

# IL-33 signaling contributes to the pathogenesis of myeloproliferative neoplasms

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**Myeloproliferative neoplasms (MPNs) are characterized by the clonal expansion of one or more myeloid cell lineage. In most cases, proliferation of the malignant clone is ascribed to defined genetic alterations. MPNs are also associated with aberrant expression and activity of multiple cytokines; however, the mechanisms by which these cytokines contribute to disease pathogenesis are poorly understood. Here, we reveal a non-redundant role for steady-state IL-33 in supporting dysregulated myelopoiesis in a murine model of MPN. Genetic ablation of the IL-33 signaling pathway was sufficient and necessary to restore normal hematopoiesis and abrogate MPN-like disease in animals lacking the inositol phosphatase SHIP. Stromal cell-derived IL-33 stimulated the secretion of cytokines and growth factors by myeloid and non-hematopoietic cells of the BM, resulting in myeloproliferation in SHIP-deficient animals. Additionally, in the transgenic *JAK2<sup>V617F</sup>* model, the onset of MPN was delayed in animals lacking IL-33 in radio-resistant cells. In human BM, we detected increased numbers of IL-33-expressing cells, specifically in biopsies from MPN patients. Exogenous IL-33 promoted cytokine production and colony formation by primary CD34<sup>+</sup> MPN stem/progenitor cells from patients. Moreover, IL-33 improved the survival of *JAK2<sup>V617F</sup>*-positive cell lines. Together, these data indicate a central role for IL-33 signaling in the pathogenesis of MPNs.**

## Introduction

Myeloproliferative neoplasms (MPNs) comprise a heterogeneous group of malignant clonal hematopoietic diseases including, among others, BCR-ABL1-negative polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), as well as BCR-ABL1-positive chronic myelogenous leukemia (CML). While the BCR-ABL1 fusion protein results in a constitutively activated tyrosine kinase activity in CML, BCR-ABL1-negative MPNs often harbor mutations in the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Both types of genetic alterations lead to abnormal proliferation of myeloid cells in the absence of overt signs of morphological dysplasia (1).

Although the role of cytokines and growth factors in normal hematopoiesis is widely recognized, their precise contribution to MPN pathogenesis is still unclear. In vitro, cells from MPN patients are characterized by an intrinsic independence and/or hypersensitivity to growth-factor stimulation and cytokine stimulation (2, 3). In CML cells, the BCR-ABL1 protein stimulates constant autocrine production of IL-3, IL-6, G-CSF, and TNF (3–5). The long-term

outcome of CML patients has substantially improved with the use of the tyrosine kinase inhibitor imatinib, yet its efficacy may be hampered by the action of cytokines (6). The *JAK2<sup>V617F</sup>* mutation is often found in BCR-ABL1-negative MPNs and similarly results in constitutively activated kinase activity, mimicking persistent signaling via multiple cytokine pathways. While JAK inhibitors have been successful in treating *JAK2*-mutated MPNs, elimination of the mutant clone and resistance may pose an important problem, and patients with WT *JAK2* MPNs are not amenable to this therapy (7). The abnormal expression and activity of a number of proinflammatory cytokines are associated with BCR-ABL1-negative MPNs (8), possibly initiating clonal evolution (9) or promoting progression to myelofibrosis (10) and resistance against *JAK2* inhibitors (11). Thus, cytokines and growth factors support the aberrant hematopoiesis associated with MPNs and provide mechanisms for drug resistance.

While several studies suggest that concomitant inflammation supports MPN pathogenesis and development, it remains elusive whether inflammation precedes and may thus initiate the myeloproliferation. Indeed, previous inflammatory conditions have been linked to myeloid malignancies (12). Moreover, recent epidemiologic studies have reported an association between MPNs and either certain autoimmune diseases (13) or antecedent chronic inflammatory conditions of infectious origin (14). Microbe-sensing receptors — and TLRs in particular — may initiate such inflamma-

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tion, and monocytes from MPN patients may display an increased responsiveness to TLR ligands, leading to excessive cytokine production (15). Therefore, and although strong causative evidence is currently lacking, microbial infections driving sustained inflammation may potentially trigger MPNs.

IL-33 is a recently identified member of the IL-1 family of cytokines that binds to a heterodimeric receptor complex consisting of ST2 (IL-1RL1) and IL-1 receptor accessory protein. IL-33 signaling is then further transduced via recruitment of the adapters MyD88 and IL-1 receptor-associated kinase 4 (IRAK4), which are shared with TLRs. IL-33 is predominantly expressed by endothelial and epithelial cells (16, 17), and it has primarily been implicated in the induction of type-2 immune responses by binding to ST2, which is expressed on a variety of cells such as Th2 CD4<sup>+</sup> T cells, mast cells, and basophils (16). While IL-33 has recently emerged as a cytokine with pleiotropic functions in diverse types of inflammatory processes — including inflammatory bowel disease (18) and virus-induced inflammation (19, 20) — its function in tumorigenesis is poorly characterized.

Here, we used a mutant allele of the inositol polyphosphate-5-phosphatase D (*Inpp5d*, which encodes SHIP, an SRC homology 2 domain-containing inositol-5-phosphatase) called *styx* to investigate the mechanisms underlying spontaneous development of MPN-like disease. Since microbial cues can be sufficient to drive inflammatory disease in genetically susceptible strains (21, 22), we assessed the suppressive effect of a genetic blockade of selected inflammatory pathways in the pathogenesis of MPN-like disease in *Inpp5d<sup>styx/styx</sup>* mice (herein referred to as *styx* mice). We identified a critical role for MyD88 and IRAK4 in determining the phenotype. Our data indicate that microbial-derived signals are dispensable, whereas the IL-33/ST2 pathway is non-redundant for initiating uncontrolled myelopoiesis in *styx* mutants. In addition, IL-33 contributes to the development of *JAK2<sup>V617F</sup>*-dependent MPNs in mice and promotes colony formation of human primary CD34<sup>+</sup> MPN stem/progenitor cells. IL-33 is also increased in BM biopsies of MPN patients. Together, these results reveal a central functional involvement of the IL-33/ST2 pathway in MPN pathogenesis.

