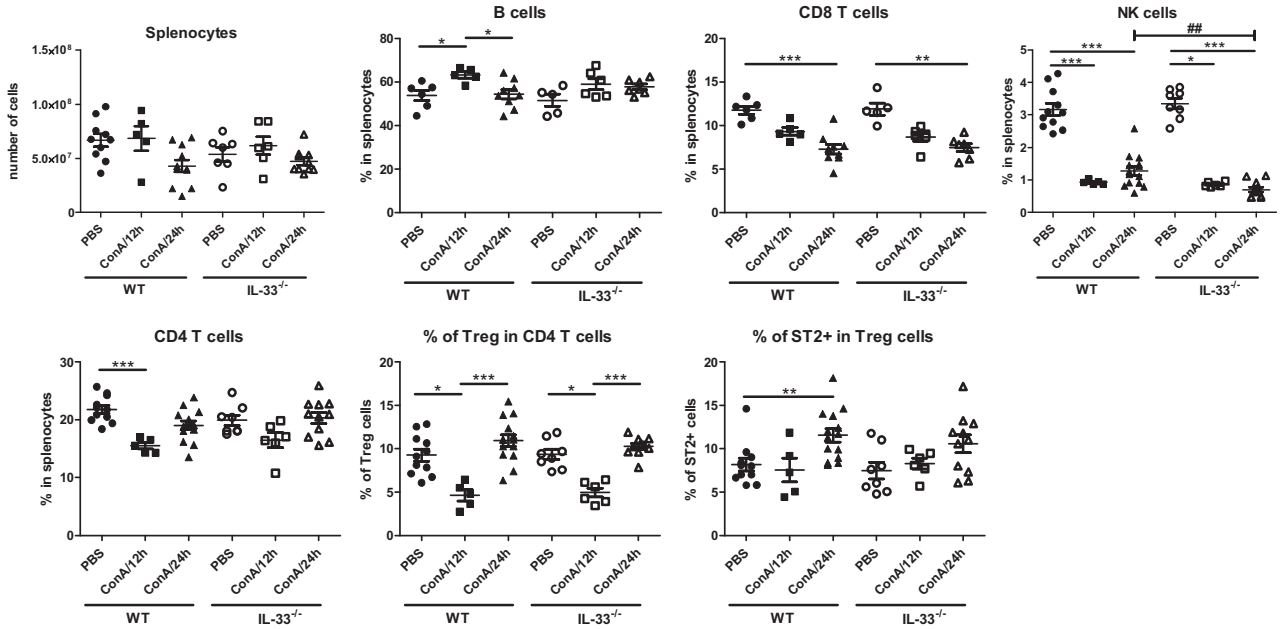

Fig. 5. Quantification of splenocytes in WT and IL-33$^{-/-}$ mice during Con A hepatitis. Quantification and characterization of the splenocytes (CD4$^+$, CD8$^+$, B cells, NK cells, and Treg cells) of WT and IL-33$^{-/-}$ mice following Con A-induced (12 or 24 h postinjection) hepatitis; comparison with PBS control mice ($n \geq 5$ for all groups). See *Statistical analysis* in text for definition of significance symbols.

CD8$^+$ T cells (Fig. 6*C*). Thus IL-33 had an effect on Treg cells by selecting and overactivating the ST2$^+$ Treg cell population and favoring lymphoid cell survival during Con A activation in vitro.

