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Interleukin-33 contributes to disease severity in *Dengue virus* infection in mice

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Summary

The excessive inflammation often present in patients with severe dengue infection is considered both a hallmark of disease and a target for potential treatments. Interleukin-33 (IL-33) is a pleiotropic cytokine with pro-inflammatory effects whose role in dengue has not been fully elucidated. We demonstrate that IL-33 plays a disease-exacerbating role during experimental dengue infection in immunocompetent mice. Mice infected with dengue virus serotype 2 (DENV2) produced high levels of IL-33. DENV2-infected mice treated with recombinant IL-33 developed markedly more severe disease compared with untreated mice as assessed by mortality, granulocytosis, liver damage and pro-inflammatory cytokine production. Conversely, ST2$^{-/-}$ mice (deficient in IL-33 receptor) infected with DENV2 developed significantly less severe disease compared with wild-type mice. Furthermore, the increased disease severity and the accompanying pathology induced by IL-33 during dengue infection were reversed by the simultaneous treatment with a CXCR2 receptor antagonist (DF2156A). Together, these results indicate that IL-33 plays a disease-exacerbating role in experimental dengue infection, probably driven by CXCR2-expressing cells, leading to elevated pro-inflammatory response-mediated pathology. Our results also indicate that IL-33 is a potential therapeutic target for dengue infection.

Keywords: dengue; inflammation; interleukin-33; mouse.

Abbreviations: ALT, alanine aminotransferase; ATCC, American Type Culture Collection; BMDM, bone-marrow-derived macrophages; CXCR2, CXC-chemokine receptor 2; DAPI, 4′,6-diamidino-2-phenylindole; DENV, dengue virus; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylene diaminetetraacetic acid; FCS, fetal calf serum; IFN, interferon; IL-33, interleukin-33; LD_{50}, median lethal dose; MOI, multiplicity of infection; MPO, myeloperoxidase; NI, non-infected; NK, natural killer; PBS, phosphate-buffered saline; PFU, plaque-forming units; p.i., post-infection; rmIL-33, recombinant murine interleukin-33; ST2, a subunit of the IL-33 receptor; Th2, T helper type 2; TNF-α, tumour necrosis factor-α; v/v, volume/volume; w/v, weight/volume; WT, wild-type

Introduction

Dengue, caused by infection with any of the dengue virus (DENV) serotypes, is a major public health concern in tropical and sub-tropical regions. Although most DENV infections are asymptomatic, approximately 100 million people are infected annually.[1] Severe forms of dengue are characterized by an intense inflammatory response associated with plasma leakage, thrombocytopenia, bleeding and hypovolaemic shock.[2] The development of severe dengue involves a complex interplay between viral, host and environmental factors.[3–5] DENV can interact with and infect leucocytes such as dendritic cells,[6] monocytes and macrophages,[7] resulting in cell activation and production of inflammatory mediators. Nevertheless, the pathogenesis of severe forms of dengue is not fully understood, although pro-inflammatory cytokines are thought to play an important role.[8]

Interleukin-33 (IL-33) is a tissue-derived nuclear cytokine member of the IL-1 cytokine family, which also includes IL-1β and IL-18. It is abundantly expressed in endothelial cells, epithelial cells and fibroblast-like cells, both during homeostasis and inflammation.[9] Interleukin-33 signals via a heterodimeric receptor, which consists of ST2 and IL-1R accessory protein. Deletion of the *ST2* gene typically abrogates the biological effects of IL-33. The major targets of IL-33 *in vivo* are tissue-resident immune cells such as mast cells, group 2 innate lymphoid cells and regulatory T cells. Other cellular targets include T helper type 2 (Th2) cells, eosinophils, basophils, dendritic cells, Th1 cells, $CD8^+$ T cells, natural killer (NK) cells, invariant natural killer T cells, B cells, neutrophils and macrophages.[9] Interleukin-33 is crucial for the induction of type 2 immune responses by promoting the expression of IL-5 and IL-13 involved in the induction of acute and chronic inflammation.[10,11] The up-regulation of IL-33 production has been associated with a number of inflammatory disorders including allergic asthma, rheumatoid arthritis, allergic rhinitis and ulcerative colitis.[11] Both IL-33 and its precursor pro-IL-33 are released by necrotic cells during tissue injury and both have biological activity, suggesting that IL-33 may act as an alarmin in the induction of inflammation.[11,12]

We report the detrimental role of IL-33 during experimental dengue infection using immunocompetent mice.[13] Production of IL-33 was elevated during DENV infection and IL-33 treatment aggravated DENV-induced disease severity. Concordantly, $ST2^{-/-}$ mice developed less severe disease to the infection. Furthermore, the CXCR2 receptor antagonist DF2156A reversed the disease-exacerbating effects of IL-33.

Materials and methods

Animals and housing

Eight- to 10-week-old male wild-type (WT) C57BL/6 mice were purchased from Janvier (Le Genest-St-Isle, France). $ST2^{-/-}$ male mice (on the C57BL/6 genetic background) were originally provided by Dr Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) and bred at the Transgenose Institute animal facility (UPS44 CNRS, Orleans, France). All animals were kept under specific pathogen-free conditions. All experimental procedures were approved and complied with the French government's ethical and animal experiment regulations and the Comité National de Réflexion Ethique sur l'Expérimentation Animale, CNRS, Orléans, France (CLE CCO 2009-013).

Virus

The mouse-adapted DENV-2 strain P23085 was obtained from the State Collection of Viruses, Moscow, Russia, and adapted as previously described.[14] Sequence of portions of the E and NS1 genes of the mouse-adapted virus was deposited at GenBank under the accession number AY927231, and identity of the DENV-2 P23085 strain was confirmed *in vitro* and *in vivo*.[2,15] Virus stocks were generated by propagating the adapted virus stocks in LLC-MK2 cells [kidney, Rhesus monkey; American Type Culture Collection (ATCC), Manassas, VA] to a maximum of two passages. Cell culture media (Dulbecco's modified Eagle's medium; DMEM; Thermo Fisher Scientific, Paisley, UK) containing DENV were harvested and stored at −80°.

In vivo infection

Mice were handled and kept in a biosafety level 2 laboratory animal facility (Transgenose Institute, UPS44, CNRS). Mice were inoculated intraperitoneally with one median lethal dose (LD_{50}) of mouse-adapted DENV-2 P23085 [200 plaque-forming units (PFU)] diluted in 100 µl of endotoxin-free phosphate-buffered saline (PBS). Mortality was monitored every 12 hr up to day 14 and body weight was recorded up to day 7 post-infection (p.i.). Some mice were treated with recombinant murine IL-33 (rmIL-33; 200 ng/mouse intraperitoneally) on days 0 and 3 p.i.) (BioLegend, San Diego, CA, USA) or DF2156A (10 mg/kg, orally, diluted in carboxymethyl cellulose, on days 0 and 3 p.i.) (Dompé S.p.A., Milan, Italy). On day 4 or 6 p.i., depending on the experimental approach, mice were killed with a ketamine (100 mg/kg)/xylazine (10 mg/kg) solution to recover blood and organs. Samples were stored at −80° before processing and analysis.

