IL-33 exacerbates antigen-induced arthritis by activating mast cells

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IL-33, a cytokine of the IL-1 family, is closely associated with type II T cell responses. Here, we report an unexpected proinflammatory role of IL-33 in inflammatory arthritis. IL-33 was expressed in synovial fibroblasts from patients with rheumatoid arthritis (RA). Expression was markedly elevated *in vitro* by inflammatory cytokines. Mice lacking ST2, the IL-33 receptor α-chain, developed attenuated collagen-induced arthritis (CIA) and reduced *ex vivo* collagen-specific induction of proinflammatory cytokines (IL-17, TNFα, and IFNγ), and antibody production. Conversely, treatment of wild-type (WT) but not ST2$^{-/-}$ mice with IL-33 exacerbated CIA and elevated production of both proinflammatory cytokines and anti-collagen antibodies. Mast cells expressed high levels of ST2 and responded directly to IL-33 to produce a spectrum of inflammatory cytokines and chemokines *in vitro*. *In vivo*, IL-33 treatment exacerbated CIA in ST2$^{-/-}$ mice engrafted with mast cells from WT but not from ST2$^{-/-}$ mice. Disease exacerbation was accompanied by elevated expression levels of proinflammatory cytokines. Our results demonstrate that IL-33 is a critical proinflammatory cytokine for inflammatory joint disease that integrates fibroblast activation with downstream immune activation mainly via an IL-33-driven, mast-cell-dependent pathway. Thus, this IL-1 superfamily member represents a therapeutic target for RA.

Rheumatoid arthritis | ST2

IL-33 is a newly described cytokine of the IL-1 family (1) that also includes IL-1α, IL-1β, and IL-18 (2). Like IL-1 and IL-18, IL-33 is produced as a pro-IL-33 molecule that is putatively cleaved by caspase-1 to form a mature 18-kDa protein. Both human and mouse IL-33 are 55% identical at the amino acid level (1). IL-33 mRNA is broadly expressed in many tissues but is restricted in cellular distribution to smooth muscle cells, epithelial cells, fibroblasts, keratinocytes, dendritic cells, and activated macrophages. The receptor for IL-33 is a heterodimer consisting of ST2 and IL-1RAcP (3, 4). ST2, a member of the IL-1 receptor family, is selectively expressed on Th2 but not Th1 cells and has been implicated in type II but not type I functions (5–7). Thus, IL-33 is closely associated with the activation and production of type II cytokines from *in vitro* polarized Th2 cells (1). *In vivo*, IL-33 injection elicits type II cytokine production and eosinophilia in naïve mice (1, 8). However, studies in ST2$^{-/-}$ mice demonstrate that ST2 is not required for IL-4-induced Th2 cell development or function (9–11), suggesting that ST2/IL-33 may drive type II responses via an alternative pathway or that the function of IL-33/ST2 could extend beyond type II responses. Mast cells express a high density of ST2 and produce a variety of proinflammatory cytokines *in vitro* in response to IL-33 (1, 12–14). Here, we explored directly the role of IL-33 and mast cells in inflammatory arthritis.

Rheumatoid arthritis (RA) is characterized by chronic inflammatory infiltration of the synovium, leading to eventual cartilage and bone destruction (15–17). Elevated levels of proinflammatory cytokine production are a key feature of synovial inflammation (18). Moreover, successful therapeutic targeting of cytokines in RA, particularly TNFα and IL-6, has demonstrated their critical pathogenic importance. Th1 responses were considered to predominate within RA synovial T cell subsets and thereby contribute significantly to dysregulated cytokine production (19, 20). However, recent studies demonstrate that IL-17-producing CD4$^+$ T cells (Th17) play a key pathogenic role in RA (21, 22). B cells are also critically involved in disease pathogenesis by cytokine production, antigen presentation, and autoantibody synthesis that subsequently triggers joint damage either by directly targeting self-tissues or forming immune complexes with autoantigens (23–25). Recently, a critical role for mast cells in RA biology was proposed (26–29). Mast cells are abundantly present in synovial tissue (26). Their degranulation and proinflammatory cytokine production correlates with disease severity (27). Mice lacking mast cells are resistant to antibody-induced arthritis (28, 29). However, factors that drive mast cell activation are not well defined. Here, we describe the previously unrecognized expression of IL-33 in RA synovial tissues and provide further direct evidence that IL-33 promotes articular inflammation acting, at least in part, via mast cell activation.