## Results

*Development of MPN-like disease is directed by MyD88 but independent of microbiota-derived signals.* As a model of MPN, we used a *Inpp5d styx* allele that was identified in an *N*-ethyl-*N*-nitrosourea (ENU) genetic screen. The *styx* point mutation results in a thymine-to-adenine transversion in the donor splice site of intron 5 of the *Inpp5d* gene, causing an aberrant transcript lacking exon 5, which in turn leads to loss of SHIP protein expression (Supplemental Figure 1, A–D; supplemental material available online with this article; doi:10.1172/JCI77347DS1). SHIP is a negative regulator of the PI3K pathway in hematopoietic cells, and its deficiency leads to increased numbers of granulocyte-macrophage progenitors (GMPs) in the BM and spleen (23). Homozygous *styx* mice entirely recapitulate the myeloproliferative-like phenotype of the described *Inpp5d* KO strains (23, 24), showing hyperproliferative BM, associated splenomegaly, and myeloid cell infiltration into several organs, including the spleen, the ileum, and particularly the lung (Supplemental Figure 1E). As a consequence, *styx* mutants display a chronic progressive and fatal wasting disease with kinetics similar to *Inpp5d* KO strains (Figure 1A; ref. 23).

Given the possible association between certain infections and malignant transformation of the myeloid lineage (14), we investigated the putative role for environmental inflammatory cues in the development of dysregulated myeloproliferation in the *styx* model. We followed a genetic strategy to systematically disrupt signaling molecules relevant for microbe sensing, focusing on TLRs. Because of its pure C57BL/6J background, the *styx* strain appeared particularly suitable for such an approach, in contrast to the non-C57BL/6 *Inpp5d* KO strains generated so far (23–25).

We found that genetic disruption of *Myd88*, a central protein adapter that relays signals from most TLRs, abrogated fatal MPN-like disease in *styx* mutants (Figure 1, A and B). Accordingly, *styx* mice lacking IRAK4, a protein directly downstream of MyD88 in the signaling cascade, were also rescued from the MPN phenotype (Figure 1A). However, single deletion of *Tlr2* or *Tlr4*, critical sensors of microbial products derived from commensal bacteria, failed to prevent disease progression on the *styx* background. *styx Unc93b1<sup>3d/3d</sup>* double mutants with impaired endosomal TLR signaling died earlier compared with *styx* mice, likely from an increased susceptibility to opportunistic infections (Supplemental Figure 2A).

We further hypothesized that MPN development may be provoked by synergistic signaling via multiple TLRs converging toward MyD88 and IRAK4. However, *styx* mice rederived into a germ-free environment, and consequently exposed to a greatly reduced quantity of TLR ligands, developed MPN-like disease at the same rate and severity as mice kept under specific pathogen-free (SPF) conditions (Supplemental Figure 2).

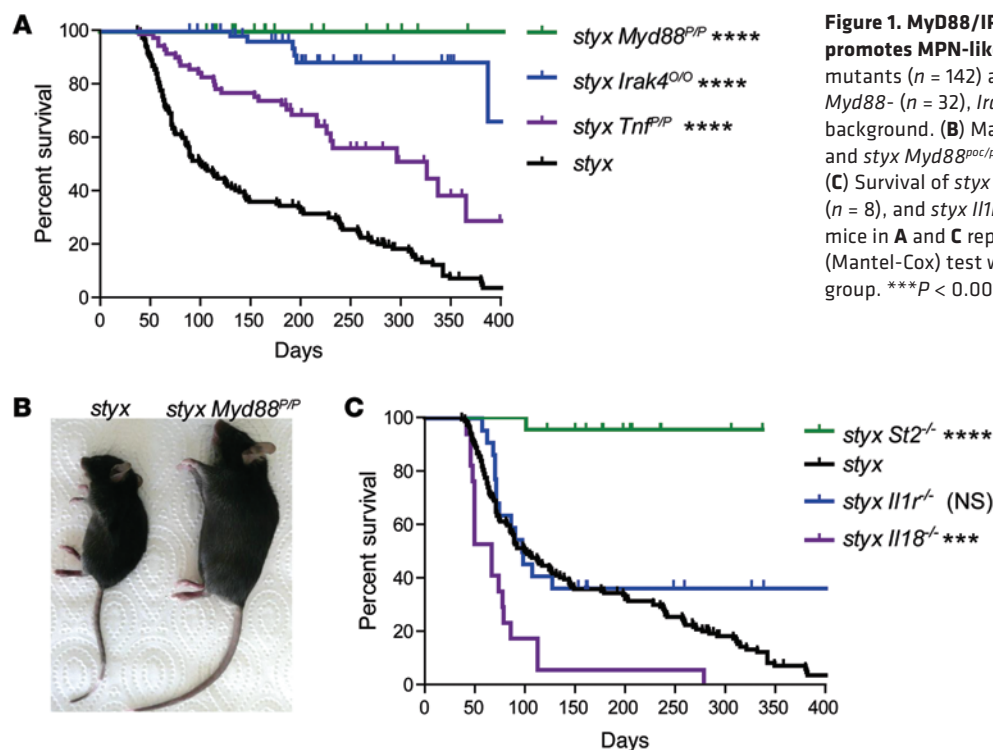
TLR signaling triggers host cell responses with production of effector cytokines such as TNF. Introduction of a mutation disrupting TNF binding to its receptor significantly extended lifespan but failed to completely rescue *styx* mice. This reveals a partial contribution of TNF to disease development and associated immunopathology in our model (Figure 1A).

In brief, progression of MPN-like disease in *styx* mutants is controlled by signaling through MyD88 and IRAK4, but not TLRs. Moreover, the commensal microbiota is not required to induce the observed phenotype.

*A non-redundant contribution of the IL-33/ST2 pathway to MPNs.* MyD88 and IRAK4 not only transduce signaling from TLRs, but they are also located downstream of the receptors for the cytokines IL-1, IL-18, and IL-33. Abrogation of signaling through the IL-1 or IL-18 pathways did not limit SHIP-controlled myeloproliferation (Figure 1C).

However, *styx* mice with bi-allelic deficiency in the MyD88-dependent IL-33 receptor ST2 were found to be protected from disease development (Figure 1C), demonstrating that signaling from ST2 was necessary to induce MPN-like disease in *styx* mutants.