In vitro infection

AML12 cells (mouse hepatocytes, ATCC) were propagated in a 1 : 1 mixture of DMEM and Ham's F12 medium (Gibco) supplemented with 10% volume/volume (v/v) fetal calf serum (FCS) (Gibco) and antibiotics (penicillin and streptomycin; Sigma-Aldrich, St Louis, MO, USA). Bone marrow was harvested from the femur and tibia of C57BL/6 mice. Cells were washed, counted and differentiated into bone-marrow-derived macrophages (BMDM) for 7 days in DMEM supplemented with 20% (v/v) horse serum (Gibco) and 30% L929 cell conditioned medium. Non-adherent THP-1 cells (human leukaemic monocytes, ATCC) and HepG2 cells (human hepatocellular carcinoma, ATCC) were propagated in RPMI-1640 (Gibco) or DMEM, respectively, supplemented with 10% v/v fetal calf serum (Gibco) and antibiotics. AML12, THP-1 and HepG2 cells, and BMDMs were plated at 1×10^6 cells/well and inoculated for 1 hr with DENV-2 P23085 at a multiplicity of infection of 0·1. Infected wells were washed twice and maintained in cell-culture medium until supernatant collection at the various time-points, and followed by an MTT assay to determine cell viability (Sigma-Aldrich). Briefly, cells were incubated for 4 hr with 300 µl of a 0·1 mg/ml MTT solution in serum-free cell-culture medium. Medium was removed, the crystals formed in living cells were dissolved in 100 µl dimethylsulphoxide per well. The relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader. Viability of non-treated control cells was defined as 100%.

Quantification of viral load

Viral stocks, cell culture and tissue samples were assayed for viral load as previously described.[16,17] Tissue samples were prepared as 10% weight/volume (w/v) homogenates in DMEM. Samples were serially diluted, adsorbed in an LLC-MK2 monolayer for 1 hr and overlaid with medium 199 (Gibco) supplemented with 3% v/v FCS in 1·5% w/v carboxymethyl cellulose, (Sigma-Aldrich). Cultures were fixed after 7 days and stained with crystal violet 1% (w/v) for counting of viral plaques and the results were expressed as PFU per 100 mg of tissue or per ml of blood/cell culture sample.

Evaluation of blood parameters

Blood was obtained from the brachial plexus in heparin-containing syringes and stored in heparinized tubes. Haematocrit, platelets, lymphocytes, granulocytes and monocytes were evaluated in a Haematology analyser (MS9–5; MS Laboratories International, Osny, France). Results are presented as percentage of haematocrit, lymphocytes, granulocytes and monocytes, or as platelet number per µl of blood.

Histopathology

The liver was perfused, dissected, washed in Hanks' balanced salt solution (Gibco), fixed in 4% (v/v) neutral-buffered formalin (Merck, Kenilworth, NJ, USA), dehydrated, and embedded in paraffin. The tissues were cut into 5-µm sections, stained with haematoxylin & eosin and examined with a Nikon Eclipse E400 microscope at × 200 magnification (scale bars = 50 µm). Histological score was performed blinded as previously described.[15] Tissue damage was estimated by the sum of the morphological findings: 0, no lesion present; ½, individual necrotic cells seen at the first cell layer adjacent to the central vein, and hyaline degeneration present; 1, necrotic cells extending two or three cell layers from the central veins; 2, necrotic cells extending three to six cell layers from the central veins, but limited in peripheral distribution; 3, the same as 2, but with necrosis extending from one central vein to another; 4, more severe than 3, with extensive centrilobular necrosis throughout the section. An overall score for hepatic lesion was computed for each liver section based on assessment of at least three lobules.

Determination of myeloperoxidase activity

For myeloperoxidase (MPO) analysis, as an indirect index of neutrophil accumulation and/or activation, organs were perfused with PBS and homogenized in PBS containing 0·5% hexadecyl trimethyl ammonium bromide and 5 mM ethylenediaminetetraacetic acid (EDTA) using a Dispomix tissue homogenizer (Medic Tools AG, Zug, Switzerland), as described previously,[2] Results are expressed as relative units (optical density at 492 nm) and were corrected for the activity of other peroxidases, which were not inhibited by 3-amino-1,2,4-triazole.

ELISA and alanine aminotransferase measurement

The concentrations of IL-33 (mouse and human), CXCL1/KC, CXCL2/MIP-2, CXCL6/GCP-2, IL-6, IL-1β, interferon-γ (IFN-γ), IL-17A, IL-12p40/p70, IL-5, tumour necrosis factor-α (TNF-α) and IL-2 were analysed by ELISA (all reagents from R&D Systems, Minneapolis, MN, USA) on homogenates of PBS-perfused tissues, cell culture supernatants or sera according to the manufacturer's instructions. The sensitivity of the assay was < 20 pg/ml. Alanine aminotransferase (ALT) concentration was assayed in non-haemolysed serum samples following the manufacturer's protocol (Quibasa/Bioclin, Belo Horizonte, Brazil).

Flow cytometry

Spleen was collected and dissociated using nylon cell strainers. Liver was perfused and treated by enzymatic digestion for 20 min at 37° in RPMI-1640 medium containing 1 mg/ml collagenase type VIII (Sigma-Aldrich) and 1 μg/ml DNase type I (Sigma-Aldrich). Cells were resuspended in a 36% Percoll™ gradient, layered onto 72% Percoll and centrifuged for 30 min at 750 g without brake at 22°. Hepatic leucocytes were washed in PBS containing 2% FCS and 2 mM EDTA (Gibco). Leucocytes (1×10^6 cells/tube) were first stained with purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™, Clone 2.4G2), followed by staining with 4′,6-diamidino-2-phenylindole (DAPI) for cell viability or for extracellular molecular expression patterns using monoclonal antibodies against mouse CD45 (fluorescein isothiocyanate-conjugated, Clone 30-F11), CD3e (Peridinin chlorophyll protein-Cychrome 5-conjugated, Clone 145-2C11), CD4 (Pacific Blue-conjugated, Clone RM4-5), CD8a (allophycocyanin-conjugated, Clone 53-6.7), CD69 (phycoerythrin-conjugated, Clone H1.2F3), Ly6G (allophycocyanin-Cy7-conjugated, Clone 1A8), CXCR2 (phycoerythrin-conjugated, Clone # 242216; R&D Systems) or NK1.1 (fluorescein isothiocyanate-conjugated, Clone PK136). All antibodies and isotype controls were purchased from BD Biosciences (Franklin Lakes, NJ, USA), unless otherwise indicated. The frequency of positive cells was analysed using a gate that included lymphocytes, granulocytes and/or monocytes/macrophages, followed by doublets exclusion (singlets) and selection of viable cells (DAPI negative staining). Limits for the quadrant markers were always set based on negative populations and isotype control antibodies. Cells were acquired on a BD FACSCanto II cytometer (BD Biosciences) and analysed using the FlowJo 7.5.3 software (FlowJo, LLC. Ashland, Oregon, USA). Representative flow cytometry gating strategy is shown in the Supplementary material (Figure. S1).

Statistical analysis

Results are shown as means ± SEM. Data values were first analysed for normality using D'Agostino–Pearson and Shapiro–Wilk tests. Differences were compared using one-way analysis of variance (ANOVA) or two-way ANOVA depending on the number of variables considered in the experiment. Analyses were followed by Tukey or Dunn's multiple comparisons tests using the GraphPad Software 5.0 (GraphPad, La Jolla, CA). All data are representative of at least two experiments. $P < 0{\cdot}05$ were considered significant.