Results

Expression and Induction of IL-33 in RA Synovium. We first investigated the expression of IL-33 and ST2 in the synovia of RA patients. Synovial membranes were obtained from RA patients and stained with anti-IL-33 and anti-ST2 antibodies. Positive staining was evident in all RA tissues examined in lining layer and in the interstitial sub lining layer areas (Fig. 1 *A* and *B*). We next obtained primary synovial fibroblasts (passage 3–6) from RA patients and examined IL-33 expression. Resting fibroblasts expressed little or no IL-33, whereas expression was markedly enhanced by the addition of TNFα and IL-1β, recapitulating the *in vivo* cytokine milieu (Fig. 1*C*). The effect of TNFα and IL-1β on IL-33 expression was confirmed at the mRNA level by RT-PCR. Whereas resting fibroblasts did not express detectable IL-33 mRNA, TNFα induced the up-regulation of IL-33 expression, particularly in combination with IL-1β (Fig. 1*D*).

ST2$^{-/-}$ Mice Develop Impaired Collagen-Induced Arthritis. To investigate the potential functional role of IL-33 in inflammatory arthritis, we used the murine model of collagen-induced arthritis

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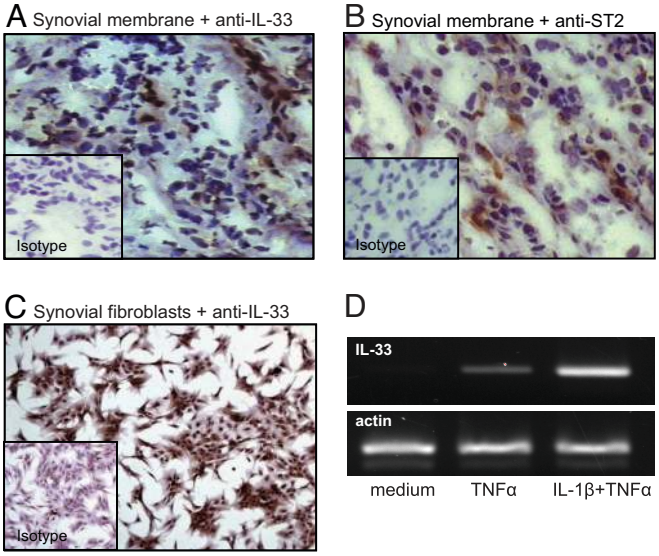

Fig. 1. Expression and regulation of IL-33 in RA synovium. (*A* and *B*) Synovial membranes from RA patients were stained with anti-IL-33 (*A*) and anti-ST2 (*B*) antibodies or isotype-matched control IgG. (*C*) Fibroblasts were stained for IL-33 as in *A*. Primary fibroblasts were purified from the synovial membrane and cultured with the cytokines indicated. (*D*) Fibroblasts were cultured for 16 h and IL-33 mRNA detected by RT-PCR. Pictures are representative of tissue samples from five RA patients.

