

IL-33 is a crucial amplifier of innate rather than acquired immunity

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IL-33, a member of the IL-1-related cytokines, is considered to be a proallergic cytokine that is especially involved in Th2-type immune responses. Moreover, like IL-1 α , IL-33 has been suggested to act as an “alarmin” that amplifies immune responses during tissue injury. In contrast to IL-1, however, the precise roles of IL-33 in those settings are poorly understood. Using IL-1- and IL-33-deficient mice, we found that IL-1, but not IL-33, played a substantial role in induction of T cell-mediated type IV hypersensitivity such as contact and delayed-type hypersensitivity and autoimmune diseases such as experimental autoimmune encephalomyelitis. Most notably, however, IL-33 was important for innate-type mucosal immunity in the lungs and gut. That is, IL-33 was essential for manifestation of T cell-independent protease allergen-induced airway inflammation as well as OVA-induced allergic topical airway inflammation, without affecting acquisition of antigen-specific memory T cells. IL-33 was significantly involved in the development of dextran-induced colitis accompanied by T cell-independent epithelial cell damage, but not in streptozocin-induced diabetes or Con A-induced hepatitis characterized by T cell-mediated apoptotic tissue destruction. In addition, IL-33-deficient mice showed a substantially diminished LPS-induced systemic inflammatory response. These observations indicate that IL-33 is a crucial amplifier of mucosal and systemic innate, rather than acquired, immune responses.

asthma | colitis | cytokine | interleukin-33 | sepsis

IL-33, which is a member of the IL-1 family of cytokines that includes IL-1 and IL-18, induces Th2-type immune responses such as eosinophil-rich inflammation in the intestines and lungs associated with elevated numbers of blood eosinophils and increases in serum concentrations of IgE, IgA, IL-5, and IL-13 in mice, dependent on IL-13 (1). IL-33 is important for Th2 cytokine-associated host defense against nematode infection through signals from IL-33 receptor (IL-33R), which consists of ST2 (also called T1, DER-4, Fit-1, or IL-1RL1) and IL-1R accessory protein (IL-1RAcP) (2, 3). As a Th2-inducing cytokine, IL-33 is considered to be involved in the development of allergic diseases such as atopic asthma (2, 3). IL-33 is also known to ameliorate the development of Th1 cytokine-associated atherosclerosis in apolipoprotein E-deficient mice (4) and accelerate Th17 cell-mediated murine arthritis (5). Thus, it is now thought that IL-33 acts not only as a Th2-inducing cytokine, but also as a proinflammatory cytokine, like IL-1 and IL-18, in various immune responses. Using ST2-deficient (ST2^{-/-}) mice and mice treated with anti-ST2 Ab or soluble ST2-Fc-fusion proteins, however, it was reported that the roles of ST2 were not identical in certain immune responses such as allergic airway inflammation (6–8) and LPS-induced endotoxin shock (9, 10). Therefore, we generated IL-33^{-/-} mice to comprehensively examine the roles of IL-33 in the following disease models: allergic airway inflammation; LPS-induced endotoxin shock, contact, and delayed-type hypersensitivity; experimental autoimmune encephalomyelitis; Con A-induced hepatitis; streptozocin-induced diabetes; and T cell-independent dextran sodium sulfate-induced colitis.

Results and Discussion

IL-33 in Allergic Airway Inflammation. Allergic airway inflammation induced by OVA is a well-established mouse model of human asthma. IgE, mast cells, and IL-1 are responsible for the development of airway inflammation in mice sensitized with OVA in the absence of alum (IgE-dependent protocol), but they are not essential for that event in the presence of alum (IgE-independent protocol) (11). At present, the role of IL-33/ST2 in allergic airway inflammation using ST2^{-/-} mice remains controversial (6–8). That is, OVA-induced airway inflammation developed normally in ST2^{-/-} mice that had been sensitized twice with OVA emulsified in alum (6–8), whereas it was attenuated in ST2^{-/-} mice that had been sensitized once with OVA emulsified in alum (8) and exacerbated in wild-type or Rag-1^{-/-} mice that had undergone adoptive transfer of ST2^{-/-} DO11.10 Th2 cells (7).

During airway inflammation induced by two sensitizations with OVA emulsified in alum, IL-33^{-/-} mice, which were newly generated (Fig. S1), showed attenuated eosinophil influx into the bronchoalveolar lavage (BAL) fluid, airway hyperresponsiveness, and pulmonary inflammation (Fig. 1A–C). The number of lymphocytes in BAL fluids was also reduced in IL-33^{-/-} mice compared with IL-33^{+/+} mice, although their proportions of CD3⁺CD4⁺ST2⁺ cells were comparable (IL-33^{+/+}: 2.2 $\pm$ 0.5%, n = 9 and IL-33^{-/-}: 2.0 $\pm$ 0.4%, n = 9) after the last OVA challenge. In contrast, the IL-4 and IL-5 levels in the BAL fluid and serum OVA-specific IgE production were only slightly (i.e., not significantly) reduced in IL-33^{-/-} mice after the last OVA challenge (Fig. S2A and B). Interestingly, IL-33 deficiency showed no effect on OVA-specific spleen cell proliferation or cytokine secretion (Fig. S2C).

We also found that IL-33 deficiency significantly diminished inflammatory cell influx into the BAL fluid during airway inflammation induced by an extract derived from house dust mites (HDM) (Fig. 24). HDM is a major source of allergens in allergic patients and can provoke allergic airway inflammation resembling human asthma in mice by facilitating barrier disruption, inflammation, and allergen sensitization of the airways through TLR4-dependent innate and acquired immunity (12–14).

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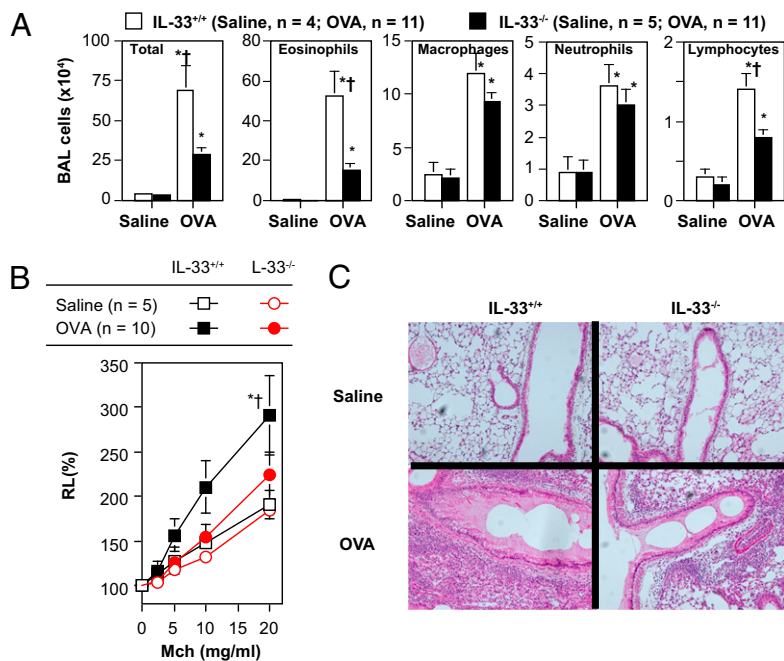


Fig. 1. IL-33 is required for development of an IgE/mast cell-independent OVA-induced allergic airway hypersensitivity response. Mice were sensitized twice with OVA emulsified in alum on days 0 and 14. The mice were challenged intranasally with OVA or saline alone on days 28, 29, and 30. The number of cells in BAL fluids (A), airway hypersensitivity to methacholine (Mch) (B), and lung sections stained with hematoxylin-eosin (C) (100 $\times$; representative data from three to five mice are shown) at 24 h after the last OVA or PBS inhalation. Data show the mean $\pm$ SE: * P < 0.05 vs. corresponding values for saline-treated mice; $^{\dagger}P$ < 0.05 vs. OVA-treated IL-33^{-/-} mice.

These findings suggest that IL-33 is important for inducing antigen-dependent Th2-associated local airway inflammation but is mostly dispensable for antigen-specific Th2 cell differentiation.