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 cell 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 proinflammatory effect on innate immunity by activating ST2, a receptor present in several types of immune cells (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 cell 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 *1*) a high dose of Con A used in our model minimized IL-33 activation, and *2*) 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 a 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$^{-/-}$ mice). The significantly raised expression of CXCR3 in liver of IL-33-deficient mice compared with WT mice at 24 h of Con A administration reinforced the recruitment of NK cells. The NK and NKT cells and lymphocytes express CXCR3 in mouse liver (6) and play a role in recruitment of these cells during liver inflammation (15). These results are consistent with the smaller number of cells infiltrating the liver 24 h after Con A administration reported by Volarevic et al. (44) in mice treated with IL-33 (1 μg/mouse) and the smaller number of activated NK and NKT cells and less severe liver damage in these IL-33-treated Balb/c mice. In contrast to our findings, these authors showed that prior treatment with IL-33 decreased the numbers of CD4$^+$ and CD8$^+$ T cells. In another study, the pretreatment with rIL-33 (10 μg/mouse) did not protect C57Bl/6 mice against Con A-hepatitis (11). The differences in the results obtained may be due to the low dose of Con A or the mice used by Volarevic et al. (44) and the prior activation of Treg cells by IL-33, both of which may decrease the activation and recruitment of CD4$^+$ and CD8$^+$ T cells.

During later stages of Con A-induced inflammation, a regulatory mechanism dependent on IL-33 may control CD25$^+$ NKT and NK cells, as these activated cells were found to be essential to trigger liver injury. The IL-33/ST2 axis promotes Treg cell function in cancer growth and metastasis (20) and in colon inflammation (38). Treg cells were reported to be protective (protective effect induced by prior treatment with galectin-9 or GP96) against Con A-induced hepatitis in previous studies (21, 25). We identified the ST2$^+$ Treg cell population