Dysregulated BM hematopoiesis is a key feature of MPNs in humans and is also the primary etiology for the myeloproliferative disease accompanying SHIP deficiency (23). We thus assessed hematopoietic stem cell (HSC) and myeloid progenitor cell populations in homozygous *styx*, *styx St2<sup>-/-</sup>*, WT, and *St2<sup>-/-</sup>* mice by flow cytometry, according to Wilson et al. (Supplemental Figure 3, A and B; ref. 26). Percentages of HSCs (Figure 2, A and B) and myeloid progenitors (Figure 2, C and D) were significantly increased in the BM of *styx* mice, compared with WT



**Figure 1. MyD88/IRAK4-dependent IL-33/ST2 signaling promotes MPN-like disease.** (A) Survival of single *styx* mutants ( $n = 142$ ) and of *styx* mice backcrossed on a *Myd88*<sup>-</sup> ( $n = 32$ ), *Irak4*<sup>-</sup> ( $n = 61$ ), and *Tnf*<sup>-</sup> ( $n = 68$ ) deficient background. (B) Macroscopic phenotype of 14-week-old *styx* and *styx Myd88<sup>poc/poc</sup>* male mice that are *Myd88* deficient. (C) Survival of *styx* ( $n = 142$ ), *styx St2<sup>-/-</sup>* ( $n = 22$ ), *styx Il1r1<sup>-/-</sup>* ( $n = 8$ ), and *styx Il18<sup>-/-</sup>* ( $n = 22$ ) mice. Survival curves for *styx* mice in A and C represent the same group of mice. Log-rank (Mantel-Cox) test was used, using *styx* mice as a reference group. \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

mice. However, genetic blockade of IL-33/ST2 signaling prevented the aberrant hematopoiesis associated with SHIP deficiency, since percentages of BM HSC and myeloid progenitor cell populations were comparable in *styx St2<sup>-/-</sup>* mice and WT or *St2<sup>-/-</sup>* controls (Figure 2, A-D).

In the periphery, effective hematopoiesis with granulocytosis and monocytosis were observed in *styx* but not *styx St2<sup>-/-</sup>* mice, as measured by complete blood count (CBC) analysis (Figure 2E). We next functionally analyzed *styx*, *styx St2<sup>-/-</sup>*, WT, and *St2<sup>-/-</sup>* BM lineage-negative (*lin*<sup>-</sup>) cells for their capacity to form myeloid colonies. SHIP-deficient HSCs are known for their increased capacity to form myeloid colonies due to their enhanced sensitivity to cytokines (23, 24). Accordingly, *styx lin*<sup>-</sup> cells exhibited markedly increased colony formation compared with *St2<sup>-/-</sup>* and WT controls. However, colony numbers were similarly augmented for *styx St2<sup>-/-</sup>* cells (Figure 2F). This implies that although disruption of IL-33/ST2 signaling normalizes hematopoietic subsets in *styx* mice, *styx St2<sup>-/-</sup>* cells retain an intrinsic hyperproliferative ability.

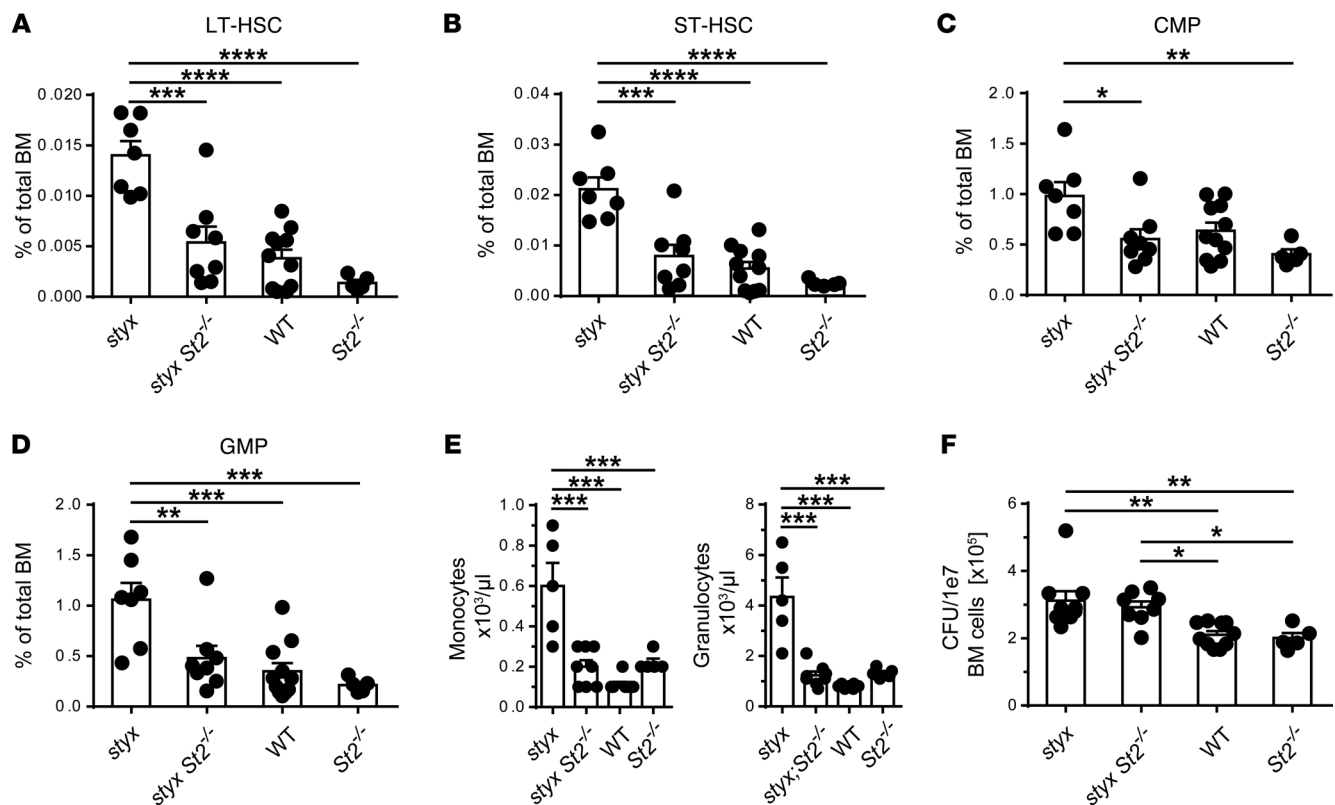
Taken together, these results reveal that steady-state engagement of the IL-33/ST2 pathway acts as a non-redundant trigger for the accumulation of BM HSCs and myeloid progenitors, and for the concomitant MPN-like phenotype observed upon SHIP loss of function.