Indeed, IL-33 can directly enhance eosinophil activation in vitro (15) and induces airway inflammation without Th2 cell activation dependent on IL-13 (16). In support of this notion, IL-33 induced

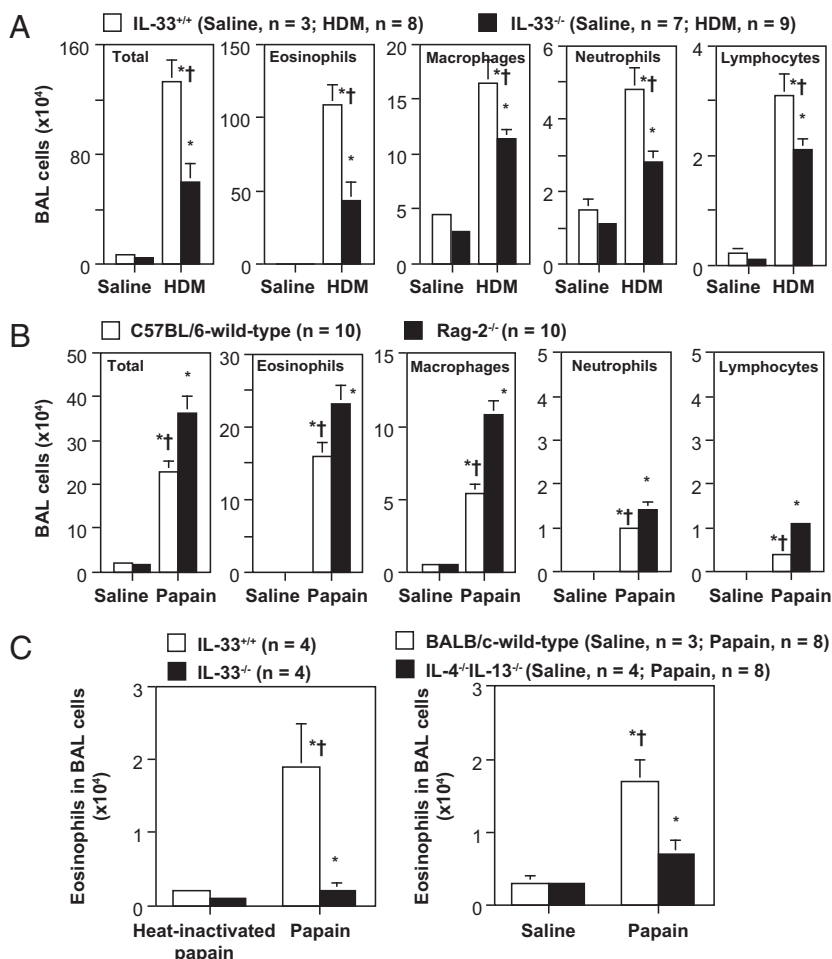


Fig. 2. IL-33 is essential for the development of innate-type airway inflammation. The number of cells in BAL fluids during airway inflammation induced by HDM in IL-33^{+/+} and IL-33^{-/-} mice (A) and induced by papain in C57BL/6-wild-type and Rag-2^{-/-} mice (B), IL-33^{+/+} and IL-33^{-/-} mice (C, Left), and BALB/c-wild-type and IL-4^{-/-}IL-13^{-/-} mice (C, Right) at 24 h after the last OVA, HDM, papain, heat-inactivated papain, or saline inhalation. Data show the mean $\pm$ SE: * P < 0.05 vs. corresponding values for saline- or heat-inactivated papain-treated mice; $^{\dagger}P$ < 0.05 vs. HDM- or papain-treated gene-deficient mice.

airway eosinophilia even in T/B cell-deficient Rag-2^{-/-} mice (Fig. S3), indicating that acquired immune cells are not essential for the setting. Likewise, we found that inhalation of a protease allergen, papain, which is regarded as a cause of occupational asthma (17), induced strong airway eosinophilia in naïve Rag-2^{-/-} mice and naïve wild-type mice (i.e., without prior immunization with papain) (Fig. 2B), although papain-induced airway inflammation has been considered to be mediated by Th2 cells when mice were first sensitized with papain (18).

Although IL-33 proteins were constitutively expressed in the lungs of naïve mice (Fig. S1C), papain inhalation resulted in increased IL-33 mRNA expression in the lungs of wild-type mice but not Rag-2^{-/-} mice (Fig. S4A). The increased papain-induced inflammation seen in Rag-2^{-/-} mice may be caused by the lack of Treg cells, as described (19). T/B cell-independent papain-induced innate-type airway inflammation developed in both C57BL/6 and BALB/c mouse strains, although the BALB/c strain was less sensitive to both papain and IL-33 than the C57BL/6 strain (Fig. S4C and D). Interestingly, papain-induced airway eosinophilia was profoundly impaired in IL-33^{-/-} mice and IL-4^{-/-}IL-13^{-/-} mice (Fig. 2C), suggesting that IL-4 and/or IL-13 derived from IL-33-stimulated innate cells (i.e., Siglec-F⁺ cells, but not Gr1⁺, c-Kit⁺, FcεRIα⁺, DX5⁺, or CD11c⁺ cells; Fig. S5), but not T cell-derived IL-4 and/or IL-13, is important for the event. These findings indicate that IL-33 is crucial for induction of innate-type allergic airway inflammation, whereas it is mostly indispensable for papain-induced airway eosinophilia.

IL-33 in Type IV Hypersensitivity. Type IV hypersensitivity responses such as contact hypersensitivity (CHS) developed normally in TLR4^{-/-} mice (20) but were abolished in Rag-2^{-/-} mice (Fig. S6A), indicating that acquired, rather than innate, immune cells are an important effector for the induction of CHS. Moreover, it is thought that Th2 cytokines are involved in the pathogenesis of

CHS (11), suggesting a contribution of IL-33 to the induction of CHS. After FITC and 2,4-dinitrofluorobenzene challenge, ear swelling was also normally induced in IL-33^{-/-} mice but attenuated in IL-1α/β^{-/-} mice (Fig. S6B and C). IL-33^{-/-} mice also showed normal responses in terms of the levels of skin DC migration and FITC-specific proliferation, IL-4 production by LN cells after FITC sensitization, the degree of skin inflammation, myeloperoxidase (MPO) activity, eosinophil peroxidase (EPO) activity, and serum FITC-specific IgG1 and IgG2a levels after FITC challenge (Fig. S7A–E).

Moreover, methylated BSA (mBSA)-induced delayed-type hypersensitivity (DTH), which is another type IV hypersensitivity that is mediated by Th17 cells (21) and suppressed by Th1 cells (22), was also normally induced in IL-33^{-/-} mice but impaired in IL-1α/β^{-/-} mice (Fig. S8A). IL-33^{-/-} mice also showed normal levels of Ag-specific T cell responses, a normal Th17/Treg proportion after Ag sensitization and normal serum levels of mBSA-specific IgG1 and IgG2a after Ag challenge (Fig. S8B–D). Therefore, IL-33 seems to be unnecessary for the development of these T cell-dependent acquired immune responses.

IL-33 in Autoimmunity. ST2 was shown to contribute to the development of a Th17 cell-mediated autoimmune disease, collagen-induced arthritis (5). However, myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), which is another Th17 cell-mediated autoimmune disease, developed normally in IL-33^{-/-} mice but was attenuated in IL-1α/β^{-/-} mice (Fig. S9A). The levels of MOG-specific LN cell proliferation and IL-17 and IFN-γ production, the proportions of Th17 and Treg cells among LN cells after MOG sensitization, and the levels of MOG-specific IgG1 and IgG2a in sera after MOG challenge were comparable between IL-33^{-/-} and IL-33^{+/+} mice (Fig. S9B–D). These observations indicate that IL-33 is not essential for the pathogenesis of this Th17 cell-mediated EAE.