Results

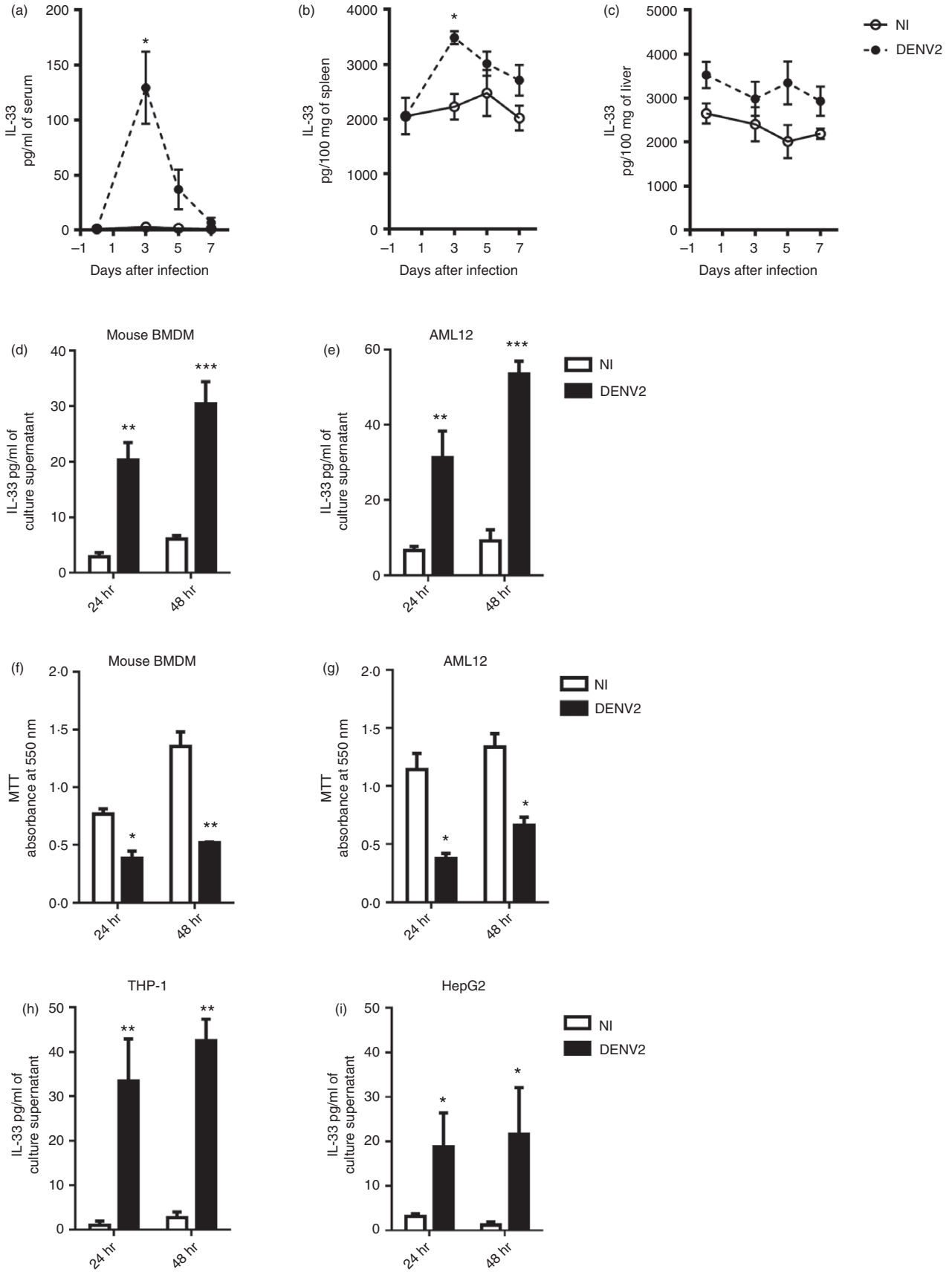
IL-33 is expressed during DENV2 infection *in vivo* and *in vitro*

Our analyses were initiated by studies using the mouse-adapted DENV2 P23085 strain, henceforth referred to as DENV2, to assess the role of the IL-33–ST2 axis in dengue. Previous studies showed that infection with DENV2 led to the development of a severe disease in adult immunocompetent mice that resembles severe dengue in humans.[2,14–18] To determine whether IL-33 was induced by DENV2 infection, WT mice were inoculated with one LD_{50} (200 PFU) of DENV2 and killed at various time-points p.i. Concentrations of IL-33 in the serum, spleen and liver homogenates were measured by ELISA. Interleukin-33 expression in the serum and in the spleen was markedly increased on day 3 and returned to non-infected control levels by day 7 (Fig. 1a,b); levels in the liver remained constant throughout the evaluated time-points, although slightly higher than those found in non-infected controls (Fig. 1c).

DENV2 also induced IL-33 expression *in vitro*. Both BMDM (Fig. 1d) and AML12 cells (Fig. 1e) produced high levels of IL-33 upon infection with DENV2. Cell viability, assessed using the MTT assay, was decreased in DENV2-infected BMDM (Fig. 1f) and AML12 cells (Fig. 1g), suggesting that IL-33 production may be related to cell death, as already reported.[19] We also observed that human monocyte (THP-1) and hepatocyte (HepG2) cell lines produced IL-33 following DENV2 infection using the adapted P23085 strain (Fig. 1h,i).

These results show that IL-33 is expressed during DENV2 infection *in vivo*. Furthermore, IL-33 production was observed systemically and in the mouse organs that are involved in dengue physiopathology, being also corroborated by the expression in *in vitro* cultures of the relevant mouse and human cell types.

Figure 1. Interleukin-33 (IL-33) is expressed during dengue virus serotype 2 (DENV2) infection *in vivo* and *in vitro*. Wild-type (WT) C57BL/6 mice were inoculated with 1 median lethal dose (LD_{50}) [200 plaque-forming units (PFU)] of DENV2 and killed immediately or on days 3, 5 or 7 post-infection (p.i.). IL-33 levels were measured in the serum (a) and homogenates of spleen (b) and liver (c) by ELISA. Mouse bone-marrow-derived macrophages (BMDM) (d) and AML12 cells (e) were infected with DENV2 (0·1 multiplicity of infection) and the concentrations of IL-33 in the culture supernatant was determined by ELISA. BMDM and AML12 cells from the same experiment were also used in an MTT assay for cell viability (f, g). Concentration of IL-33 was also determined in the culture supernatant of human cell lines THP-1 (h) and HepG2 (i) following the same infection protocol. Data are mean ± SEM, representative of at least two independent experiments. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ relative to non-infected (NI) control.


(a)
IL-33 pg/ml of serum
Days after infection
(b)
IL-33 pg/100 mg of spleen
Days after infection
(c)
IL-33 pg/100 mg of liver
Days after infection
NI
DENV2
(d)
Mouse BMDM
IL-33 pg/ml of culture supernatant
24 hr
48 hr
(e)
AML12
IL-33 pg/ml of culture supernatant
24 hr
48 hr
NI
DENV2
(f)
Mouse BMDM
MTT absorbance at 550 nm
24 hr
48 hr
(g)
AML12
MTT absorbance at 550 nm
24 hr
48 hr
NI
DENV2
(h)
THP-1
IL-33 pg/ml of culture supernatant
24 hr
48 hr
(i)
HepG2
IL-33 pg/ml of culture supernatant
24 hr
48 hr
NI
DENV2

Treatment with IL-33 is deleterious while ST2 deficiency attenuates DENV2 infection

We then investigated the role of IL-33 in disease development *in vivo*. Wild-type or ST2$^{-/-}$ mice were inoculated with one LD_{50} (200 PFU) of DENV2 with or without treatment of IL-33 (200 ng, intraperitoneally, on day 0 and 3). Infected WT mice treated with IL-33 (DENV2 + rmIL-33) developed significantly higher mortality rate compared with untreated WT mice (DENV2), In contrast, infected ST2$^{-/-}$ mice (DENV2 ST2$^{-/-}$) had reduced mortality compared with the infected WT mice (Fig. 2a). These results