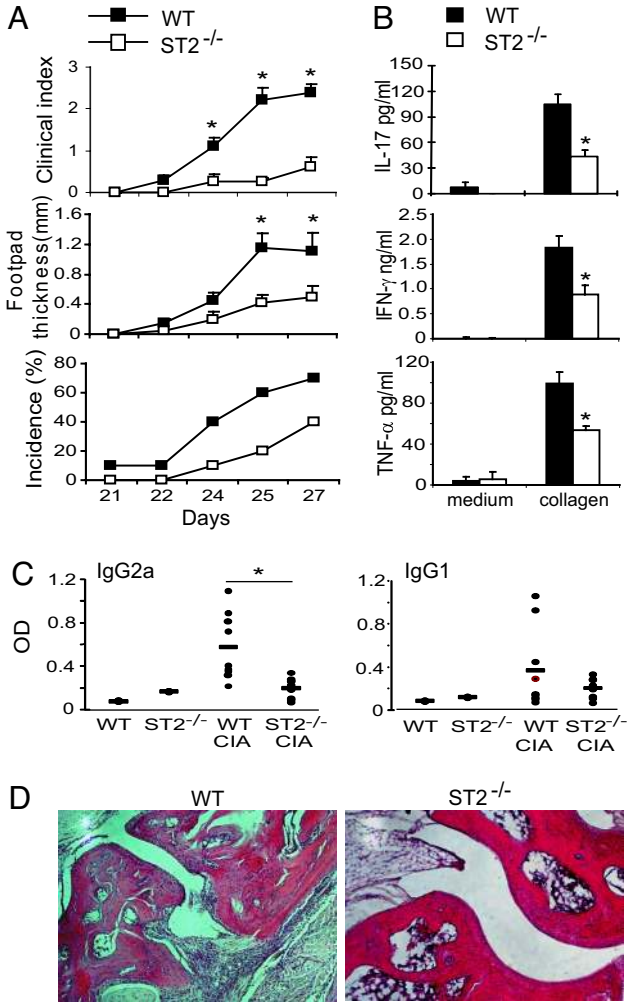

Fig. 2. $ST2^{-/-}$ mice developed impaired CIA. (*A*) Groups of WT and $ST2^{-/-}$ DBA/1 mice were immunized with CII and the disease development (clinical score, footpad thickness, and incidence) was monitored. (*B*) DLNs of the mice in *A* were collected on day 27 and stimulated with CII for 3 days and the cytokine concentrations in the culture supernatant were determined by ELISA. (*C*) Serum anti-CII antibodies of individual samples were determined by ELISA. (*D*) On day 27, mice were killed and arthritic paws removed and stained with hematoxylin and eosin. Original magnification was ×50. Data are means ± SEM (*, $P < 0.01$ WT vs. $ST2^{-/-}$ mice by Mann–Whitney *U* test, $n = 10$). Data are representative of three experiments. Similar results were obtained when mice were immunized and boosted on day 21 with CII.

(CIA). We generated $ST2^{-/-}$ DBA/1 mice by back-crossing $ST2^{-/-}$BALB/c mice with DBA/1 mice for 10 generations. Groups of male $ST2^{-/-}$DBA/1 and WT mice were immunized intradermally with bovine type II collagen (CII) in complete Freund's adjuvant (CFA) without further antigenic challenge and disease development was monitored thereafter. $ST2^{-/-}$ mice developed significantly attenuated disease compared with WT mice (Fig. 2*A*). This observation was manifest in reduced disease severity in involved animals. Furthermore, the reduction in clinical disease score was accompanied by significantly decreased IL-17, IFNγ, and TNFα production to recall collagen by draining lymph node (DLN) cultures from $ST2^{-/-}$ mice compared with WT mice (Fig. 2*B*). $ST2^{-/-}$ mice also expressed significantly reduced concentrations of serum anti-CII IgG2a compared with WT mice (Fig. 2*C*). Collagen-specific IgG1 levels were similar between groups. Histological examination revealed marked reduction of infiltration of mononuclear and polymorphonuclear cells and of synovial hyperplasia in the joints of $ST2^{-/-}$ mice compared with WT mice (Fig. 2*D*). These results indicate that ST2 signaling contributes to the pathogenesis of CIA and that this process is associated with proinflammatory cytokine synthesis and collagen-specific type I and Th17 T cell responses.

IL-33 Exacerbates CIA. We next examined the effect of recombinant IL-33 on the development of CIA. DBA/1 mice were immunized with CII/CFA and challenged with CII in PBS on day 21. The mice were injected i.p. daily with IL-33 (1 μg per mouse) or PBS for 5 days from day 21. Mice treated with IL-33 developed significantly more severe disease as assessed by clinical score and footpad thickness compared with the PBS control (Fig. 3*A*). There was no difference in arthritic incidence, suggesting that IL-33 acts primarily as a disease severity modulator in this experimental protocol. Similar results were obtained in mice immunized with CII/CFA but without antigen challenge on day 21 (data not shown). DLN cultures stimulated with CII from IL-33-treated mice produced significantly more IL-17, IFNγ, TNFα, IL-5, and IL-12 (Fig. 3*B*) than PBS control mice. IL-4 was not detectable (data not shown). Consistent with these results, IL-33 administration also significantly increased serum CII-specific antibodies, IgG1, and IgG2a (Fig. 3*C*). T cell proliferation was similar between the two groups (data not shown). Histological examination revealed that IL-33 treatment markedly exacerbated mononuclear and polymorphonuclear cell infiltration into the joint and synovial hyperplasia, accompanied by marked cartilage and bone erosion (Fig. 3*D*). As a specificity control, we also demonstrated that IL-33 treatment did not affect the incidence or severity of CIA in $ST2^{-/-}$ mice [supporting information (SI) Fig. S1] nor their inflammatory cytokine production (data not shown), confirming that IL-33 acted via a ST2-dependent pathway. Moreover, IL-33 treatment alone (without CII immunization) did not induce any articular disease (data not shown). Together, these data clearly indicate that IL-33 potently enhances the development of CIA and attendant articular pathology.