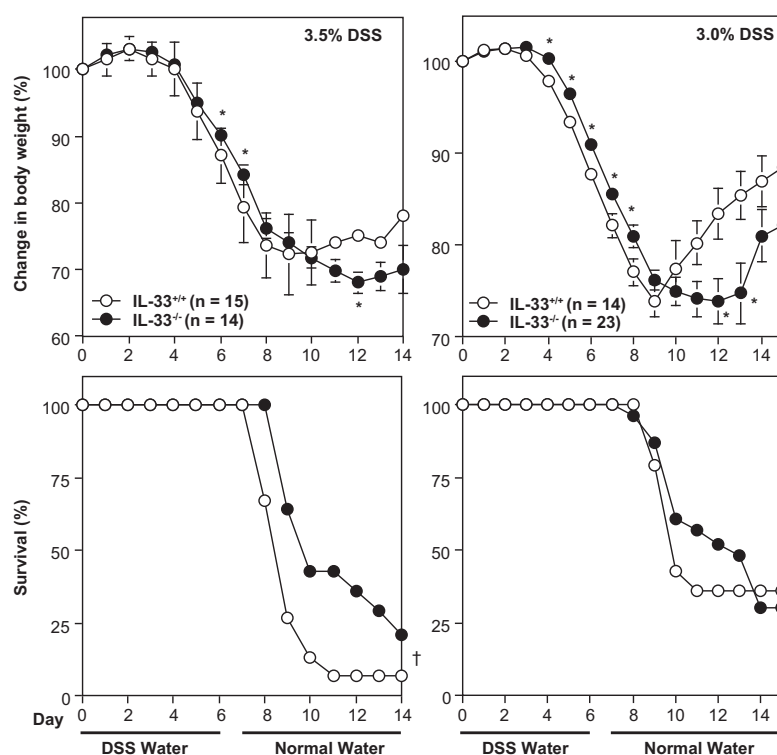


Fig. 3. IL-33 is crucial for T/NKT cell-independent DSS-induced colitis. Mice were provided sterile water containing 3.5% or 3.0% DSS ad libitum for 7 d, followed by 14–15 d of regular drinking water. The change in body weight and survival during 3.5% or 3.0% DSS-induced colitis are compared. Data show the mean $\pm$ SE: * and † $P < 0.05$ vs. corresponding values for IL-33^{+/+} mice.

IL-33 in Tissue Injury. Like pro-IL-1 α , pro-IL-33, but not caspase-cleaved IL-33 (23–25), has biological activity and is suggested to act as an alarmin, an endogenous danger signal that alerts immune cells, in necrosis-, rather than apoptosis-, associated tissue injury during trauma and/or infection (23, 24). Streptozocin-induced diabetes and Con A-induced hepatitis are well-established models of apoptotic tissue injury via TRAIL and Fas on T cells and/or NKT cells (26, 27). Hyperglycemia during streptozocin-induced diabetes and the serum levels of GOT and GPT activities during Con A-induced hepatitis were comparable between IL-33^{+/+} and IL-33^{-/-} mice (Fig. S10), again indicating that IL-33 is not essential for the development of T/NKT cell-mediated tissue injury during these acquired immune responses.

Development of dextran sodium sulfate (DSS)-induced colitis is known to be triggered by enterobacteria via TLR (28), independently of T cells, NK cells, NKT cells, and mast cells (29–31). We found that IL-33^{-/-} mice showed higher viability than IL-33^{+/+} mice until approximately day 12 during 3.5% and 3.0% DSS-induced colitis, whereas the final viability on days 14 and 15 was nearly the same (Fig. 3). After treatment with 3.0% DSS, body weight loss, inflammation, and decreased MPO activity in the colon were significantly more pronounced in IL-33^{-/-} mice compared with IL-33^{+/+} mice until day 8 (Fig. 4 A–C). These observations suggest that IL-33 is important for induction of local inflammation at the onset of DSS-induced colitis. However, body weight recovery was markedly delayed in IL-33^{-/-} mice compared with IL-33^{+/+} mice after changing from drinking water containing 3.0% DSS to plain drinking water (Fig. 3). On day 15, the lesion was almost recovered but was still observed in a small proportion of the colons of both IL-33^{+/+} and IL-33^{-/-} mice (Fig. 4A), and the severity of tissue damage in the colon lesions was similar in IL-33^{+/+} and IL-33^{-/-} mice (Fig. 4B). On day 15, the MPO activity in the homogenates of whole colons from both strains returned to the level seen in naïve mice (Fig. 4C). The levels of IL-33 and proinflammatory mediators (i.e., IL-1 β , TNF, KC, and MIP-2) that are involved in neutrophilia were similarly increased in the colon of both IL-33^{+/+} and IL-33^{-/-} mice compared with naïve mice on day 8 after DSS treatment (Fig. S11). The expression levels of IL-1 β , KC, and MIP-2 in the colon of IL-33^{+/+} mice on day 15 were higher than on day 8 (Fig. S11A), whereas the expression levels of IL-33 and TNF were reduced on day 15 (Fig. S11). In the setting, KC and MIP-2 expression was significantly decreased in the colon of IL-33^{-/-} mice on day 15 after DSS treatment (Fig. S11). Normal expression of potent proinflammatory cytokines such as TNF and IL-1 β may induce significant inflammation in IL-33^{-/-} mice during DSS-induced colitis. However, these observations suggest that IL-33 deficiency leads to delayed local inflammation by reducing neutrophil-chemoattractant factors, resulting in delayed resolution of tissue damage during DSS-induced “innate” colitis.

IL-33 in Endotoxin Shock. The LPS-induced systemic inflammatory response is characterized by dysfunction of multiple organs, including the liver and lung. LPS-stimulated macrophages release IL-33 (32), and IL-33 enhances IL-6 (Fig. S12A) and TNF production (33) by LPS-stimulated macrophages, suggesting involvement of IL-33 in the setting. Indeed, IL-33^{-/-} mice were resistant to endotoxin shock in comparison with IL-33^{+/+} mice after LPS injection (Fig. 5A). We also found that production of IL-6, IL-1 α , and IL-1 β , but not TNF, by thioglycolate-induced peritoneal macrophages of IL-33^{-/-} mice was reduced compared with IL-33^{+/+} mice at 9 and/or 48 h after LPS stimulation (Fig. 5B). LPS-mediated IL-6 and TNF production by macrophages is known to be differentially regulated by nuclear I κ B proteins such as I κ BNS (34) and I κ B ζ (35), which was originally identified as an IL-1-, but not TNF-, inducible nuclear protein (36, 37). Therefore, like IL-1, IL-33 may be involved in host defense against bacteria and in innate inflammatory responses triggered

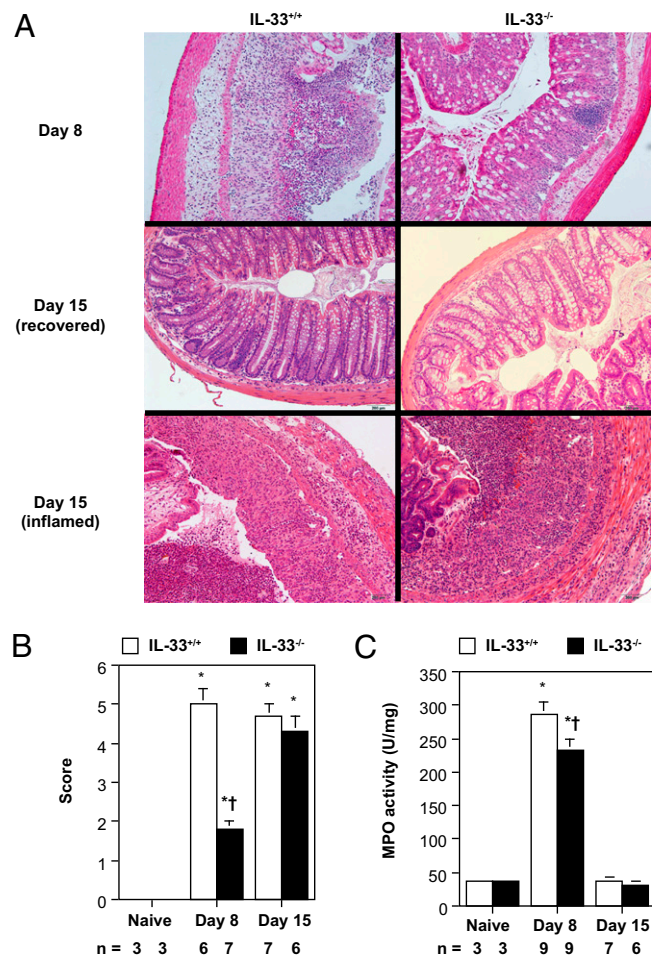


Fig. 4. IL-33 is involved in the induction of inflammation during DSS-induced colitis. Colon sections (A) (hematoxylin-eosin, 100 $\times$. Representative data from three to five mice are shown), score of the severity of colitis (B) (only inflamed sites were evaluated on day 15), and the levels of MPO activity (C) in colon homogenates from IL-33^{+/+} and IL-33^{-/-} mice on day 8 or 15 during 3.0% DSS-induced colitis or in naïve mice. Data show the mean $\pm$ SE: * P < 0.05 vs. corresponding values for naïve mice; † P < 0.05 vs. corresponding values for IL-33^{+/+} mice.

by bacteria by inducing production of IL-6 and IL-1, rather than TNF, by LPS-stimulated macrophages via distinct nuclear protein activation.