*Stromal IL-33 engages ST2 signaling on both the radio-resistant and the radio-sensitive compartments to induce MPN-like disease.* To determine the source of IL-33 and the compartment(s) expressing ST2 in our model system, we next generated various sets of BM chimeric mice. Lethally irradiated *St2<sup>-/-</sup>* or control WT hosts reconstituted with *styx* BM developed MPN-like disease with similar kinetics (Figure 3A). However, genetic abrogation of IL-33/ST2 signaling on a hematopoietic SHIP-deficient compartment significantly delayed, but did not prevent, disease development (Figure

3B and Supplemental Table 1). These findings demonstrate that ST2 can be engaged on both stromal and hematopoietic cells and that the 2 compartments can compensate for each other to induce MPN-like disease in our model.

Importantly, lethally irradiated *Il33<sup>-/-</sup>* mice reconstituted with *styx* BM did not develop MPN-like disease (Figure 3A and Supplemental Table 1), thereby indicating a non-hematopoietic origin of IL-33 for uncontrolled myelopoiesis. Since the BM is the primary site of hematopoiesis in adult mice, we investigated whether BM stromal cells produce IL-33. IHC analysis showed that IL-33 protein in BM was expressed at similar levels in *styx*, *styx St2<sup>-/-</sup>*, WT, and *St2<sup>-/-</sup>* mice. Furthermore, IL-33 expression was restricted to few cells — most of which had characteristic histological features of endothelial cells (ECs) — lining the BM sinusoids (Supplemental Figure 3). Thus, although IL-33 is not constitutively expressed in ECs along the vascular tree (17), our data demonstrate that it is expressed in some BM sinusoidal ECs.

*ST2 is expressed on BM stromal and hematopoietic cells, but not on myeloid progenitors.* To assess how IL-33 produced by radio-resistant cells induces MPNs in *styx* mutants, we analyzed the expression pattern of the ST2 receptor on BM cells of *styx*, *styx St2<sup>-/-</sup>*, WT, and *St2<sup>-/-</sup>* mice. While surface ST2 was not detectable on BM HSCs and myeloid progenitors (Supplemental Figure 4), we identified a heterogeneous *lin*<sup>+</sup> population of myeloid origin cells that expressed ST2 that was CD11b<sup>int</sup>, Ly6C<sup>-</sup>, and Ly6G<sup>-</sup> (Figure 4, A and B). Further phenotypic analysis revealed that the majority of these cells most likely represented early myeloid cells and were CD11b<sup>int</sup>, FcεRI<sup>-</sup>, and c-KIT<sup>-</sup>, while the minority were basophils, defined as CD11b<sup>int</sup>, FcεRI<sup>+</sup>, c-KIT<sup>+</sup>, and CD49b<sup>+</sup> (Supplemental Figure 5A). These two ST2<sup>+</sup> populations accounted for 5%–6% and 0.7% of total hematopoietic cells in the BM, respectively (data not shown). Importantly, ST2<sup>+</sup> BM cells in *styx* versus



**Figure 2. IL-33/ST2 signaling alters BM HSC and myeloid progenitor cell populations, promoting systemic myeloproliferation.** Flow cytometric analysis of  $lin^{-}$  BM cells show different frequencies of (A) long-term HSC (LT-HSC) and (B) short-term HSC (ST-HSC), (C) common myeloid progenitors (CMP) and (D) GMPs in the indicated mouse strains. (E) CBC analysis of myeloid cells. (F) CFU of the indicated  $lin^{-}$  BM cells after 7 days of culture in the presence of IL-3, FLT3-ligand, and SCF. Data are mean  $\pm$  SEM. Pooled data from 3 (A and D) or 2 (E and F) independent experiments are represented ( $n = 5-10$  mice per group). (A-F) One-way ANOVA with Bonferroni post hoc test was used; only statistically significant data are presented. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; and \*\*\*\* $P < 0.0001$ .

WT mice did not differ in their relative distribution or their ST2 expression levels (data not shown).

Since our BM chimera experiments indicated that the radio-resistant compartment was also able to promote MPN-like disease in an ST2-dependent manner, we correspondingly assessed ST2 expression on BM stromal cells. We found that ECs (defined as  $CD45^{-}$ ,  $lin^{-}$ ,  $CD51^{-}$ , and  $CD31^{+}$ ) and mesenchymal stromal cells (MSCs; specified as  $CD45^{-}$ ,  $lin^{-}$ ,  $CD31^{-}$ ,  $CD51^{+}$ , and  $CD140a^{+}$ ; ref. 27) in the BM expressed ST2, although ST2 expression was marginal on MSCs (Figure 4C and Supplemental Figure 5B).

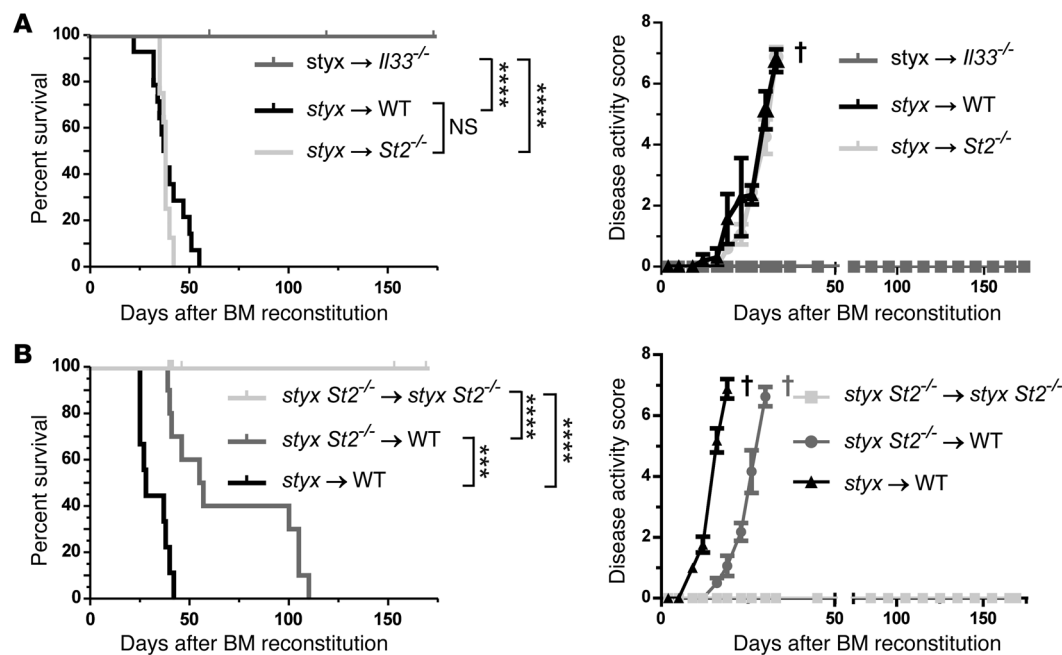
Together, our data suggest that the BM microenvironment contains the minimal requirements to promote myeloproliferative disease on a SHIP-deficient background via activation of IL-33/ST2 signaling. Furthermore, the expression pattern of ST2 indicates that IL-33/ST2 signaling likely has an indirect effect on *styx* HSCs and myeloid progenitors.