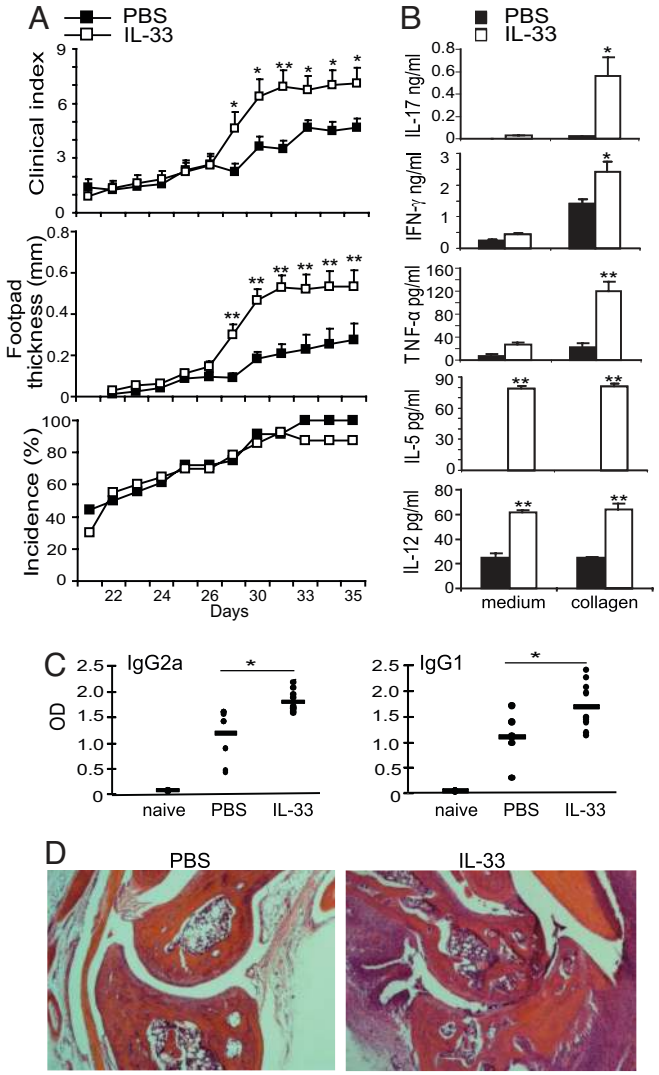

Fig. 3. IL-33 exacerbated CIA. (*A*) Collagen-immunized and -challenged DBA/1 mice ($n = 10$) were injected i.p. with IL-33 (1 μg/ml) or PBS daily from days 21–25. Mice were monitored for disease progression as in Fig. 2. (*B*) DLN cells (2×10^6 per milliliter) were stimulated with or without CII for up to 3 days and the levels of cytokines in the supernatants were determined by ELISA. (*C*) Serum anti-CII antibody of individual samples was determined by ELISA. (*D*) On day 35, mice were killed and arthritic paws removed and stained with hematoxylin/eosin or toluidine blue as in Fig. 2*D*. Data are mean ± SEM (*, $P < 0.05$; **, $P < 0.01$ compared with PBS controls). Data are representative of three experiments.

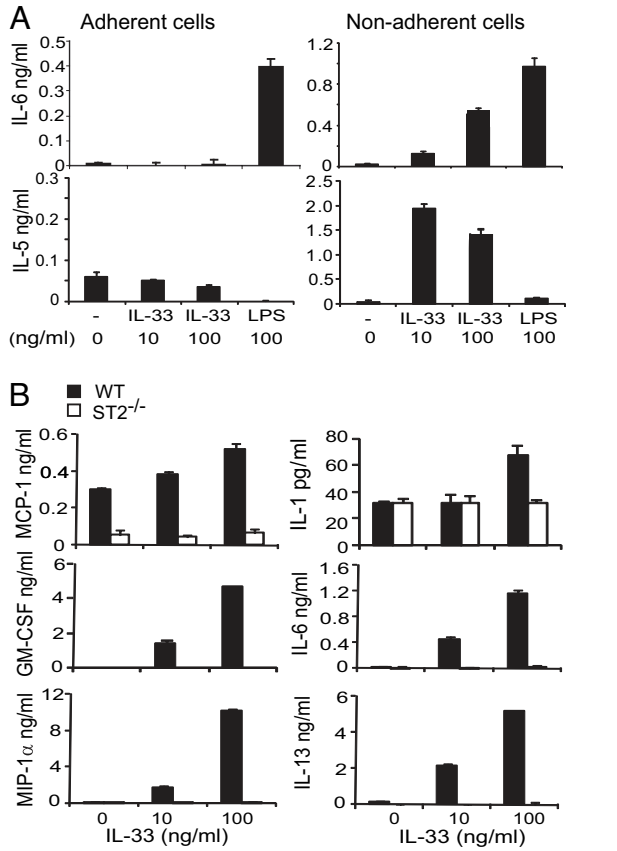

Fig. 4. Induction of inflammatory cytokine production by IL-33 *in vitro*. (*A*) Spleen and LN cells (4×10^6 per milliliter) from CIA mice were incubated in 24-well plates at 37°C for 3 h before removing nonadherent cells. Adherent and nonadherent cells (2×10^6 per milliliter) were treated with IL-33 or LPS (1 μg/ml) or medium alone for 48 h. IL-6 and IL-5 concentrations in the culture supernatant were measured by ELISA. (*B*) BMMCs from WT or ST2$^{-/-}$ DBA/1 mice were cultured with IL-33 for 48 h and the cytokine and chemokine concentrations in the supernatant analyzed by Luminex as above. Data are mean ± SD and are representative of three experiments.