Unlike endotoxin shock induced by a single LPS injection (Fig. 5A), the susceptibility to a lethal dose of LPS was comparable in IL-33^{+/+} and IL-33^{-/-} mice that had been injected once with a low dose of LPS (Fig. S12C) or had been made tolerant to LPS by repeated injection of a low dose of LPS (Fig. S12D). These observations suggest that IL-33 is important for acute responses, rather than secondary responses and tolerance, to LPS.

However, as in the case of OVA-induced airway inflammation (6–8), the contribution of ST2 to that response is controversial. Production of each of IL-6, IL-12, and TNF by LPS-stimulated macrophages was suppressed by blockade of ST2 signals using soluble ST2-Fc fusion proteins, and BALB/c mice treated with soluble ST2-Fc fusion proteins were resistant to LPS-induced endotoxemia (9). Conversely, production of these cytokines by LPS-stimulated BALB/c-ST2^{-/-} macrophages was increased, and BALB/c-ST2^{-/-} mice were highly susceptible to LPS-induced endotoxemia (10). On the other hand, it was reported that LPS-induced TNF production was normal in BALB/c-ST2^{-/-} macrophages (33). Thus, in contrast to treatment with soluble ST2-

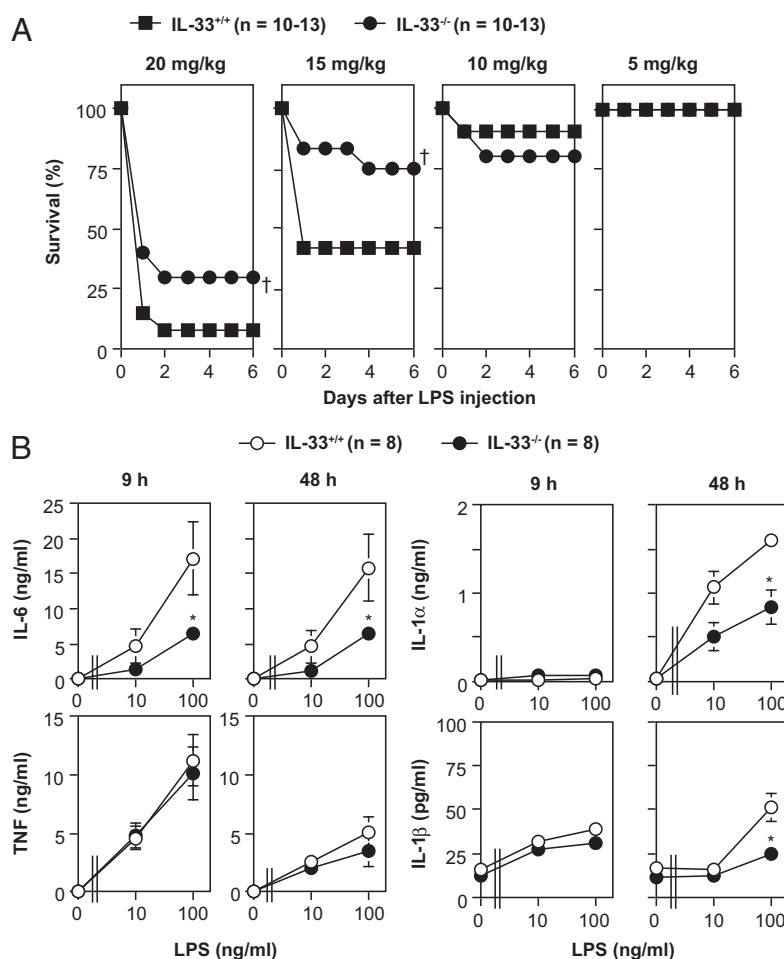


Fig. 5. IL-33 is important for LPS-induced endotoxin shock. (A) Mice were injected intraperitoneally with LPS (5–20 mg/kg; $n = 10$ –13), and survival was monitored. (B) Thioglycolate-induced peritoneal macrophages were stimulated with LPS for 9 or 48 h. IL-1 α , IL-1 β , IL-6, and TNF levels in the culture supernatants were determined by ELISA. Data show the mean $\pm$ SE; *, $^{\dagger}P < 0.05$ vs. corresponding values for IL-33^{+/+} mice.

Fc fusion proteins, ST2 deficiency may result in increased formation of IL-1R (IL-1R1 and IL-1RAcP) due to failed formation of IL-33R (ST2 and IL-1RAcP), causing cytokine hyperproduction by ST2^{-/-} macrophages in response to IL-1. Notably, ST2^{-/-} macrophages produced larger amounts of cytokines than wild-type macrophages after IL-1 α or IL-1 β treatment (10). Although IL-1R1^{-/-} mice showed normal susceptibility to LPS-induced endotoxin shock (38), IL-1R antagonist-deficient mice, which have excessive IL-1-signaling, showed heightened susceptibility (39). Thus, IL-1 is not required for induction of LPS-induced endotoxin shock, but excessive IL-1 production leads to amplified susceptibility to LPS, as seen in ST2^{-/-} mice. Distinct phenotypes were also seen in IL-18^{-/-} and IL-18R α ^{-/-} mice: EAE developed normally in IL-18^{-/-} mice but was dramatically reduced in IL-18R α ^{-/-} mice (40), suggesting a contribution by a ligand(s) other than IL-18, i.e., IL-1F7 (41), in the setting. As is well known, the function of the IL-1 family of cytokines is elaborately controlled by functional/decoy receptors and antagonists (41). Therefore, like IL-18R, ST2 may be a component of receptors for other ligand(s) besides IL-33. Alternatively, IL-33 may bind to other functional receptors besides ST2. Accordingly, for elucidation of the precise roles of IL-33 in immune responses, studies using IL-33^{-/-}, rather than ST2^{-/-}, mice have been highly anticipated.

Taken all together, IL-33 seems to play a critical role in various types of innate-type inflammation in the lung and gut,

whereas it is dispensable for acquired immune responses. Of note, IL-33 is indispensable for innate airway inflammation induced by papain. We were also able to show that IL-33 is crucial as an alarmin for innate-type, but not acquired-type, tissue injury-related inflammatory responses such as LPS-induced endotoxin shock.

Methods

LPS-Induced Sepsis. LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich)-induced sepsis was evaluated as described (38).

DSS-Induced Colitis. Mice were provided sterile water containing 3.0% or 3.5% DSS (reagent grade, MW = 36,000–50,000; MP Biomedicals) ad libitum for 7 d, followed by 14 d of plain drinking water.

Other Methods. The details are provided in *SI Methods*.