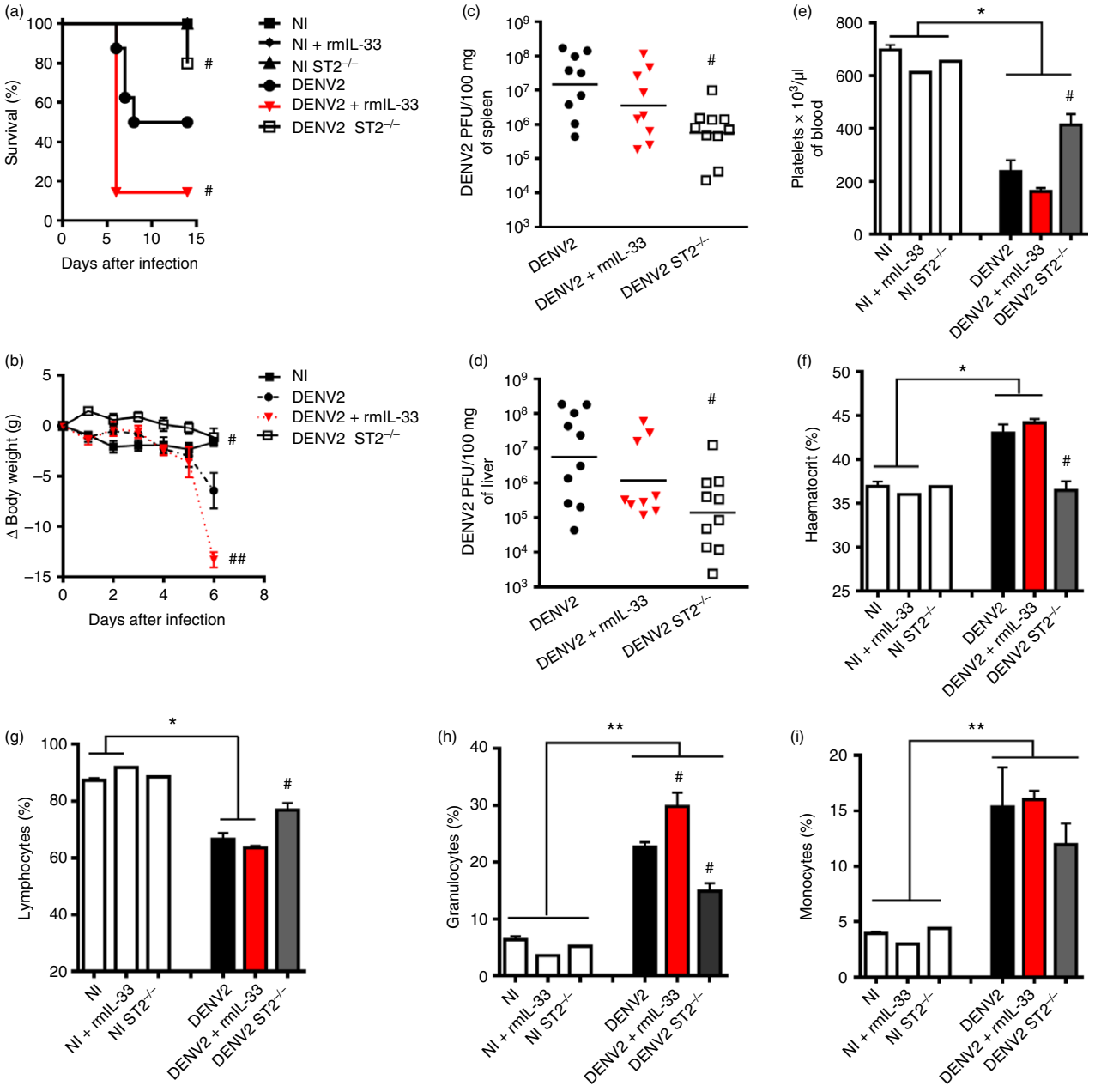


Figure 2. Interleukin-33 (IL-33) treatment is deleterious while ST2 deficiency is protective in dengue virus serotype 2 (DENV2) infection. Untreated wild-type (WT) C57BL/6 (DENV2), recombinant murine (rm) IL-33-treated mice (DENV2 + rmIL-33, 200 ng/mouse) and ST2-deficient mice (DENV2 ST2$^{-/-}$) were inoculated with 1 median lethal dose (LD_{50}) [200 plaque-forming units (PFU)] of DENV2 and monitored for survival (a) and weight loss (b) for 14 days post-infection (p.i.), together with relevant control groups. In parallel experiments, groups of mice were killed on day 6 p.i. Viral loads in the spleen (c) and in the liver (d) were determined. Platelet count (e), haematocrit index (f) and percentages of circulating lymphocytes (g), granulocytes (h) and monocytes (i) in the blood were also determined. Data are mean ± SEM, exception in (a) (percentage of survival) and (c, d), where the geometric mean is presented. Data are representative of at least two independent experiments each. NI, non-infected. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ relative to NI controls. $^{\#}P < 0{\cdot}05$ relative to DENV2 group.

are corroborated by weight measurement. The WT mice receiving IL-33 had markedly increased weight loss on day 6 p.i. compared with untreated WT mice; and ST2$^{-/-}$ mice did not lose weight during the evaluated period (Fig. 2b).

We also determined the DENV2 viral load in the liver and spleen. Mice were inoculated with DENV2 with or without IL-33 and killed on day 6 p.i. Plaque assay shows that infected mice had up to 10^8 DENV2 PFU in the liver