IL-33 Induces Inflammatory Cytokine Production by Mast Cells. We next investigated putative target cells mediating the arthritis-relevant activities of IL-33. Spleen and LN cells from WT DBA/1 mice with CIA were collected and separated into adherent and nonadherent fractions, and then were cultured with either IL-33 or LPS. Both populations of cells responded to LPS to produce high levels of both IL-6 and IL-5. However, whereas nonadherent cells responded to IL-33 to produce significant amounts of IL-5 and IL-6, adherent cells did not (Fig. 4*A*). These results indicated that it was unlikely that IL-33 manifests direct effects on macrophages and fibroblasts. With respect to likely responder populations in the nonadherent fraction, we considered it unlikely that either Th1 or Th17 cells were targets because IL-33 had no direct effect on the induction on Th17 cells *in vitro* (Fig. S2) and Th1 cells do not express ST2 (5, 6). Moreover, the high levels of IFNγ detected during IL-33-induced exacerbation of CIA mitigated against Th2 cells playing a major role in arthritic pathology. Mast cells express high density of cell-surface ST2 (12) and are reported to respond directly to IL-33 to produce inflammatory cytokines and chemokines *in vitro* (1, 13, 14). We therefore investigated the potential role of mast cells in this system by using WT and ST2$^{-/-}$ DBA/1 mice. Bone marrow-derived mast cells (BMMCs) were generated from either WT or ST2$^{-/-}$DBA/1 mice as described previously (12, 14). The c-*kit*$^+$ST2$^+$FcεRI$^+$ BMMCs were activated with or without graded doses of IL-33 for 2 days and cytokines secreted in the culture supernatants were determined by ELISA and Luminex. IL-33 induced the production of a range of proinflammatory cytokines (IL-1β, IL-6, IL-13, and GM-CSF) and chemokines (MCP-1 and MIP-1α) by BMMCs from WT but not ST2$^{-/-}$ DBA/1 mice (Fig. 4*B*). In agreement with an earlier report using C57B/6 mice (14), IL-33 did not induce IL-5 production by mast cells from DBA/1 mice but rather enhanced IL-13 production (Fig. 4*B*). Other members of the IL-1 family, IL-1β and IL-18, did not modulate cytokine production by IL-33-activated mast cells, suggesting that this effect is relatively specific within the IL-1 superfamily to IL-33 (Fig. S3). These data are consistent with the notion that mast cells could represent a pathway through which IL-33 mediates enhanced articular inflammation.

IL-33 Exacerbates CIA in ST2$^{-/-}$ Mice Engrafted with WT Mast Cells. To directly demonstrate a role for mast cells in IL-33-exacerbated

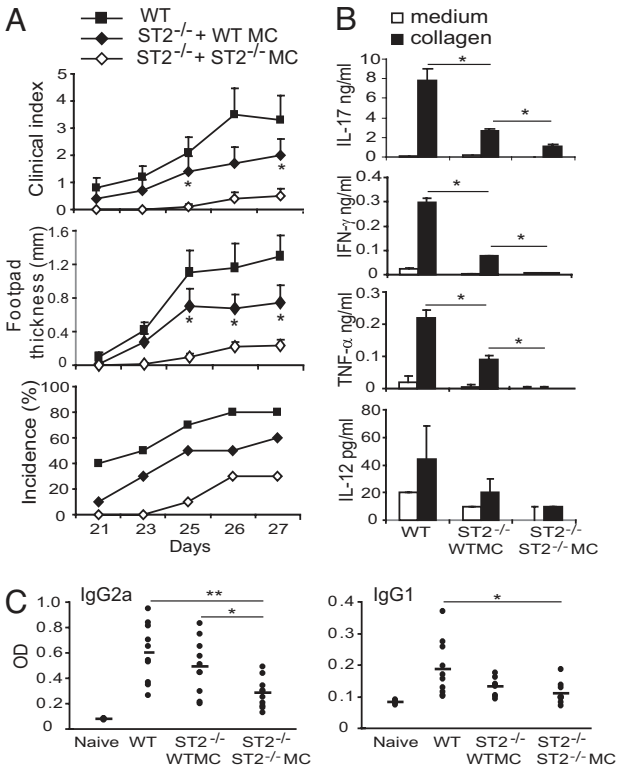

Fig. 5. IL-33 exacerbated CIA in ST2$^{-/-}$ mice engrafted with mast cells from WT but not ST2$^{-/-}$ mice. Groups of ST2$^{-/-}$ mice were injected i.v. with ST2$^{-/-}$ or WT BMMCs (1×10^7). CIA was induced 4 weeks later as in Fig. 2*A*. A group of WT mice was also included as positive control and immunized as engrafted ST2$^{-/-}$ mice. The mice were injected i.p. with IL-33 (1 μg per mouse) daily from 21 days after immunization for five days. (*A*) Mice were monitored for clinical score, footpad thickness, and disease incidence. (*B*) DLN cells were collected and cultured *in vitro* with collagen for 3 days, and cytokine production was determined by ELISA. (*C*) Serum anti-CII antibody concentration from individual mice was measured by ELISA. Data are mean ± SEM, $n = 6$ and are representative of two experiments.