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Supporting Information

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SI Methods

Generation of IL-33-Deficient Mice. A BAC clone, RP23-355N22, which contains the translational start site of the IL-33 gene, was obtained from BACPAC Resources (<http://bacpac.chori.org>). The targeting vector was constructed as described (<http://www.cdb.riken.jp/arg/protocol.html>). The second exon was replaced with a cassette consisting of the *GFP* gene and the *neomycin resistance* gene (*neo^r*) flanked by loxP sequences (<http://www.cdb.riken.jp/arg/cassette.html>). The locations of 5' and 3' homologous recombinations of the targeting vector are shown in Fig. S1A. The targeting vector was electroporated into ES cells (TT2, derived from CBA × C57BL/6 mice). Chimera mice were obtained from two distinct clones out of three independently identified targeted ES cells and mated with C57BL/6 female mice (1). For confirmation of correct targeting, Southern blot hybridization was performed with 5' probes located outside of the regions used in the targeting vector (Fig. S1B). As we reported (2), constitutive expression of IL-33 proteins could be detected in homogenates of lungs from IL-33-sufficient, but not IL-33-deficient, mice by Western blot analysis (Fig. S1C). IL-33-deficient mice were obtained at the expected Mendelian ratio from the intercross of heterozygous mice on the C57BL/6 background (N2). IL-33-deficient mice were fertile and did not show any gross phenotypic abnormalities under specific pathogen-free housing conditions. Genotyping of mice was performed by PCR of tail genomic DNAs using the following PCR primers: ex2-F (5'-cactaagactactcagcctcag), WT-R (5'-cggtgatgctgtgaagtctg), and KO-R (5'-gtgttctgctgtgtagtctg). The ex2-F and WT-R primers were used for detection of wild-type alleles, and the ex2-F and KO-R primers were used for detection of mutant alleles. Detailed information regarding the IL-33-deficient mice (accession number CDB0631K) is available at <http://www.cdb.riken.jp/arg/mutant%20mice%20list.html>.

Mice. C57BL/6-wild-type mice (SLC Japan), BALB/c-wild-type mice (SLC Japan), C57BL/6-Rag2-deficient mice (Taconic), and BALB/c-4-get mice (The Jackson Laboratory) were used. C57BL/6-IL-1 α / β ^{-/-} mice and BALB/c-IL-4^{-/-}IL-13^{-/-} mice were kindly provided by Yoichiro Iwakura and Andrew McKenzie, respectively. All mice were housed under specific pathogen-free conditions at the National Research Institute for Child Health and Development, and the animal protocols were approved by the Institutional Review Boards of the National Research Institute for Child Health and Development and the Institute of Medical Science, University of Tokyo.

Contact Hypersensitivity (CHS). FITC-induced CHS and 2,4-dinitrofluorobenzene (DNFB)-induced CHS were examined as described (3, 4). Briefly, 2 d after shaving the dorsal hair with clippers, mice were sensitized with 200 μ L of a 2.0% FITC isomer I suspension (Sigma) in a mixture of acetone and dibutyl phthalate (1:1) or 25 μ L of 0.5% DNFB solution (Wako) in a mixture of acetone and olive oil (1:4). Five days after sensitization, the mice were challenged with 40 μ L of 0.5% FITC isomer I solution in a mixture of acetone and dibutyl phthalate (1:1) (the left ear, 20 μ L on each surface) and 40 μ L of the vehicle alone (the right ear, 20 μ L on each surface) or with 20 μ L of a 0.2% DNFB solution in a mixture of acetone and olive oil (1:4) (the left ear, 20 μ L on the outside skin surface) and 20 μ L of the vehicle alone (the right ear, 20 μ L on the outside skin surface). Ear thickness was measured before and after FITC or DNFB challenge by using an engineer's calipers (Ozaki) by an investigator who was blinded to the mouse genotypes. At 24 h after

FITC challenge, ear tissues were harvested for histological analysis and measurement of the myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities in ear skin homogenates, as described below. One week after FITC challenge, sera were collected for measurement of the FITC-specific Ig levels, as described below.

Skin DC Migration. Skin DC migration was determined as described (5, 6). Mice were treated epicutaneously with 40 μ L of a 0.5% (wt/vol) FITC isomer I solution in a mixture of acetone and dibutyl phthalate (1:1) (the left ear, 20 μ L on each surface) and the vehicle alone (the right ear, 20 μ L on each surface). Twenty-four hours later, submaxillary lymph nodes (LNs) were collected separately from both the FITC-treated left and vehicle-treated right ears. After incubation with anti-CD16/CD32 mAb (2.4G2; BD Biosciences), LN cells were incubated with PE-anti-mouse CD11c mAb (N418; eBioscience) and APC-anti-mouse I-A/I-E mAb (M5/114.15.2; eBioscience). The proportion of FITC⁺ cells among 7-aminoactinomycin D-negative, MHC class II^{hi}, CD11c⁺ cells was determined by using a FACSCalibur (BD Biosciences).

Delayed-Type Hypersensitivity (DTH). Methyl-BSA (mBSA)-induced CHS was examined as described (4, 7). Briefly, 200 μ L of 1.25 mg/mL mBSA (Sigma) emulsified with complete Freund's adjuvant (CFA; Difco) was injected s.c. to the backs of mice. Seven days later, the mice were challenged intradermally by injection of 20 μ L of 10 mg/mL mBSA in PBS to one footpad and 20 μ L of PBS alone to another footpad as a control. Footpad thickness was measured before and after mBSA or PBS challenge by using an engineer's calipers (Ozaki) by an investigator who was blinded to the mouse genotypes. One week after the challenge, sera were collected for measurement of the mBSA-specific Ig levels, as described below.

Airway Inflammation. OVA-induced airway inflammation was examined as described (8, 9). For the IgE-independent protocol (OVA with alum), mice were immunized intraperitoneally with 200 μ L of 0.5 mg/mL OVA (grade V; Sigma) emulsified with alum (Alum Immject; Pierce) (1 mg/mL OVA:alum = 1:1) on days 0 and 14. The mice were then challenged intranasally with 20 μ L of 10 mg/mL OVA in saline or saline alone on days 28, 29, and 30. For the IgE-dependent protocol (OVA without alum), mice were treated intraperitoneally with 200 μ L of 50 μ g/mL OVA in saline on days 0, 2, 4, 6, 8, 10, and 12. The mice were then challenged intranasally with 20 μ L of 10 mg/mL OVA in saline or saline alone on days 40, 43, and 46. House dust mite (HDM)-induced airway inflammation was established as described elsewhere (10, 11). Mice were treated intranasally with 20 μ L of 1.25 mg/mL HDM extract (Greer Laboratories) in saline or saline alone on 5 d per week for up to three consecutive weeks. For IL-33-induced airway inflammation, mice were treated intranasally with 20 μ L of 25 μ g/mL recombinant human IL-33 (PeproTech) in saline or saline alone once per day for 3 d. For papain-induced airway inflammation, mice were treated intranasally with 20 μ L of 5 mg/mL papain (Wako) or heat-inactivated papain (at 100 °C for 15 min) in saline or saline alone once per day for 3 d. Twenty-four hours after the last Ag or saline inhalation, bronchoalveolar lavage (BAL) fluids and lungs were collected for examination of the BAL cell profiles and lung histology, respectively. After centrifugation, the BAL cells were resuspended in 200 μ L of Hanks' buffer, and the total cell number and leukocyte profile were determined by using a hemocytometer (XT1800iV; Sysmex). Lung function during OVA-induced airway

inflammation was determined by an invasive approach (Elan Series Mouse RC Site; Buxco Electronics), as described (9, 12).

Experimental Autoimmune Encephalomyelitis (EAE). MOG-induced EAE was examined as described (13, 14). Briefly, mice were immunized s.c. with 200 μ L of 1.5 mg/mL MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified with CFA, which consisted of incomplete Freund's adjuvant (Difco) and 5 mg/mL *Mycobacterium tuberculosis* H37RA (Difco), by injection to one flank on day 0 and the other flank on day 7. The mice were injected i.v. with 200 μ L of 2.5 μ g/mL pertussis toxin (Allexis) on days 0 and 2. After the first MOG treatment, the severity of EAE was monitored daily and graded on a scale of 0–5 by an investigator who was blinded to the mouse genotypes. The scale was as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and fore limb paralysis; 5, moribundity and death.

Streptozocin-Induced Diabetes. Streptozocin-induced diabetes was examined as described (15), with minor modification. Briefly, mice were injected intraperitoneally with 4 mg/mL streptozocin in 0.05 M citrate buffer (pH 4.5) (50 mg/kg) once per day for 5 d. On days 0, 10, 17, and 24, blood was collected by cutting the tail vein, and the blood glucose levels were measured with an Accu-CHEK Aviva system (Roche Diagnostics).