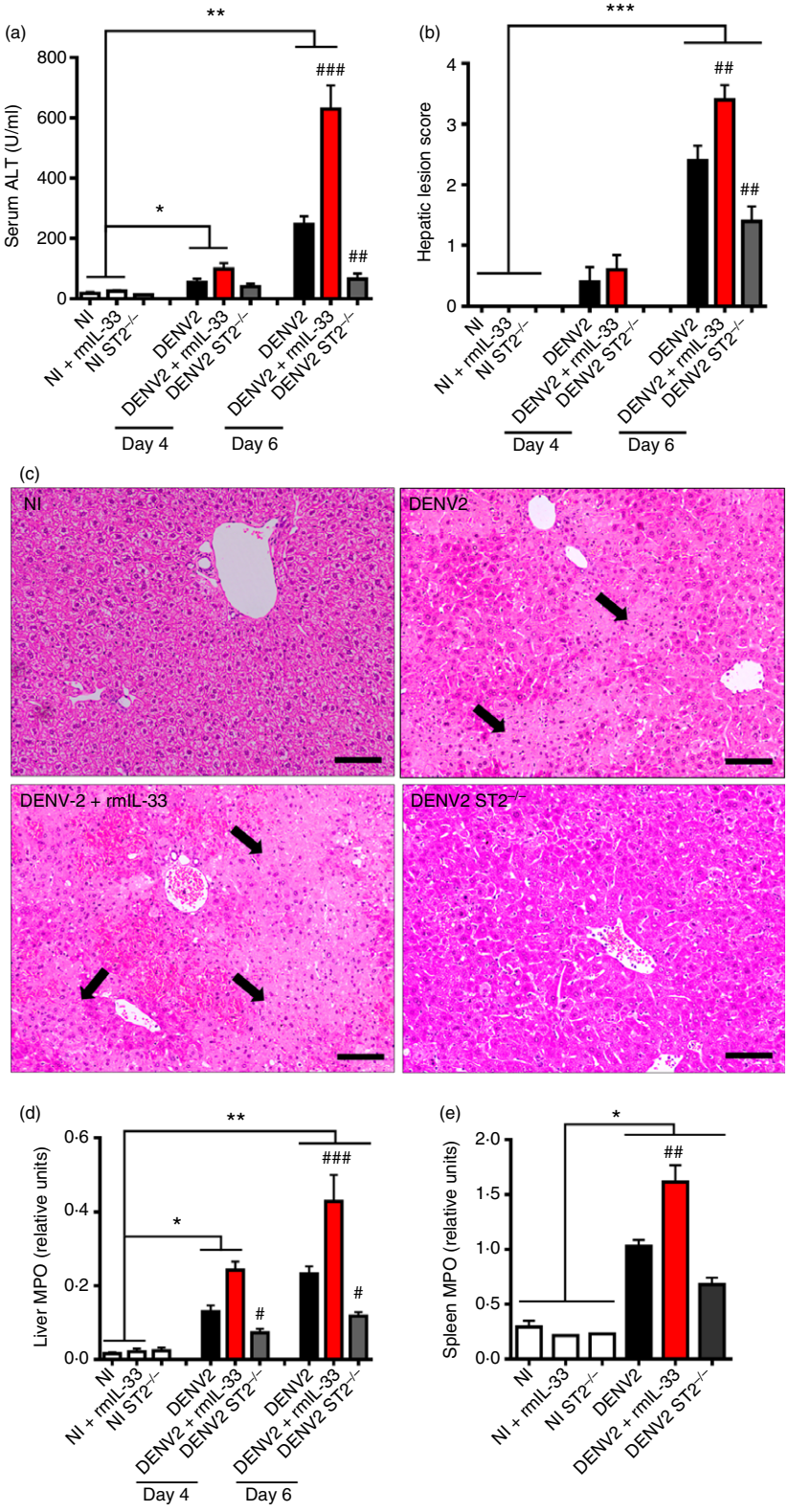


Figure 3. Interleukin-33 (IL-33) treatment increases the severity of dengue virus serotype 2 (DENV2) -induced tissue damage, which is reduced in ST2$^{-/-}$ mice. Untreated wild-type (WT) C57BL/6 (DENV2), recombinant murine (rm) IL-33-treated mice (DENV2 + rmIL-33, 200 ng/mouse) and ST2-deficient mice (DENV2 ST2$^{-/-}$) were inoculated with 1 median lethal dose (LD_{50}) [200 plaque-forming units (PFU)] of DENV2 and killed on days 4 and 6 post-infection (p.i.). Alanine aminotransferase (ALT) levels were measured in serum (a), and liver samples were processed for histological analysis (b,c). A semi-quantitative hepatic lesion score in (b) from the analysis of representative liver haematoxylin & eosin-stained sections (c), at a magnification of 200 × (scale bars = 50 μm). Darker pink areas correspond to healthy tissue whereas pale pink areas (arrows) are necrotic/damaged hepatocytes. Liver samples were also homogenized and processed for measurement of myeloperoxidase (MPO) activity (d, e). Quantitative data are mean ± SEM, representative of at least two independent experiments. NI, non-infected. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ relative to NI controls. $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ relative to DENV2 group.

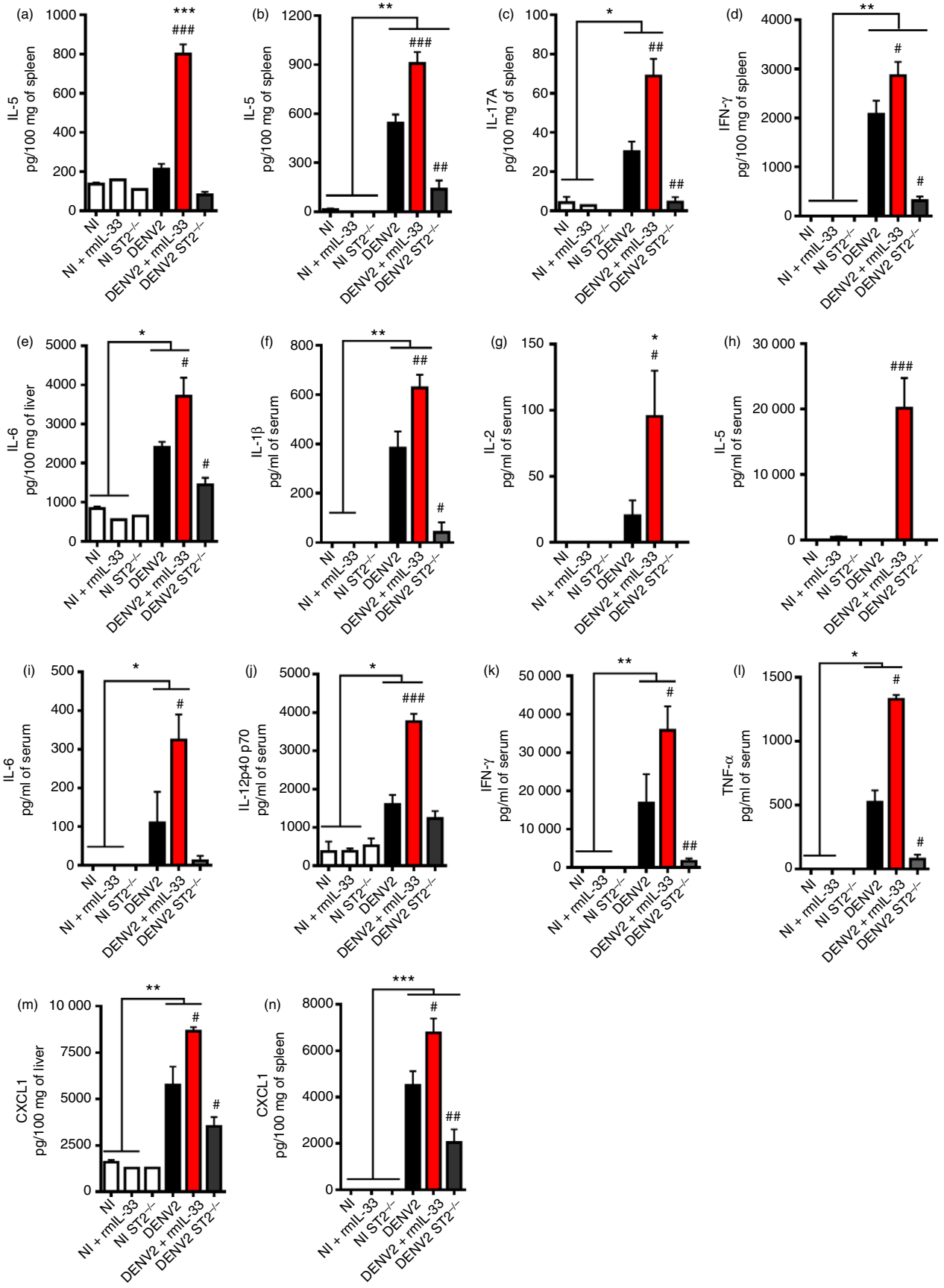


Figure 4. Interleukin-33 (IL-33) treatment increases pro-inflammatory cytokine production *in vivo*. Untreated wild-type (WT) C57BL/6 (dengue virus serotype 2; DENV2), recombinant murine (rm) IL-33-treated mice (DENV2 + rmIL-33, 200 ng/mouse) and ST2-deficient mice (DENV2 $ST2^{-/-}$) were inoculated with 1 median lethal dose (LD_{50}) [200 plaque-forming units (PFU)] of DENV2 and killed on days 4 and 6 post-infection (p.i.). Cytokine and chemokine concentrations were measured in the spleen (a–d, n), liver (e, m) and serum (f–l) by ELISA, on day 4 in sera or on day 6 p.i. in solid organ samples. Data are mean ± SEM, representative of at least two independent experiments. NI, non-infected. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ relative to NI controls. $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ relative to DENV2 group.

and spleen, and that IL-33 treatment did not significantly alter viral load in these organs. In contrast, $ST2^{-/-}$ mice showed markedly reduced DENV2 loads in both the spleen (Fig. 2c) and the liver (Fig. 2d) compared with infected WT mice.