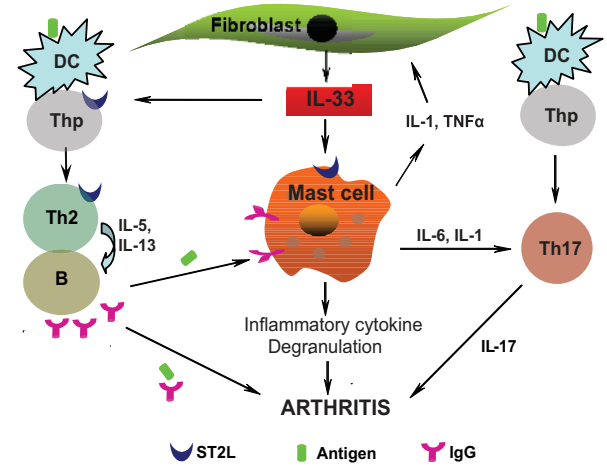

Fig. 6. Schematic representation of the pathogenic role of IL-33 in CIA. Tissue stroma cells including fibroblasts produce IL-33 which could be further enhanced by proinflammatory cytokines such as IL-1β and TNFα. IL-33 can promote inflammatory responses by at least three pathways. (*i*) IL-33 can directly activate mast cells to secrete proinflammatory cytokines which in turn cause joint inflammation. (*ii*) IL-1β and IL-6 secreted by IL-33-activated mast cells can promote collagen-specific Th17 cell development and function. (*iii*) IL-33 can also stimulate collagen-primed CD4$^+$ T cells to produce IL-5 and IL-13 that enhance B cell activation, leading to increased IgG production. The antibodies further exacerbate joint inflammation by triggering mast cell degranulation and the formation of immune complexes with collagen.

arthritis, ST2$^{-/-}$ DBA/1 mice were adoptively transferred with BMMCs from either WT or ST2$^{-/-}$DBA/1 mice. Four weeks later, CIA was induced as in Fig. 2 and IL-33 was administered i.p. daily 21–25 days after CIA induction. Age-matched WT DBA/1 mice were included as positive controls. ST2$^{-/-}$ mice engrafted with ST2$^{-/-}$ BMMCs developed minimal disease compared with WT mice. ST2$^{-/-}$ mice engrafted with WT BMMCs developed significantly more severe CIA than those engrafted with ST2$^{-/-}$ BMMCs, although this did not reach the levels of severity observed in WT mice (Fig. 5*A*). The disease severity paralleled the cytokine and antibody profiles observed in these mice. DLN cells from ST2$^{-/-}$ mice reconstituted with WT BMMCs produced significantly more IL-17, IFNγ, and TNFα compared with ST2$^{-/-}$ mice engrafted with ST2$^{-/-}$ BMMCs (Fig. 5*B*). The WT cell-reconstituted mice also produced more serum IgG2a compared with ST2$^{-/-}$ mice engrafted with ST2$^{-/-}$ cells (Fig. 5*C*). Histological examination revealed that IL-33 administration increased leukocyte infiltration into the joints in WT BMMC-engrafted ST2$^{-/-}$ mice compared with ST2$^{-/-}$ BMMC-reconstituted ST2$^{-/-}$ mice (data not shown). In these experiments, the only cells capable of responding to IL-33 in the ST2$^{-/-}$ host were the engrafted WT BMMCs. These data therefore confirm that mast cells play a pivotal role in the proinflammatory effect of IL-33 in CIA.

Discussion

Data reported here demonstrate a previously unrecognized propathogenic role of IL-33 in inflammatory arthritis. IL-33 was detected in RA synovial tissues. In animal model studies, IL-33 enhanced collagen-initiated inflammatory responses by a putative mechanism schematically depicted in Fig. 6. A major pathway by which IL-33 contributes to the inflammatory process is likely to be via the activation of mast cells that express a high density of ST2, the IL-33R α-chain.