Con A (ConA)-Induced Hepatitis. ConA-induced hepatitis was examined as described (16). Briefly, mice were i.v. administered 2 mg/mL ConA (Sigma) in saline (20 mg/kg). One day later, sera were collected, and the levels of GOT and GPT were measured by using Transaminase CII-test Wako (Wako).

Ag-Specific LN or Spleen Cell Responses. The FITC-, mBSA-, OVA- and MOG-specific LN cell proliferative responses were examined as described (4, 6, 14). Briefly, for FITC-specific LN cell responses, mice were treated epicutaneously with 2.0% FITC on both the left and right ears (20 μ L on one surface of each ear). Five days later, submaxillary LNs were collected. For mBSA-specific LN cell responses, the backs of mice were injected s.c. with 200 μ L of 1.25 mg/mL mBSA emulsified with CFA. Five days later, inguinal LNs were harvested. For OVA-specific LN and spleen cell responses, mice were immunized intraperitoneally with 200 μ L of 0.5 mg/mL OVA emulsified with alum (1 mg/mL OVA:alum = 1:1) on days 0 and 14. Seven days later, mesenteric LNs and the spleen were collected. For MOG-specific LN cell responses, the backs of mice were s.c. injected with 200 μ L of 1.5 mg/mL MOG35-55 peptide emulsified with CFA. Seven days later, inguinal LNs were harvested.

LN or spleen cells (4×10^5 cells per well in 96-well flat-bottom plates) were cultured in the presence and absence of 40 μ g/mL FITC, mBSA, OVA, or MOG at 37 °C for 72 h. Cell proliferative responses were determined by pulsing with 0.25 μ Ci/mL [^{3} H]-labeled thymidine for 6 h.

The intracellular cytokine profiles during mBSA- and MOG-specific LN cell responses were determined as described (14) with minor modification. LN cells (5×10^6 cells per well in a 24 well-plate) were cultured in the presence of 40 μ g/mL mBSA or MOG at 37 °C for 72 h, and then stimulated with 1 μ g of ionomycin (Sigma) and 0.1 μ g/mL PMA in the presence of 1 μ M monensin (Sigma) for 5 h. After washing, the cells were incubated with anti-CD16/CD32 mAb (2.4G2; BD Biosciences) in FACS buffer (Hanks' buffer containing 2% FCS) for FcR blocking on ice for 15 min, and then incubated with APC-conjugated anti-mouse CD4 mAb (GK1.5; eBioscience) on ice for 30 min. After washing, the cells were treated with Fix Buffer I (BD Biosciences) at room temperature for 15 min. Then the cells were washed with 0.1% saponin (Sigma) in FACS buffer and incubated with FITC anti-mouse Foxp3 mAb (FJK-16s; eBiosciences) and PE anti-mouse IL-17 mAb (TC11-18H10, BD Biosciences) at 4 °C for 30 min. IL-17 and Foxp3 expressions in CD4⁺ T cells were analyzed

on a FACSCalibur (Becton Dickinson) by using CellQuest software (Becton Dickinson).

Measurement of Cytokines. Cytokine levels in the culture supernatants of Ag-specific LN cells and BAL fluids during OVA-induced airway inflammation were determined with mouse IFN- γ , IL-1 α , IL-1 β , IL-4, IL-5, IL-13, and IL-17 ELISA kits obtained from BD Biosciences or eBioscience. The level of IL-33 in colon homogenates (prepared as described below) was measured with an ELISA kit (BioLegend).

Measurement of MPO and EPO Activities. The levels of MPO and EPO activities in tissues were examined as described (9, 12). Briefly, tissues (ear and colon) were homogenized in a 0.5% cetyltrimethylammonium chloride solution (Sigma). After centrifugation, the supernatants of the homogenates were collected. Total protein levels in the tissue homogenates were measured with a Bio-Rad DC protein assay kit. Recombinant human MPO and EPO (Calbiochem) were used as standard proteins. The MPO and EPO activities per milligram of total protein in tissue homogenates were calculated.

Measurement of Ag-Specific Ig Levels. Ninety-six-well ELISA plates (Nunc; 442404) were coated with 2 μ g/mL FITC-OVA (6), 10 μ g/mL mBSA, 10 μ g/mL OVA, or 10 μ g/mL MOG at 4 °C overnight. After the wells were blocked with PBS containing 10% FCS, optimally diluted serum samples (IgG1 = 1:10,000, IgG2a = 1:100, and IgE = undiluted for FITC-specific Igs; IgG1 = 1:50, IgG2a = 1:10, IgG2b = 1:50, and IgG3 = 1:2 for mBSA-specific Igs; IgE = undiluted for OVA-specific IgE; and IgG1 = 1:2 and IgG2a = 1:2 for MOG-specific Igs) were applied, and the plates were incubated at room temperature for 1 h. After washing, biotinylated anti-mouse IgG1 (A85-1; BD Biosciences), IgG2a (R19-15; BD Biosciences), IgG2b (R12-3; BD Biosciences), IgG3 (R40-82; BD Biosciences), or IgE (R35-118; BD Biosciences) mAb was added, followed by incubation at room temperature for 1 h. Then, after washing, HRP-conjugated streptavidin (BD Biosciences) was added, followed by incubation at room temperature for 1 h. For enzymatic reaction, TMB substrate (KPL) was used as the substrate. The reaction was stopped by addition of 1 M H₂SO₄, and then the absorbance at 450 nm was measured by using a plate reader. Data show the absorbance value at 450 nm. The levels of OVA-specific serum IgE were normalized by using an anti-OVA IgE mAb (TOS-2; kindly provided by Mamoru Kuniwa, Taiho Pharmaceutical, Saitama, Japan) as the standard antibody (12).

Quantitative PCR. Total RNA in the colon and lung specimens was isolated by using ISOGEN (Nippon Gene) and RNeasy Mini Kit (Qiagen). Using the isolated RNA, cDNA was obtained by RT-PCR with an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo) and an Applied Biosystems 7300 real-time PCR system. The relative gene expression was normalized against GAPDH gene expression. PCR primers were designed as shown in Table S1.

Histology. Tissues were fixed in Carnoy's fluid and embedded in paraffin. Then sections were prepared and stained with hematoxylin-eosin.

Score During DSS-Induced Colitis. The severity of diseases was scored as described (17).

Statistical Analyses. Data show the mean $\pm$ SE. Differences were evaluated by the Kaplan–Meier test (survival), two-way ANOVA followed by the Holm–Sidak post hoc test (airway hypersensitivity), the Mann–Whitney *u* test (score in EAE), or the two-tailed Student's *t* test (other studies, unless otherwise specified).

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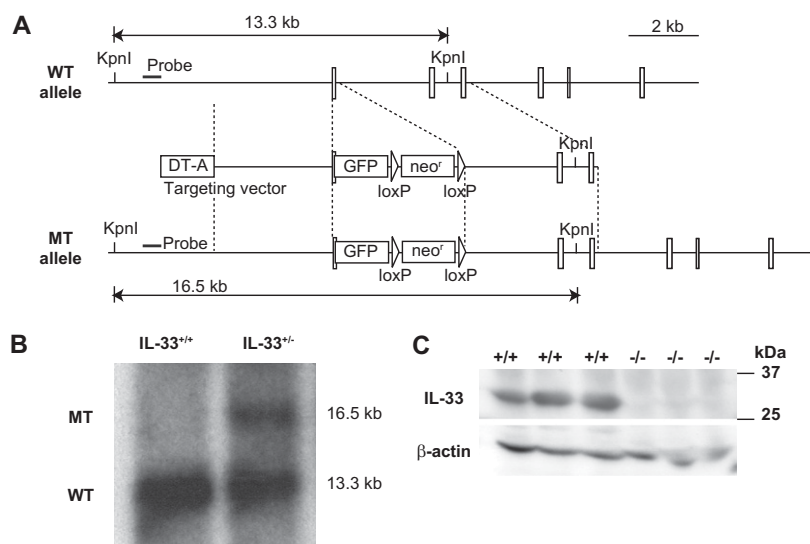


Fig. S1. Generation of IL-33-deficient mice. (A) IL-33 gene targeting strategy. The second exon containing a translational start codon was replaced with a tandemly arrayed promoter-less *GFP* gene and a floxed *neomycin resistance* gene (*neo*^r) (targeted allele). (B) Southern blot analysis of genomic DNA obtained from wild-type or mutant ES cells. The DNA probes used for Southern blot analysis are shown in A. By digestion of genomic DNA with KpnI, the probes detected endogenous wild-type (WT; 13.3 kb) and/or targeted (MT; 16.5 kb) fragments. (C) Western blot analysis of whole-lung homogenates.