*IL-33 acts indirectly on HSCs and myeloid progenitors.* In order to determine a potential indirect mode of action of IL-33/ST2 signaling in HSCs and myeloid progenitor cells, we used magnetic cell separation to purify  $lin^{-}$  cells from *styx* or WT BM and assessed their capacity to form colonies in the presence or absence of IL-33 (Figure 4D). IL-33 alone did not affect colony formation of  $lin^{-}$  BM cells. However, stimulation with IL-6 and/or GM-CSF, factors known to promote myeloproliferation and contribute to the dysregulated hematopoiesis characteristic of SHIP-deficient mice

(23, 24, 28), increased the number of colonies from *styx* and WT  $lin^{-}$  BM cells. Moreover, addition of IL-33 to IL-6 and GM-CSF did not further modulate the colony-forming ability of  $lin^{-}$  cells (Figure 4D). These results show that IL-33 does not promote colony formation of mouse HSCs and myeloid progenitor cells directly, and it does not synergize with other known myeloproliferative factors. Also, and in agreement with our ST2 expression analysis, SHIP-deficiency is not associated with an increased sensitivity of HSCs and myeloid progenitors to IL-33, as observed for IL-6 and GM-CSF (Figure 4D).

We next performed a coculture assay to dissect how IL-33/ST2 signaling indirectly supports development of MPN-like disease in *styx* BM. We assessed the contribution of stromal cells, as well as the role of the hematopoietic cell population we previously identified as constitutively expressing ST2. To this end, we measured colony formation by  $St2^{-/-}$   $lin^{-}$  cells following coculture with IL-33-stimulated primary stromal cells (MACS-purified  $CD45^{-}$  BM cells) or after supplementation with conditioned medium from IL-33-stimulated,  $St2^{+}$  BM hematopoietic cells. Under these experimental settings, we observed an increase in colony formation of *styx St2<sup>-/-</sup>* or *St2<sup>-/-</sup>*  $lin^{-}$  BM cells solely in the combined presence of exogenous IL-33 and ST2-expressing stromal or hematopoietic responders (Figure 4E).

Hence, we concluded that IL-33 can indirectly support development of MPN-like disease in *styx* mutants via the activation of the radio-resistant compartment, as also indicated by our BM chimera



**Figure 3. Stromal cell-derived IL-33 engages ST2 signaling on both the stromal and the hematopoietic compartment to induce MPN-like disease.** (A) Contribution of IL-33 and IL-33/ST2 signaling in radio-resistant cells. (B) Contribution of IL-33/ST2 signaling in radio-sensitive cells. Survival curves and disease activity index are shown for the indicated groups of BM chimeric mice. Pooled data from 2 independent experiments are represented each for (A) *styx*→WT ( $n = 14$ ), *styx*→*St2*<sup>-/-</sup> ( $n = 8$ ), *styx*→*Il33*<sup>-/-</sup> ( $n = 9$ ), and (B) *styx*→WT ( $n = 9$ ), *styx* *St2*<sup>-/-</sup>→*styx* *St2*<sup>-/-</sup> ( $n = 10$ ), and *styx* *St2*<sup>-/-</sup>→WT ( $n = 10$ ). Log-rank (Mantel-Cox) test was used. † indicates euthanasia of animals. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

experiments (Figure 3). Our results also suggest that the ST2-expressing hematopoietic population in the BM is able to sustain myeloproliferation, likely via the secretion of soluble factors. In addition, the contribution of ST2<sup>+</sup> hematopoietic cells to development of myeloproliferation in *styx* mice appears to be superior to the one provided by ST2<sup>+</sup> stromal cells, in line with our findings from the BM chimeras.

*IL-33 induces cytokine and growth-factor secretion by BM cells.* Increased levels of several growth and survival factors in the peripheral blood have been proposed to contribute to the dysregulated hematopoiesis observed in SHIP-deficient mice (29). Accordingly, analysis of the sera of 40-day-old *styx* mice that were healthy in appearance showed that the levels of cytokines critical in supporting myelopoiesis — including G-CSF, IL-6, and to a lesser extent GM-CSF, M-CSF, IL-3, and IL-5 — were already elevated in several mutants at this age (Supplemental Figure 6, A–F). Conversely, the same cytokines were present at low concentrations in age-matched *styx* *St2*<sup>-/-</sup>, WT, or *St2*<sup>-/-</sup> mice. Although we previously identified TNF as a partial mediator of MPN-like disease, it was not elevated in the serum of young *styx* mice (Figure 1A and Supplemental Figure 6G). However, serum IL-33 levels were significantly augmented in the mutants (Supplemental Figure 6H).