Mast cells are abundantly expressed in synovial tissues and have been proposed to exert proinflammatory effects primarily based on antibody transfer-induced disease models (28, 29). The mode of mast cell activation and the mechanism by which activated-mast cells mediate antigen-induced arthritis are largely unknown and likely complex. We now provide direct *in vivo* evidence that IL-33 plays a major role in mast cell activation in the context of antigen-induced arthritis. Thus, IL-33 enhanced CIA when ST2$^{-/-}$ mice were reconstituted with BMMCs from WT but not from ST2$^{-/-}$ mice (Fig. 5). Our data also provide mechanisms by which mast cells could promote inflammatory synovitis (Fig. 6). IL-33 induces mast cell production of IL-1, IL-6, IL-13, and a range of chemokines (13, 14, 30) (Fig. 4). Because both IL-1 and IL-6 play crucial roles in the induction of Th17 cells (31–33), a key pathogenic cell type in arthritis (21, 22), our studies indicate a relationship between ST2/IL-33 function and collagen-specific IL-17 production in CIA (Figs. 2*B* and 3*B*). Because we are unable to show a direct effect of IL-33 on Th17 maturation *in vitro* (Fig. S2), we propose that the effect is manifested indirectly via IL-1 and IL-6 release. This notion provides a mechanism whereby synovial fibroblasts could influence the local activation of mast cells and polarization of local autoreactive T cell responses. Finally, the chemokines produced by IL-33-activated mast cells are also likely to play an important role in the recruitment of inflammatory cells into diseased joints.

Interestingly, IL-33 was still capable of inducing some type II cytokine production (IL-5 and IL-13 but not IL-4) in the context of a Th1/Th17-dominant environment. IL-5 and IL-13 expression has been reported in established RA tissues (34, 35). These cytokines could play a role in the promotion of specific IgG and IgE synthesis and antibody isotype switching in CIA. Such

autoantibodies could bind to both low- and high-affinity Fc receptors on mast cells and subsequently trigger mast cell degranulation (Fig. 6) (28, 29) or form immune-complexes with antigens (23, 24). Our data are compatible with the cytokine milieu described in RA synovial fluid obtained in early disease in which type II cytokines predominate (34, 35). By this mechanism, the target tissue environment including fibroblast activation and consequent IL-33 release could operate to promote the transition from acute to chronic disease via mast cell-dependent pathways. Additional mechanisms, which do not involve mast cell activation, but which could entail transient Th2 activation, may also explain why WT mast cell engraftment in $ST2^{-/-}$ mice did not fully restore the CIA-enhancing effect of IL-33 to the level of WT mice (Fig. 5).

Synovial fibroblasts may be the main source of IL-33 (36). We found that resting synovial fibroblasts expressed little or no IL-33 whereas expression was markedly enhanced by the presence of proinflammatory cytokines, such as TNFα and IL-1β, which are abundantly produced during CIA and RA (15). Thus, IL-33-mast cell interactions that lead to the enhanced production of IL-1β and TNFα may represent a self-amplification circuit in the proinflammatory response during the development and perpetuation of arthritis. Such a model is compatible with the notion that the synovial microenvironment is critical to the maintenance and amplification of local inflammatory responses.

The identification of a proinflammatory role of IL-33 in inflammatory arthritis extends our understanding of the functional diversity of members of the IL-1 family in RA. IL-1 promotes the inflammatory process mainly by amplifying macrophage and Th17 cell functions whereas IL-18 promotes arthritis by enhancing Th1 responses (20, 31, 37). In contrast, IL-33 predominantly triggers mast cell activation and downstream autoantibody production. Thus, IL-33 may synergize with IL-1 and IL-18 by further amplifying Th1/Th17 responses in acute and chronic phases of the disease. The demonstration here that IL-33 is expressed in the synovial membrane of RA patients and that mast cells are directly associated with IL-33-mediated arthritic disease suggests that IL-33 may offer a tractable target to modulate mast cell-dependent pathology therein and beyond in a range of inflammatory diseases.

Materials and Methods

Mice. Male DBA/1 mice were obtained from Harlan Olac. $ST2^{-/-}$ BALB/c mice were generated as described previously (38). $ST2^{-/-}$ mice of the DBA/1 background were generated by backcrossing $ST2^{-/-}$ mice with DBA/1 mice for 10 generations. All mice were used at 8–10 week old and maintained at the Joint Animal Facilities, University of Glasgow. All animal experiments conducted in this study were performed in accordance to the Home Office, United Kingdom, animal guidelines.

Recombinant IL-33. Mouse IL-33 was expressed in *Escherichia coli* and IL-33 proteins were purified by Ni-NTA affinity chromatography as described previously (39). Endotoxin was removed by purification with polymyxin B chromatography. The purity of IL-33 was >97% by silver staining. Endotoxin levels were <0.1 unit/μg of protein by the Limulus Amebocyte Lysate QCL-1000 pyrogen test (Cambrex).