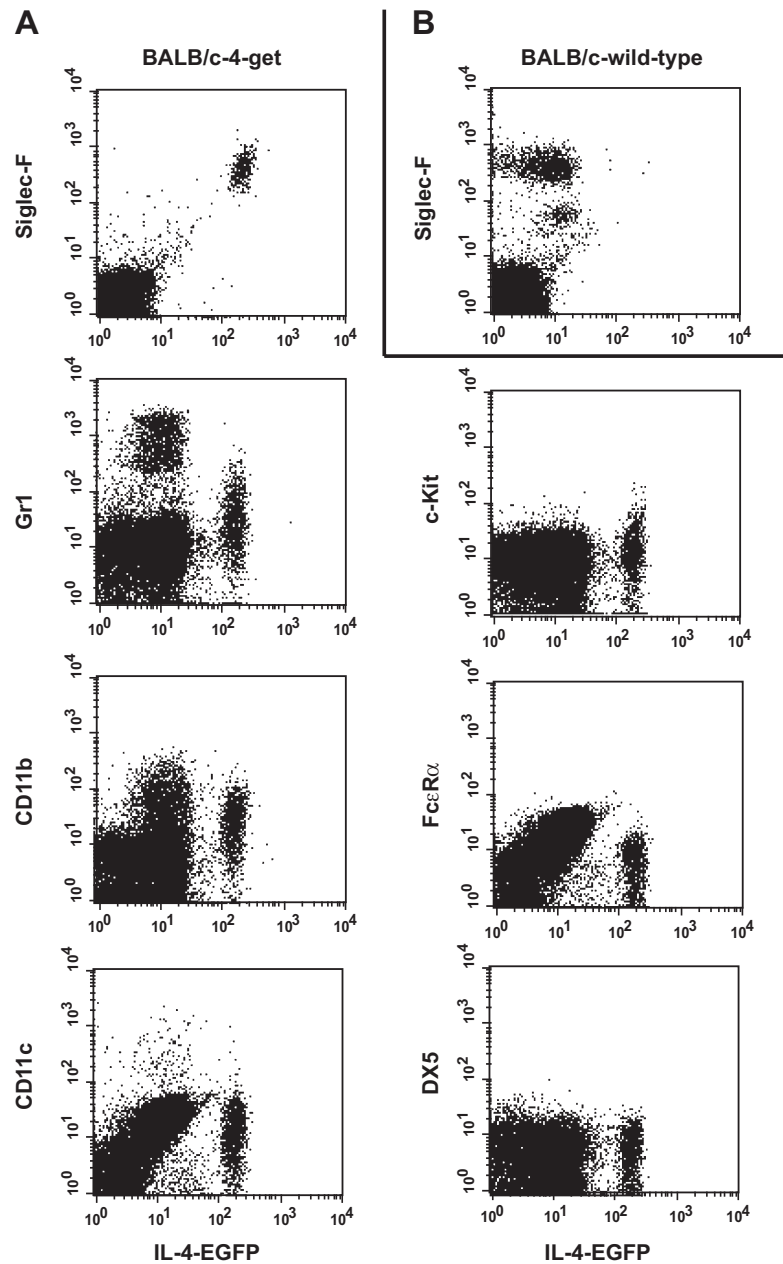
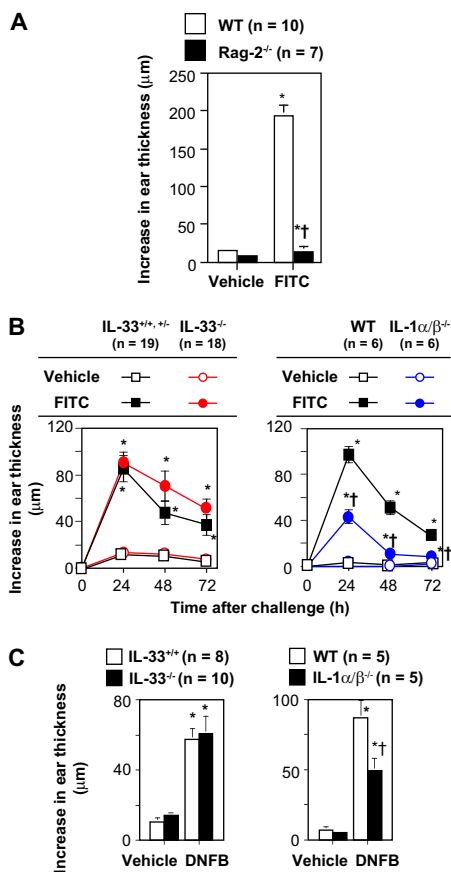


Fig. S5. Eosinophils are a producer of IL-4 in BAL cells after papain inhalation. BAL cells were harvested from BALB/c-4-get mice (A), which express EGFP with IL-4, and BALB/c-wild-type (B) at 24 h after the last papain inhalation. EGFP-IL-4-expressing cells in 7-aminoactinomycin D-, CD3- and B220-negative cells were detected by flow cytometry. Data show a representative result from two independent experiments.



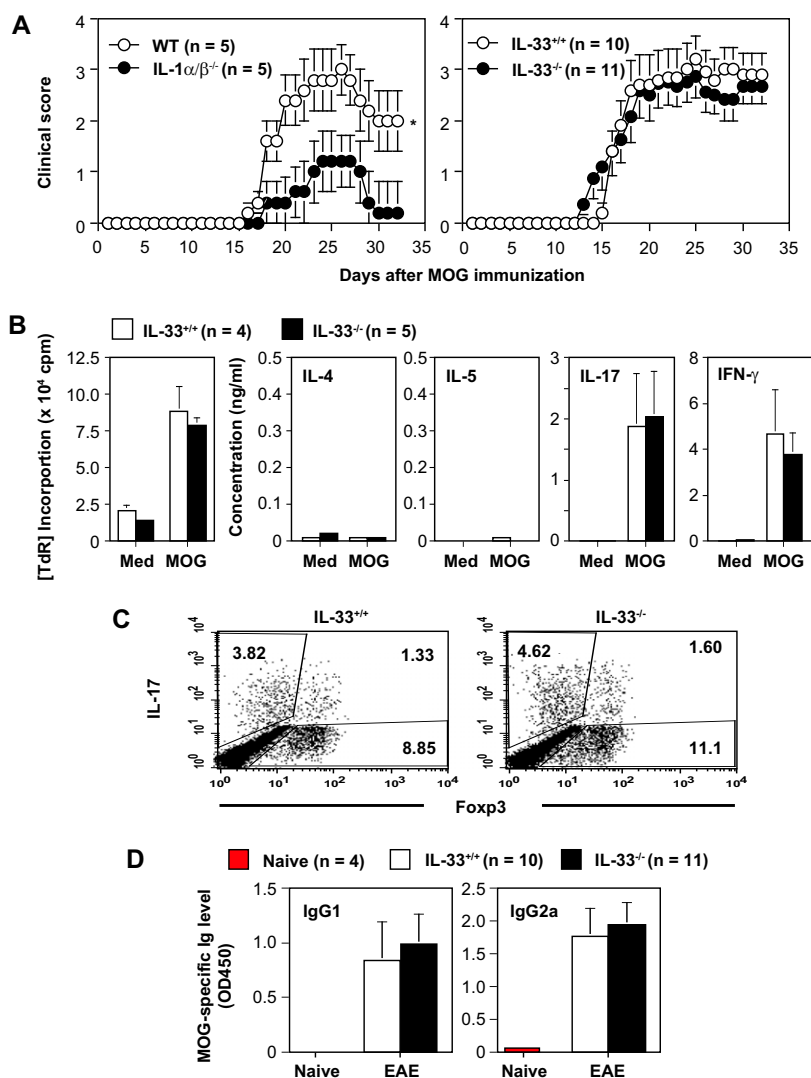


Fig. S9. The role of IL-33 in the development of EAE. (A) The severity of MOG-induced EAE. (B) MOG-specific proliferative responses and cytokine secretion of LN cells from mice sensitized with MOG emulsified in CFA. (C) Profiles of IL-17- and Foxp3-expressing CD4⁺ T cells in the culture with MOG in B. Representative FACS results are shown. (D) MOG-specific Ig levels in sera from mice 33 d after the first MOG immunization in A or naïve IL-33^{+/+} mice. The levels of MOG-specific IgG1 and IgG2a were measured by ELISA. Data show the mean $\pm$ SE: **P* < 0.01 (Mann–Whitney *u* test). No significant differences were found between the IL-33^{+/+} and IL-33^{-/-} groups.