Together, our results indicate that IL-33 treatment exacerbates DENV2-induced mortality and weight loss. Conversely, ST2 deficiency attenuates mortality, weight loss and viral load in the liver and spleen.

IL-33 increases the severity of DENV2-induced haematological alterations and tissue damage

We next evaluated the effects of the IL-33–ST2 axis on the haematological alterations induced by DENV2. Blood samples were collected on day 6 p.i. from WT and $ST2^{-/-}$ mice infected with DENV2 with or without IL-33. The WT mice infected with DENV2 showed marked thrombocytopenia (Fig. 2e) and increased haemoconcentration (Fig. 2f) compared with non-infected controls. Interleukin-33-treated mice had haematological alterations similar to those presented by infected untreated mice. $ST2^{-/-}$ mice had a modest reduction in platelet counts (Fig. 2e) and normal haematocrit indexes compared with uninfected mice (Fig. 2f). Infected WT mice treated or not with IL-33 had a mild reduction in lymphocyte counts, which was not significant in $ST2^{-/-}$ mice (Fig. 2g). Granulocyte counts revealed that all infected groups had increased granulocyte numbers compared with non-infected controls. Treatment with IL-33 further increased the number of granulocytes, whereas ST2 deficiency led to a significant decrease of granulocytes compared with infected untreated mice (Fig. 2h). Monocyte frequency was elevated in all infected groups, but no differences were observed among untreated, IL-33-treated and $ST2^{-/-}$ mice (Fig. 2i).

Infection with DENV2 induces acute tissue damage in mice, which is thought to contribute to disease development. Wild-type mice infected with DENV2 developed markedly increased serum ALT compared with uninfected controls. The serum ALT was further elevated by the treatment with IL-33. In contrast, infected $ST2^{-/-}$ mice developed minimal ALT levels (Fig. 3a). This pattern was noticeable at day 4 p.i. and was obvious by day 6.

DENV2 infection caused extensive liver damage on day 6 p.i., evident by the presence of congestion and necrotic areas (pale, light pink areas) which are absent in the non-infected group. The liver lesion was exacerbated by the treatment with IL-33, whereas the pathology was significantly reduced in the infected $ST2^{-/-}$ mice (Fig. 3b,c). Liver MPO levels, which are indicative of the recruitment of granulocytes to the tissue, were consistent with the results of serum ALT and liver damage. IL-33-treated WT mice presented elevated MPO concentrations, whereas $ST2^{-/-}$ mice had reduced MPO activity compared with infected untreated WT mice on days 4 and 6 p.i. (Fig. 3d). Similar patterns of MPO activity were also found in the spleen. MPO level was increased in the spleens of the DENV2-infected WT mice; and this was further elevated by the treatment with IL-33, while $ST2^{-/-}$ mice presented a similar level of MPO to that in the uninfected control mice (Fig. 3e).

IL-33 increases pro-inflammatory cytokine production *in vivo*

We next investigated the effects of IL-33 on cytokine and chemokine production during DENV2 infection. Both WT and $ST2^{-/-}$ mice were infected with DENV2 with or without IL-33 treatment and killed on day 4 or 6 p.i. Spleen from infected WT mice produced significantly more IL-5, IL-6, IL-17A and IFN-γ than uninfected mice. These levels were furthered increased by the treatment with IL-33 but were markedly reduced in infected $ST2^{-/-}$ mice at day 6 p.i. (Fig. 4a–d). Similar results for IL-6 were obtained from liver (Fig. 4e) and for IL-1β, IL-2, IL-5, IL-6, IL-12, IFN-γ and TNF-α in the serum on day 4 p.i. (Fig. 4f–l).

We found that the chemokine CXCL1 also followed the same pattern as the pro-inflammatory cytokines above. Liver and spleen from infected WT mice produced significantly more CXCL1 than uninfected mice. This level was further increased by the treatment of the WT mice with IL-33, but was markedly reduced in $ST2^{-/-}$ mice (Fig. 4m,n).

IL-33 increases neutrophil migration to the site of infection

As CXCL1 is a key mediator for cell migration, notably in regard to neutrophils, we next investigated the leucocyte populations recruited to the site of infection. Wild-