Generation of Bone Marrow-Derived Mast Cells (BMMCs). BMMCs were derived from cells of either $ST2^{-/-}$DBA/1 or WT mice and maintained as described previously (12, 14). Briefly, bone marrow cells from femurs of mice were cultured with IL-3 (5 ng/ml) and SCF (50 ng/ml) in complete RPMI medium 1640 (supplemented with 10% FCS, 2 mM L-glutamine, and 100 units/ml penicillin–streptomycin) in a 5% CO_2 atmosphere. After 6 weeks, BMMCs were collected and tested for c-*kit*, FcεRI, and ST2 expression by flow cytometry with fluorescent-labeled anti-c-*kit* (BD Biosciences), anti-FcεRI (eBioscience), and anti-ST2 (MD Bioscience) antibodies. For *in vitro* activation, BMMCs (2×10^6 per milliliter) were stimulated with IL-33 for 48 h. The levels of TNFα, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFNγ, MCP1α, MCP1β, MCP3, and MIP1α in the supernatant were determined by multiplex ELISA (Luminex cytokine 20-Plex; Biosource) according to the manufacturer's instructions. For *in vivo* engraftment, $ST2^{-/-}$ mice were injected intravenously with *in vitro*-generated BMMCs from either DBA1 WT or $ST2^{-/-}$ mice (1×10^7 per mouse). Four weeks later, the mice were immunized with CII to induce CIA and treated with IL-33 as detailed below.

Induction of CIA and Assessment of Arthritis. CIA was induced in mice as previously described (20). Briefly, male DBA/1 or $ST2^{-/-}$DBA/1 mice, 6–8 weeks old (10 mice per group), were immunized by intradermal injection of 50 μg of acidified bovine type II collagen (CII; Chondrex) that was emulsified in Complete Freund's Adjuvant (CFA; MD Biosciences). In some experiments, the mice were challenged i.p. on day 21 with 50 μg of CII in PBS. To investigate the effect of IL-33 in CIA, mice were injected i.p. daily with IL-33 (1 μg per mouse) from days 2–25 after the initial immunization. Control mice received the same volume of PBS. All mice were monitored for signs of arthritis as previously described (20). Clinical scores were assigned based on erythema, swelling, or loss of function present in each paw on a scale of 0 to 3, giving a maximum score of 12 per mouse. Paw thickness was measured with a dial-calliper (Kroeplin).

Histological Examination. For histological assessment, mice were killed and the hind limbs removed, fixed in 10% neutral-buffered formalin, and 5-μm sections were stained with hematoxylin/eosin (Sigma).

Collagen-Specific *in Vitro* Culture. DLNs were removed on day 42 after primary immunization. Single-cell suspensions (2×10^6 cells per milliliter) were prepared and cultured in triplicates in complete RPMI medium 1640 at 37°C in 5% CO_2. Cells were cultured with graded concentrations of CII (50 and 100 μg/ml) in 96-well plates (Nunc). Supernatants were collected after 72 h for analysis of cytokine concentration.

Measurement of Cytokines and Anti-Collagen Antibody. All cytokine concentrations were determined by ELISA with paired antibodies (BD PharMingen) or a 20-plex mouse cytokine assay according to the manufacturer's instructions (Luminex; Biosource, Invitrogen). The serum anti-type II collagen Ab titers of individual mice were detected as previously described (20).

Clinical Materials and Cell Preparation. Synovial tissue samples from patients meeting the ACR criteria for rheumatoid arthritis were obtained from the tissue bank of The Centre of Rheumatic Diseases, Royal Infirmary, Glasgow, derived by arthoscopy or arthroplasty. The study was approved by the East Glasgow Ethical Committee and informed consent was obtained from each patient. For immunohistochemistry, synovial membrane tissues were fixed in formalin for 24 h and then embedded in paraffin. Serial sections (5 μm) were stained with anti-IL-33 antibody (5 μg/ml; Axxora) or anti-ST2 antibody (15 μg/ml; R&D Systems) and then counterstained with hematoxylin (Sigma). Negative controls were stained with isotype-matched normal IgG at the same concentration. The sections were scanned with a Duoscan T2000XL microscope and pictures taken with a Fuji X digital camera (HC-300Z) at magnification of $\times$40. Synovial fibroblasts were grown from primary synovial tissue cell suspensions in 25-cm^2 flasks and used after three to five passages (fibroblast purity was >99%, analyzed by FACS with anti-CD45 antibody to detect contaminating inflammatory cells). For RT-PCR, cDNA was prepared from synovial fibroblasts by using TRIzol extraction (Life Technologies). mRNA was detected by using specific primers for IL-33 (39) and β-actin (R&D Systems).

Statistical Analysis. Clinical scores were analyzed with the nonparametric Mann–Whitney *U* test. Cytokine and collagen-specific IgG levels were compared with a Student's *t* test.

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