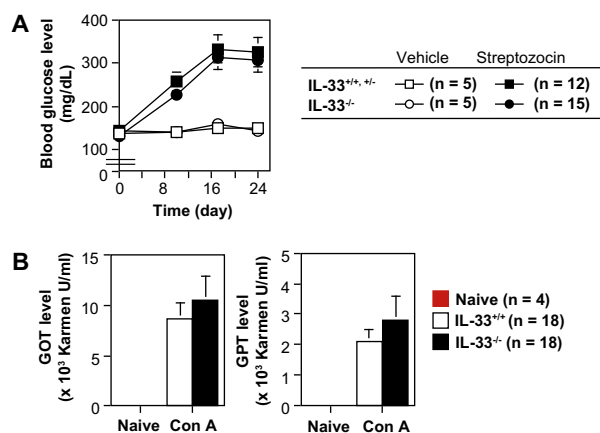


Fig. S10. The role of IL-33 in the development of T/NKT cell-dependent streptozocin-induced diabetes or Con A-induced hepatitis. (A) The levels of blood glucose in mice during streptozocin-induced diabetes. (B) GOT and GPT activity levels in sera from mice during Con A-induced hepatitis. Data show the mean $\pm$ SE. No significant differences were found between the IL-33^{+/-} and IL-33^{-/-} groups.

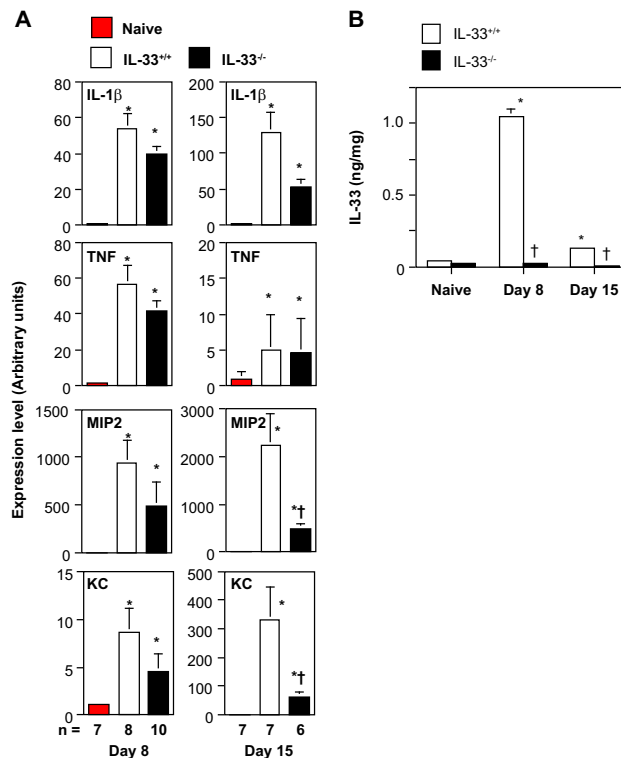


Fig. S11. IL-33 is involved in the expression of neutrophil chemoattractant factors during DSS-induced colitis. The colon was collected from mice on day 8 or 15 during 3.0% DSS-induced colitis or from naïve mice. (A) The levels of IL-1 β , TNF, MIP2, and KC expression in the colon were determined by quantitative PCR. (B) The levels of IL-33 in the colon homogenates were determined by ELISA. Data show the mean $\pm$ SE: * $P < 0.05$ vs. corresponding values for naïve mice, and † $P < 0.05$ vs. DSS-treated IL-33^{+/+} mice.

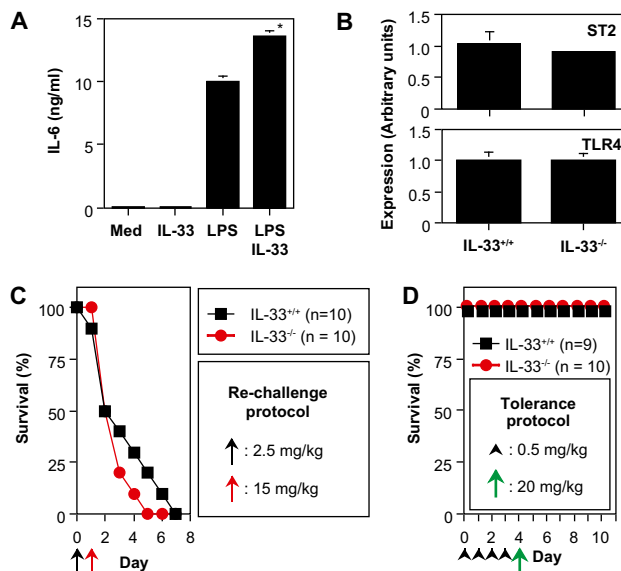


Fig. S12. IL-33 is not essential for secondary responses or tolerance to LPS. (A) TGC-induced peritoneal macrophages from C57BL/6 mice were cultured with plain medium or medium containing IL-33, LPS, or IL-33 + LPS for 48 h. IL-6 levels in the culture supernatants were determined by ELISA. Data show the mean $\pm$ SE (n = 3). * $P < 0.05$ vs. medium alone, IL-33, and LPS stimulation. (B) The expressions of ST2 and TLR4 in peritoneal macrophages from IL-33^{+/+} and IL-33^{-/-} mice were determined by quantitative PCR. Data show the mean $\pm$ SE (n = 3). (C) The survival ratios in IL-33^{+/+} and IL-33^{-/-} mice, which were first sensitized with a low dose of LPS (2.5 mg/kg; black arrow), after treatment with a lethal dose of LPS (15 mg/kg; red arrow). (D) The survival ratios in IL-33^{+/+} and IL-33^{-/-} mice, which were first made tolerant to LPS by repeated injection of a low dose of LPS (0.5 mg/kg; black arrowhead), after treatment with a lethal dose of LPS (20 mg/kg; green arrow). No significant differences were found between the IL-33^{+/+} and IL-33^{-/-} groups (B–D).

Genes	Forward (5' - 3')	Reverse (5' - 3')
<i>GAPDH</i>	CCCACTCTTCCACCTTCGATG	AGGTCCACCACCCTGTTGCT
<i>IL-1β</i>	CAACCAACAAGTGATATTCCATG	GATCCCACTCTCCAGCTGCA
<i>IL-33</i>	CAGGCCTTCTTCGTCCTTCAAC	TCTCTCCACTAGAGCCAGCTG
<i>TNF</i>	GCCTCCCTCTCATCAGTTCT	CAC TTGGTGGTTTGCTACGA
<i>KC</i>	CACGTGTTGACGCTTCCCTT	TGAACGCTCTGTGCCGAGC
<i>MIP-2</i>	AAC TGA C T G G A A A G G A G G A G C	ACTCTCAGACAGCGAGGCACAT
<i>ST2</i>	TCAACCGCCTAGTGAACACACC	CAAAGCCCAAAGTCCCATTCTC
<i>TLR4</i>	CGCTTTCACCTCTGCCTTCACTACAG	ACACTACCACAATAACCTTCCGGCTC