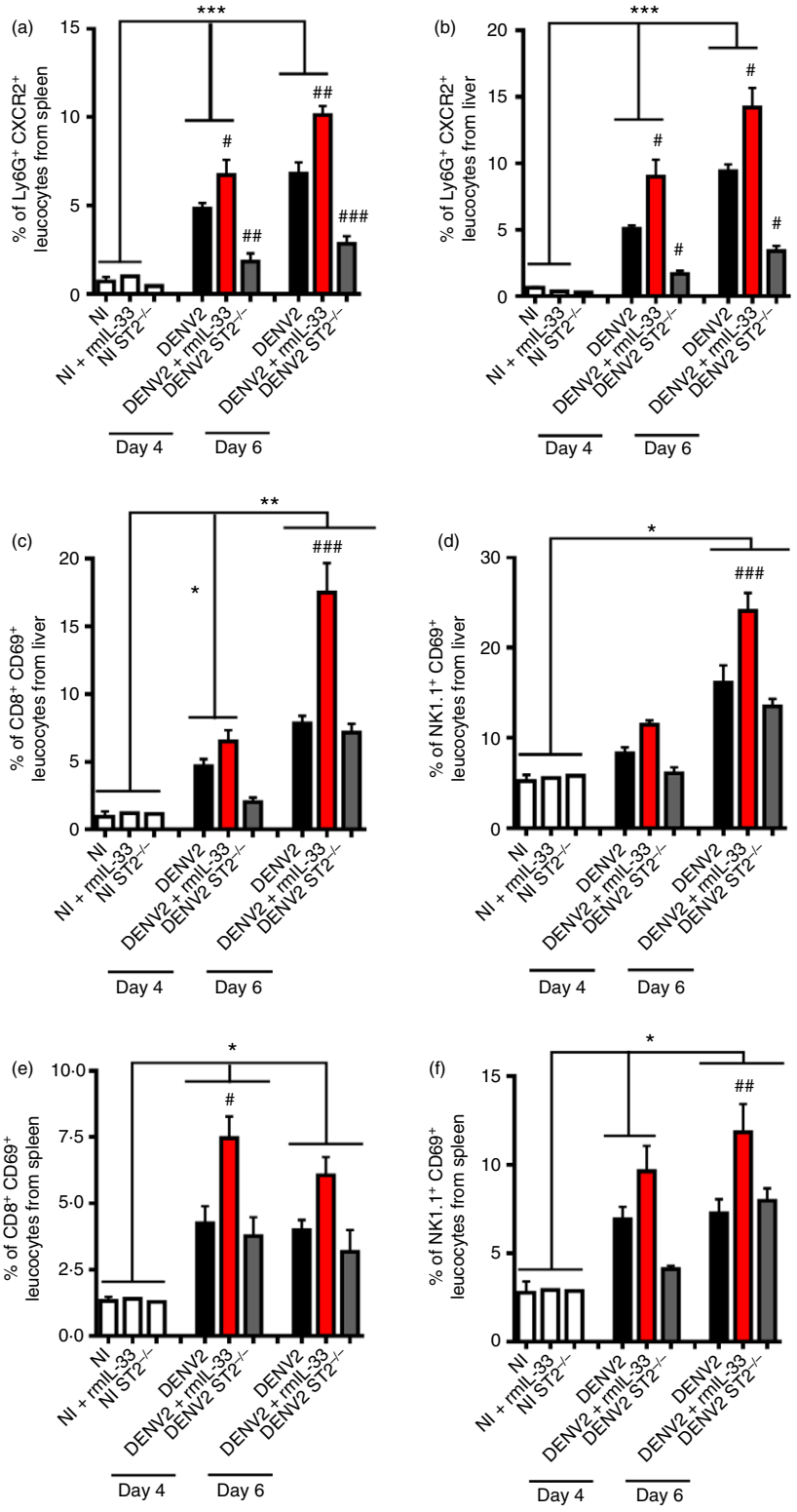


Figure 5. Interleukin-33 (IL-33) increases neutrophil migration to the site of infection. Untreated wild-type (WT) C57BL/6 (dengue virus serotype 2; DENV2), recombinant murine (rm) IL-33-treated (DENV2 + rmIL-33, 200 ng/mouse) and mice treated with both rmIL-33 and DF2156A (DENV2 + rmIL-33 + DF2156A, 10 mg/kg of body weight) were inoculated with 1 median lethal dose (LD_{50}) [200 plaque-forming units (PFU)] of DENV2 and killed on days 4 and 6 p.i. for collection of spleen and liver samples. Leucocyte populations were isolated from organ samples and stained for surface markers of neutrophils (Ly6G, CXCR2) (a, b), activated T CD8 lymphocytes (CD8, CD69) (c, e) or activated natural killer cells (NK1.1, CD69) (d, f). Data are representative of at least two independent experiments. NI, non-infected. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ relative to NI controls. $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ relative to DENV2 group.

type and $ST2^{-/-}$ mice were infected with DENV2 and spleen and liver were harvested on day 4 or 6 p.i. The leucocyte populations were then analysed by FACS. Neutrophil ($LY6G^{+}$ $CXCR2^{+}$) frequency was markedly increased in the spleen and liver of infected WT mice compared with uninfected control mice. This frequency was significantly increased by the treatment with IL-33 and was greatly reduced in the infected $ST2^{-/-}$ mice

(Fig. 5a,b). The frequencies of $CD8^+$ $CD69^+$ and $NK1.1^+$ $CD69^+$ cells in the spleen and liver of WT mice were also significantly increased by DENV2 infection. Treatment with IL-33 further increased the frequencies of activated $CD8^+$ T cells and NK cells in DENV2-infected mice, notably at day 6 p.i. However, there was no difference in the frequencies of these two cell types between the infected WT and $ST2^{-/-}$ mice (Fig. 5c–f). Representative flow cytometry dot plots for the most significant findings at day 6 p.i. are shown in the Supplementary material (Figs S2 and S3).

Hence, IL-33 produced during DENV2 infection appears to mostly influence the recruitment of neutrophils to the site of infection. Factors other than IL-33 could contribute to the movement of other immune cells.

Pharmacological inhibition of neutrophil recruitment prevents the pathogenic effects of IL-33

We next tested the possibility that the detrimental effect of IL-33 in experimental dengue is mediated by neutrophils. Neutrophil recruitment to target tissues is mostly mediated by the chemokine receptor CXCR2, which can be blocked by the compound DF2156A.[20] Infected WT mice were treated with IL-33 alone or with a combination of IL-33 and DF2156A. As observed previously, infected mice treated with IL-33 showed increased mortality and weight loss. Both the mortality and weight loss were significantly reversed by DF2156A (Fig. 6a,b). As expected, DENV2-infected mice showed haematological alterations that were exacerbated by IL-33 treatment. This exacerbation was reversed by DF2156A (Fig. 6c,d). DF2156A treatment also resulted in a significant reduction in the percentage of granulocytes in blood, to below the level of the infected untreated group (Fig. 6e). Importantly, DF2156A reversed the excessive liver damage caused by the IL-33 treatment, restoring serum ALT to levels observed in untreated infected mice (Fig. 6f). Furthermore, DF2156A reversed the excessive increase in liver MPO activity caused by IL-33 treatment (Fig. 6g). Levels of pro-inflammatory mediators, such as neutrophil-chemoattractant chemokines CXCL1, CXCL2 and CXCL6, and cytokines IL-6 and IL-17A, which were elevated in the livers of infected mice and further increased by IL-33 treatment, were also reversed by DF2156A (Fig. 6h–l). Evidence from other organ systems implicates not only CXC chemokines but also IL-17A as important activators of innate immune mechanisms, including the recruitment and survival of neutrophils.[21,22]

Together our results demonstrate that IL-33 exacerbates experimental DENV infection through the production of pro-inflammatory cytokines and chemokines, which increase the recruitment of neutrophils to the site of infection.

Discussion

The immune response to DENV infection is complex.[8,23–25] We attempted to dissect the potential regulation of this immunopathology using a murine experimental model, which, though not ideal, allows studies in immunocompetent hosts.[14,18,26] Our results show that among the many cytokines that are associated with dengue infection,[27,28] IL-33 acts as a disease-deleterious agent, the absence of whose action led to a significant attenuation of the disease in term of immunopathology and mortality. Furthermore, we reveal the mechanism by which IL-33 manifests its disease-exacerbating effect via the induction of CXC chemokines (e.g. CXCL1) in the target organs, liver and spleen, resulting in the recruitment of neutrophils into these organs. The neutrophils would cause the damage and failure of these organs via a cocktail of pro-inflammatory cytokines, including IL-1β, IL-2, IL-5, IL-6, IL-17A, IFN-γ and TNF-α, some of which are documented here. However, whether neutrophils would be the sole source of these cytokines is yet to be determined.

Given the complexity of the disease, it is most likely that several other cell types are also involved in the pathogenesis of dengue infection. We show that although IL-33 increased the frequency of $CD8^+$ $CD69^+$ T cells and $NK1.1^+$ $CD69^+$ cells in DENV2-infected mice, the absence of ST2 did not affect DENV2 alone-induced increase of these cell types. These results therefore suggest that, apart from IL-33, other factors induced by DENV2 could come into play, leading to liver and spleen damage, albeit at an attenuated level.[15,18,26] This notion is also borne out in our results using the CXCR2 inhibitor DF2156A, which significantly but not completely reversed IL-33-mediated mortality and weight loss in the DENV2-infected mice. It is noteworthy that this partial reversal was in the face of complete inhibition of the haematological alterations, cytokine production and granulocyte recruitment. Previous studies have shown that IL-33 directly increases CD69 expression in $CD8^+$ T cells and NK cells, and enhances IFN-γ production by NK cells.[29,30] Moreover, the IL-33–ST2 signalling axis in NK cells is important to the host response during certain viral infections.[31,32] Hence, cell types other than the neutrophils, such as $CD8^+$ T cells and NK cells, some of which were induced by IL-33, could also cause damage to the liver and spleen and hence the mortality of the mice.[16,17]

With regard to the kinetics between intrahepatic and intrasplenic infiltrating leucocytes, it is important to highlight that the frequencies of activated $CD8^+$ T cells and NK cells are already significantly increased in the spleen of DENV2-infected mice at day 4 p.i., remaining at the same level until day 6 p.i. These findings may not only reflect activation of $CD8^+$ T cells and NK cells within the