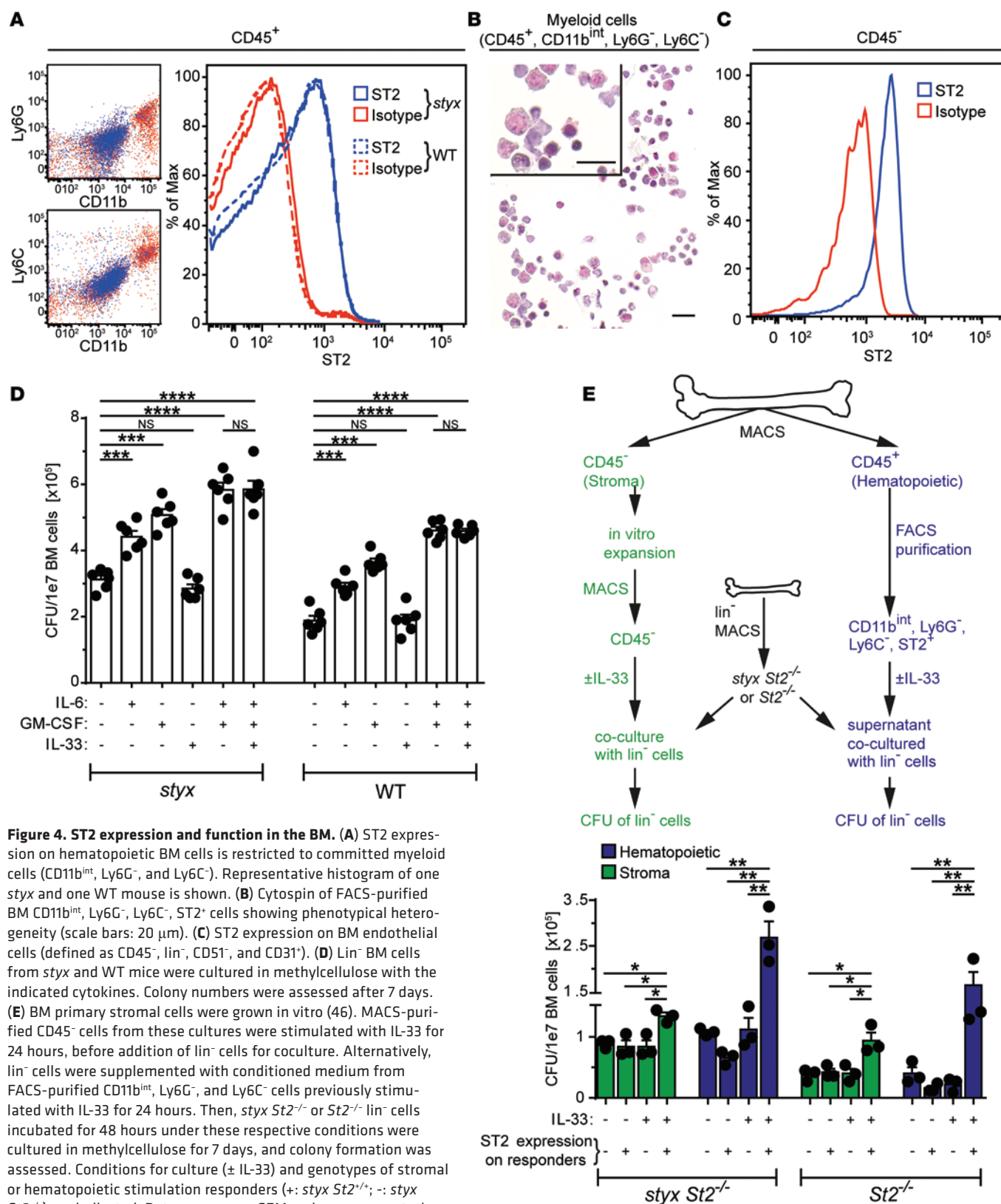
SHIP has been suggested to regulate the production of soluble factors produced by the BM niche (29). To assess the ability of the BM microenvironment to secrete cytokines and growth factors in response to IL-33 and thus promote myelopoiesis, we isolated BM cells from *styx* and WT mice, and we MACS-purified them into CD45<sup>-</sup> non-hematopoietic and CD45<sup>+</sup> hematopoietic cell fractions. Ex vivo stimulation with IL-33 increased the levels of G-CSF, GM-CSF, IL-3, and IL-6 in the superna-

tants of both CD45<sup>-</sup> and CD45<sup>+</sup> cell fractions (Figure 5). IL-5 was only secreted by CD45<sup>+</sup> hematopoietic cells. Engagement of IL-33/ST2 signaling was required, as specific cytokine secretion was abrogated in the absence of ST2 (Figure 5). Therefore, we deduced that the BM microenvironment is able to produce growth factors in response to secreted IL-33 to provoke uncontrolled myeloproliferation in *styx* mice.

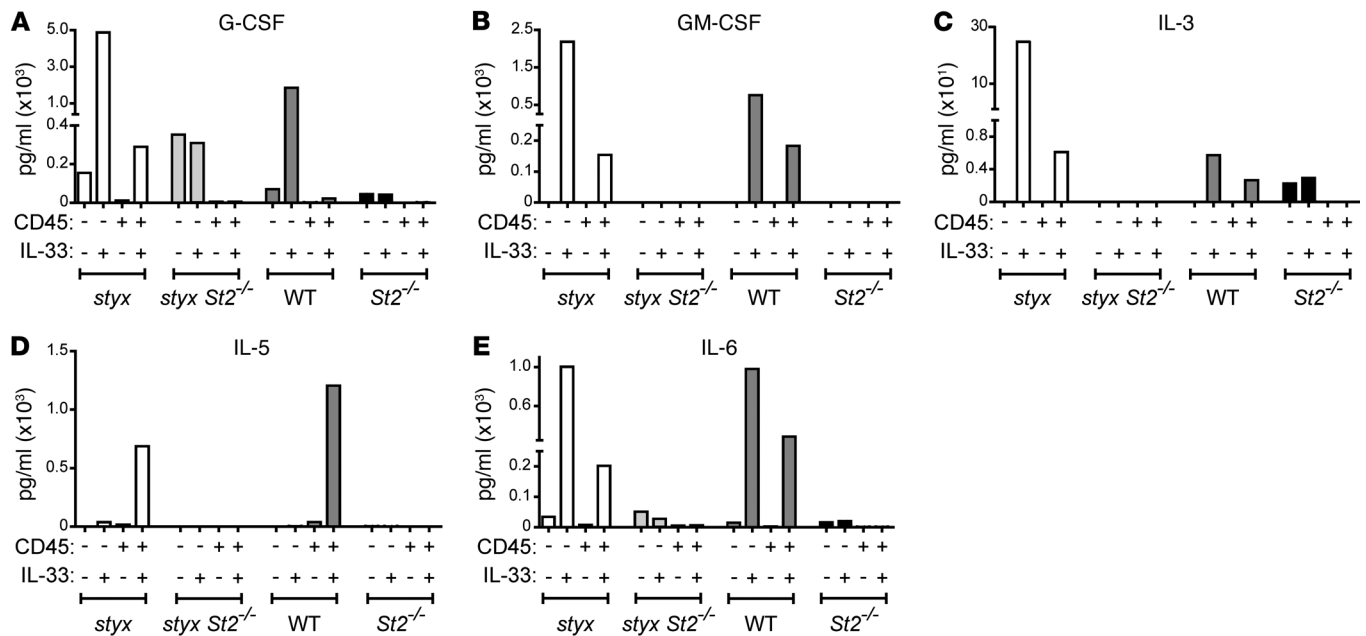
SHIP-deficient myeloid cells display a higher responsiveness to cytokines, such as GM-CSF and IL-3, that leads to a reduced susceptibility to apoptotic stimuli (23, 24). Consequently, we next evaluated whether IL-33/ST2 signaling may affect the viability of *styx* BM cells. We found that ex vivo CD11b<sup>+</sup> BM *styx* *St2*<sup>-/-</sup> cells cultured for short term (6 hours) without cytokine supplementation showed survival rates similar to control cells but reduced compared with ST2-proficient *styx* cells (Supplemental Figure 7A). Correspondingly, in vitro stimulation with IL-33 promoted the survival of *styx* but not *styx* *St2*<sup>-/-</sup> CD11b<sup>+</sup> BM cells (Supplemental Figure 7B).