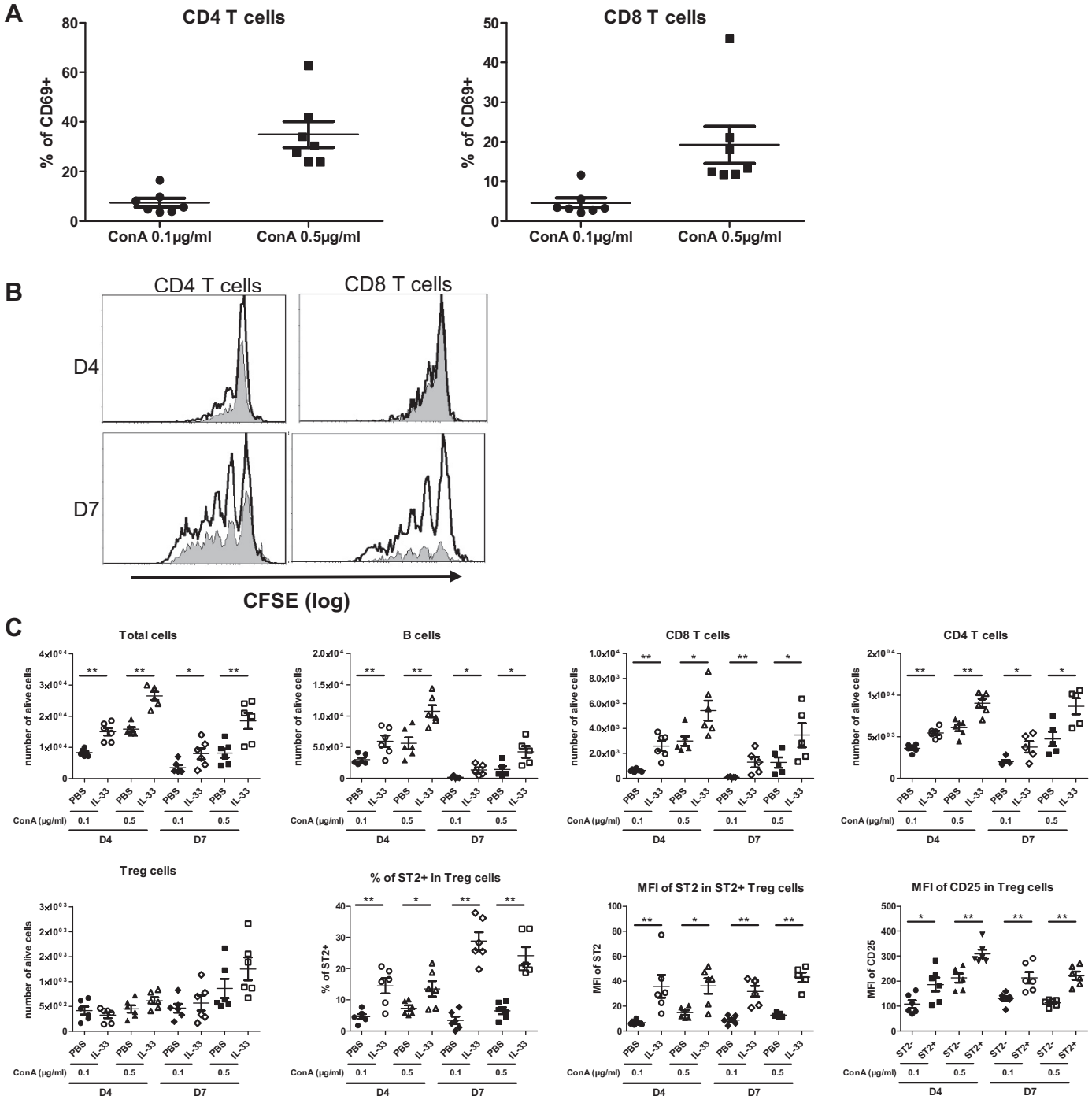

Fig. 6. Effect of IL-33 in Con A-activated splenocytes in vitro. *A*: splenocytes (105 cells/well) were activated in vitro with 2 concentrations of Con A (0.1 or 0.5 μg/ml) with or without IL-33 (10 ng/ml). *B*: expression of membrane CD69 on $CD4^+$ and $CD8^+$ T cells were quantified at 15 h, and frequency of CD69-positive cells was calculated. *C*: dilution of carboxyfluorescein succinimidyl ester (CFSE) in $CD4^+$ and $CD8^+$ T cells during Con A activation at 0.5 μg/ml. Gray shaded graphs represented Con A activation without IL-33, and black graphs represent activation in the presence of IL-33. Quantification of total cells, B cells, and $CD4^+$ 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) is shown. See *Statistical analysis* in text for definition of significance symbols.

in the liver and spleen and showed that these cells strongly infiltrated the liver within 24 h of Con A administration, independently of IL-33. In WT mice, these $ST2^+$ Treg cells displayed high levels of ST2 expression (with an MFI 25 times higher than that for NKT cells) and higher levels of CD25 expression than in IL-$33^{-/-}$ mice (with an MFI 6 times higher than that in NK cells in WT mice and twice that in IL-$33^{-/-}$ mice). In IL-$33^{-/-}$ mice, 24 h after Con A injection, 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 *1*) the larger numbers of Treg cells infiltrating the liver 24 h after Con A administration (attributable to the exogenous injection of IL-33) reported by Volarevic et al. (44) 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); *2*) an accumulation of $ST2^+$ Treg cells within tumors, associated with lower levels of NK cell activation and cytotoxicity (20); and *3*) the direct suppression of NK-mediated hepatocytotoxicity by Treg cells in hepatitis B virus-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 attributable to the limitation of NK cell activation. Our results provide a possible explanation of the 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 myeloid-derived suppressor cells (MDSC) in the Con A hepatic model cannot be excluded because Huang et al. (19) demonstrated that IL-33-stimulated macrophages produce granulocyte colony-stimulating factor, in turn boosting MDSC levels.

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 proapoptotic genes and increasing the expression of antiapoptotic 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 provide evidence 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 promotion of cell survival.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

G.N., M.I.A., C.P.-P., and M.S. conception and design of research; G.N., M.I.A., A.F., V.G., M.R., and C.P.-P. performed experiments; G.N., M.I.A., V.G., C.L.-C., C.P.-P., and M.S. analyzed data; G.N., M.I.A., C.P.-P., and M.S. interpreted results of experiments; G.N., M.I.A., and M.S. prepared figures; G.N., M.I.A., and M.S. drafted manuscript; G.N., M.I.A., and M.S. edited and revised manuscript; G.N., M.I.A., V.G., M.R., C.L.-C., A.L., J.-P.G., C.P.-P., and M.S. approved final version of manuscript.

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