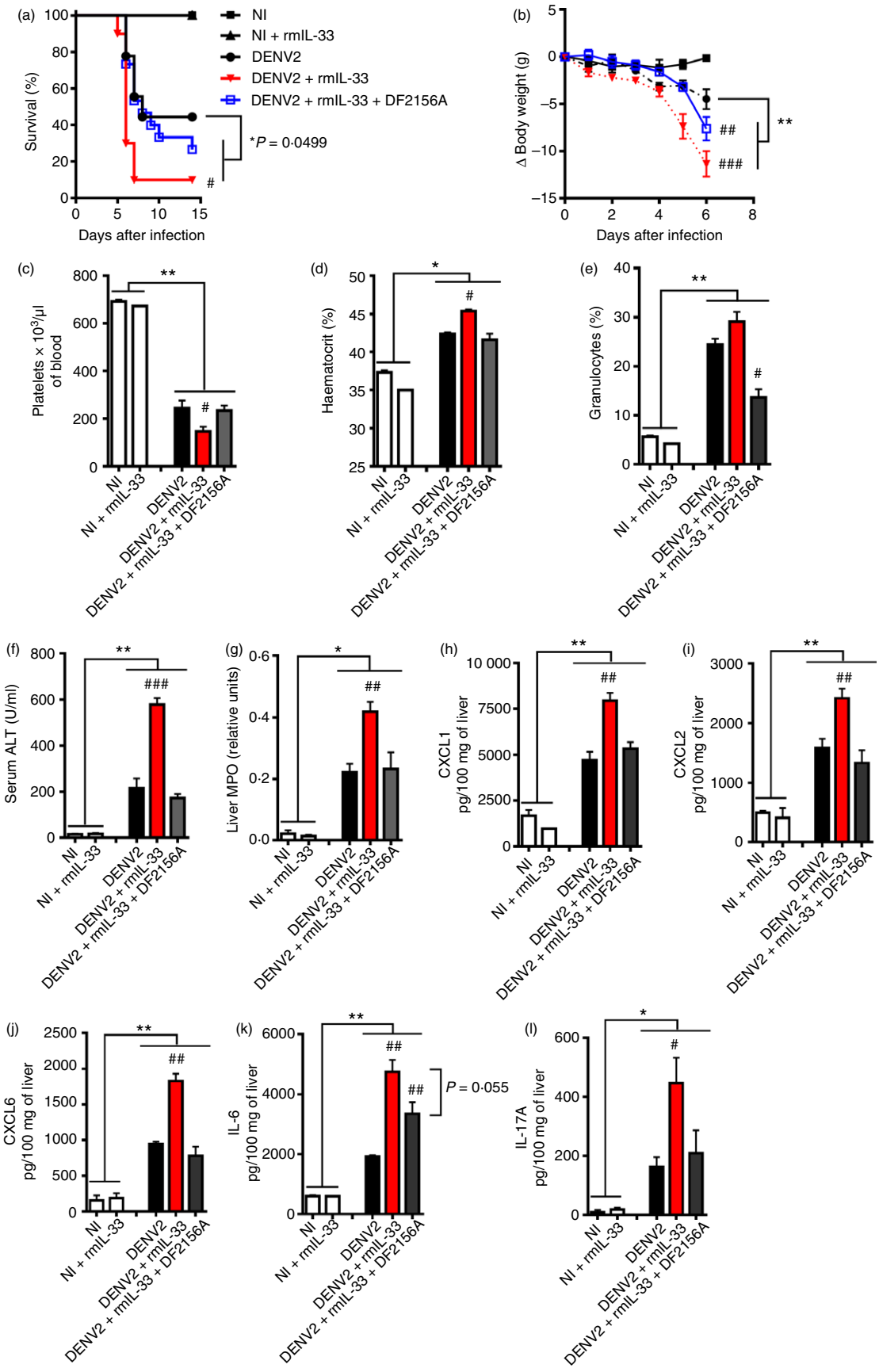


Figure 6. Pharmacological inhibition of neutrophil recruitments prevents the pathogenic effects of interleukin-33 (IL-33). Untreated wild-type (WT) C57BL/6 (dengue virus serotype 2; DENV2), recombinant murine (rm) IL-33-treated (DENV2 + rmIL-33, 200 ng/mouse) mice and mice treated with both rmIL-33 and DF2156A (DENV2 + rmIL-33 + DF2156A, 10 mg/kg of body weight) were inoculated with 1 median lethal dose (LD_{50}) [200 plaque-forming units (PFU)] of DENV2 and monitored for survival (a) and weight loss (b) for 14 days post-infection (p.i.). In parallel experiments, groups of mice were killed on day 6 p.i. for evaluation of blood parameters: platelet count (c), haematocrit index (d) and percentages of circulating granulocytes (e). Serum samples were used for assessment of alanine aminotransferase (ALT) levels (f). Liver samples were processed and assessed for myeloperoxidase (MPO) activity (g), and the concentrations of CXCL1 (h), CXCL2 (i), CXCL6 (j), IL-6 (k) and IL-17A (l) were assessed by ELISA. Data are mean ± SEM, exception (a), where percentage of survival is presented. Data are representative of at least two independent experiments. NI, non-infected. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ relative to NI controls. $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ relative to DENV2 group.

spleen but also in the bloodstream. Indeed, only at day 6 p.i. we observed a marked increase in the frequencies of these populations in the liver, notably in animals receiving rmIL-33, suggesting a delayed recruitment of these leucocytes to this organ. We believe that dengue-induced chemokines and other yet to be identified inflammatory mediators at early time-points (from day 3 p.i.) might participate in the recruitment of $CD8^+$ T cells and NK cells to the liver at later time-points p.i., which would lead to increased tissue damage. Although not evaluated here, the intensity of these phenomena might greatly rely on the amount of virus inoculum, which would drive the magnitude and kinetics of leucocyte recruitment from the bloodstream to the liver.

It is therefore plausible to suggest that IL-33 acts relatively early during the infection. This is consistent with the accepted concept that IL-33 is an important alarmin.[33,34] Our analysis was carried out on days 4 and 6 after infection. The effect of the $CD8^+$ T cells and NK cells could manifest in an even more pronounced fashion at a later phase of the infection[35,36], a hypothesis that merits to be investigated using a less severe model of DENV2 infection. Nevertheless, the limited administration of the CXCR2 inhibitor at an early stage of infection (day 0 and 3) was markedly effective in attenuating the disease pathology by reducing organ damage, mortality and weight loss. These data therefore suggest that blocking IL-33 or CXCR2 could be a potential therapeutic option in managing DENV infections.

Interleukin-33 is mainly associated with Th2 responses and is frequently associated with diseases such as asthma and allergies.[34,37] Hence, the deleterious effect of IL-33 in dengue infection whose pathology is chiefly mediated by Th1-type of response is counter-intuitive. However, IL-33 can also be a major driver of neutrophil migration[38] and the localization of these pro-inflammatory cells could be a key factor for the deleterious effect of IL-33 in dengue infection.

Earlier reports had indicated an association of the IL-33–ST2 axis with clinical dengue infections. Soluble ST2 (sST2, a spliced-variant of ST2) was associated with severe forms of paediatric[39] and adult[40] dengue infections. Furthermore, it seems that the production of the elevated levels of sST2 was the result of TNF-α stimulation.[40] However, the role of IL-33–ST2 signalling in dengue infection was unknown. Our proof-of-concept study reported here suggests that IL-33 could play an important deleterious role in clinical dengue infection and that blocking IL-33/ST2 signalling or its downstream events such as CXCR2 may be potentially therapeutic. Furthermore, the participation of IL-33 in dengue infection is suggestive that IL-33 may also play an important role in diseases caused by related flaviviruses such as yellow fever and zika viruses.

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Author contributions

RG and FYL designed the study. REM, AGB, IM, CTF and RG performed the experiments and analysed data. REM, CTF, DGS, BR, MMT, FYL and RG contributed to results analysis and discussion. BR and MMT provided reagents and animals. REM, FYL and RG wrote the paper.

Disclosure

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article

Figure S1. Representative flow cytometry gating strategy used for leucocyte phenotyping in spleen and liver samples.

Figure S2. Representative flow cytometry dot plots for phenotyping of neutrophils and CD8$^+$ T cells in spleen and liver samples at day 6 post-infection.

Figure S3. Representative flow cytometry dot plots for natural killer cells phenotyping in spleen and liver samples at day 6 post-infection.