In summary, these data support the notion that IL-33 is an important trigger for the production of effector cytokines by the stromal and hematopoietic compartments. These cytokines, in turn, promote enhanced survival and proliferation of myeloid cells, eventually resulting in MPN-like disease in *styx* mutants.

*Stromal IL-33 supports the development of JAK2<sup>V617F</sup>-dependent MPNs in vivo.* To assess the contribution of IL-33/ST2 signaling to dysregulated myelopoiesis in a different model of MPN, we adoptively transferred BM cells transgenic for the *JAK2*<sup>V617F</sup> mutation (30) into *Il33*<sup>-/-</sup> recipient mice. In these chimeras, we found a lower percentage of *JAK2*<sup>V617F+</sup> GMPs in the BM and a reduced frequency



**Figure 4. ST2 expression and function in the BM.** (A) ST2 expression on hematopoietic BM cells is restricted to committed myeloid cells (CD11b<sup>int</sup>, Ly6G<sup>-</sup>, and Ly6C<sup>-</sup>). Representative histogram of one *styx* and one WT mouse is shown. (B) Cytopsin of FACS-purified BM CD11b<sup>int</sup>, Ly6G<sup>-</sup>, Ly6C<sup>-</sup>, ST2<sup>-</sup> cells showing phenotypical heterogeneity (scale bars: 20 μm). (C) ST2 expression on BM endothelial cells (defined as CD45<sup>-</sup>, lin<sup>-</sup>, CD51<sup>+</sup>, and CD31<sup>+</sup>). (D) Lin<sup>-</sup> BM cells from *styx* and WT mice were cultured in methylcellulose with the indicated cytokines. Colony numbers were assessed after 7 days. (E) BM primary stromal cells were grown in vitro (46). MACS-purified CD45<sup>-</sup> cells from these cultures were stimulated with IL-33 for 24 hours, before addition of lin<sup>-</sup> cells for coculture. Alternatively, lin<sup>-</sup> cells were supplemented with conditioned medium from FACS-purified CD11b<sup>int</sup>, Ly6G<sup>-</sup>, and Ly6C<sup>-</sup> cells previously stimulated with IL-33 for 24 hours. Then, *styx St2<sup>-/-</sup>* or *St2<sup>-/-</sup>* lin<sup>-</sup> cells incubated for 48 hours under these respective conditions were cultured in methylcellulose for 7 days, and colony formation was assessed. Conditions for culture (± IL-33) and genotypes of stromal or hematopoietic stimulation responders (+: *styx St2<sup>-/-</sup>*; -: *styx St2<sup>-/-</sup>*) are indicated. Data are mean ± SEM and are representative of (A) *n* = 8 mice per group; (B) *n* = 2 mice per group; (C) pooled cells from 4 WT mice, repeated twice; (D) pooled data of 2 experiments, *n* = 6 mice per group; and (E) 1 experiment where each data point represents 2 pooled mice (*n* = 6 mice per group). Stromal and hematopoietic responders were pooled from 5 donors. (D and E) One-way ANOVA with Bonferroni post hoc test was used. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; and \*\*\*\**P* < 0.0001.



**Figure 5. IL-33 is a potent inducer of cytokines and growth factors promoting myelopoiesis.** BM cells from the indicated strains were MACS-purified into CD45<sup>+</sup> (enriched for hematopoietic) and CD45<sup>-</sup> (enriched for stromal) cells and cultured  $\pm$  IL-33. After 24 hours, supernatant was collected, and concentrations of (A) G-CSF, (B) GM-CSF, (C) IL-3, (D) IL-5, and (E) IL-6 were measured by LASER Bead Technology. Each condition represents the value measured from pooled cells from 7 (*styx*) or 10 (*styx St2<sup>-/-</sup>*, WT, and *St2<sup>-/-</sup>*) mice. A comparable trend was found after 12 hours of culture  $\pm$  IL-33 (not depicted).

of blood neutrophils (defined as CD11b<sup>+</sup> and Ly6G<sup>+</sup>) compared with control mice expressing IL-33 in the stroma (Figure 6, A and B). IL-33-deficiency in the radio-resistant compartment also correlated with delayed expansion of several hematopoietic lineages from donor *JAK2<sup>V617F</sup>* BM cells (Figure 6C). Taken together, these data suggest that stromal cell-derived IL-33 supports the development of *JAK2<sup>V617F</sup>*-dependent MPNs in vivo.

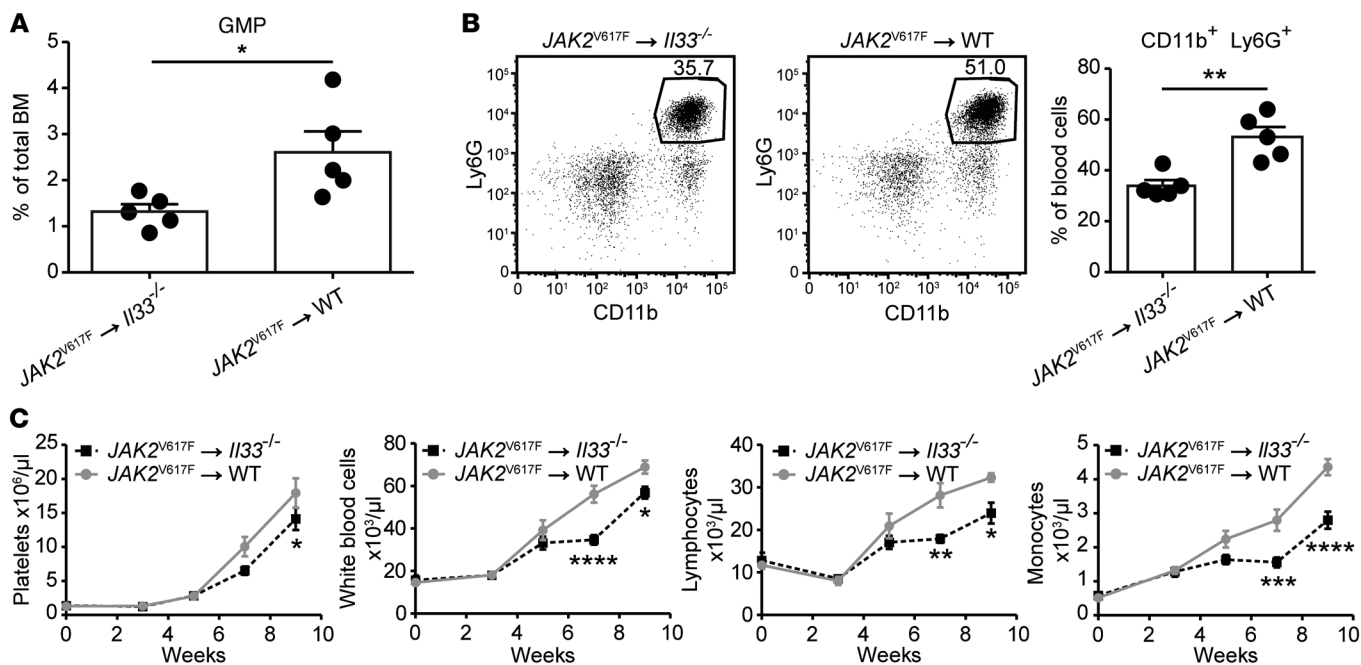
**Increased levels of IL-33 protein in the BM of MPN patients.** To address the general relevance of the findings from our mouse models of MPN, we assessed the presence of IL-33 in the BM of MPN patients. IL-33 was also expressed in human BM biopsies and was localized in the nucleus of cells that were mainly but not solely endothelial, based on their histomorphological presentation and expression of CD34 (Figure 7, A and B). Automated quantification of cells displaying nuclear IL-33 expression indicated that MPN patients newly diagnosed with ET, PMF, and PV, but not CML, display a higher percentage of IL-33<sup>+</sup> BM cells compared with controls (Figure 7C). This increase seemed specific to BCR-ABL1-negative MPN patients, as it was not detected in other types of chronic or acute leukemia affecting the myeloid or lymphoid lineages (Supplemental Figure 8A). Hence, these data suggest that the IL-33/ST2 pathway may also play a role in the pathogenesis of human MPNs.

**MPN cell lines and CD34<sup>+</sup> stem/progenitor cells from MPN patients directly respond to IL-33.** The IHC findings from patient BM biopsies raised the question as to whether IL-33 also exerts a proliferative effect on human MPN cells. In particular, the increase in IL-33 protein expression in BCR-ABL1-negative MPNs prompted us to examine the effect of IL-33 on human *JAK2<sup>V617F</sup>* cells. In vitro experiments showed that IL-33 pro-

moted the upregulation of *CSF2* (*GMCSF*) and *IL6* transcripts in several *JAK2<sup>V617F</sup>* cell lines (Figure 8A). These cell lines constitutively expressed ST2 on the surface (Supplemental Figure 8B). IL-33 did not stimulate the growth of MPN cell lines; however, it improved their survival (Figure 8B). Human BCR-ABL1<sup>+</sup> CML cell lines expressed ST2 at various intensities, and they also upregulated the transcription of growth factors when stimulated with IL-33 (Supplemental Figure 8, C and D). However, IL-33 did not influence the proliferation or the survival of CML cell lines (Supplemental Figure 8E). These results suggest that *JAK2<sup>V617F</sup>* cells are more sensitive to the effects of IL-33/ST2 signaling compared with BCR-ABL1<sup>+</sup> CML cells.

Primary human CD34<sup>+</sup> hematopoietic stem/progenitor cells (HSPCs) express ST2 receptor and secrete cytokines and chemokines when stimulated with IL-33 (31). We detected increased ST2 expression on CD34<sup>+</sup> stem/progenitor cells from BCR-ABL1-negative MPN patients (Figure 8, C and D). In agreement with our data from cell lines, *GMCSF* expression was significantly upregulated in circulating CD34<sup>+</sup> stem/progenitor cells from CML patients and CD34<sup>+</sup> HSPCs from controls following IL-33-mediated stimulation (Supplemental Figure 8F). Furthermore, IL-33 promoted colony formation of CD34<sup>+</sup> MPN stem/progenitor cells but not of CD34<sup>+</sup> control HSPCs (Figure 8E).

Collectively, these observations extend our in vivo data in SHIP-deficient mice and substantiate their relevance to human MPNs. Our findings also point toward differences as to how the IL-33/ST2 pathway may exert an effect on MPN progenitors — either via the intermediate of differentiated cells that are ST2<sup>+</sup> in the mouse model or directly on ST2-expressing CD34<sup>+</sup> stem/progenitor cells in MPN patients (Figure 9).



**Figure 6. Stromal cell-derived IL-33 supports the development of *JAK2*<sup>V617F</sup>-dependent MPNs in vivo.** BM cells from *Scl-Cre JAK2*<sup>V617F</sup> transgenic donor mice were injected into lethally irradiated *Il33*<sup>-/-</sup> or WT recipient mice. After 7 weeks, (A) percentage of GMPs in the BM and (B) frequency of neutrophils in the blood were assessed. (C) Alternatively, indicated blood parameters were evaluated every other week by CBC, starting 3 weeks after reconstitution.  $n = 5$  mice per group. For C, 1 representative of 2 independent experiments is shown. (A and B) Standard Student's *t* test, and (C) 2-way ANOVA with Bonferroni post hoc test were used. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; and \*\*\*\* $P < 0.0001$ .

## Discussion

IL-33 has hitherto essentially been described as a nuclear alarmin that is released following necrotic cell death or under conditions of cellular stress. On the other hand, IL-33 undergoes caspase-mediated inactivation during apoptosis (reviewed in ref. 16). Inflammatory cues such as IFN $\gamma$  and IL-4, as well as microbial products such as LPS, strongly induce expression of endogenous IL-33 (17, 32). Here we present evidence that IL-33 signaling can also be engaged in vivo to drive tumorigenesis and immunopathology under sterile, steady-state conditions in mice.

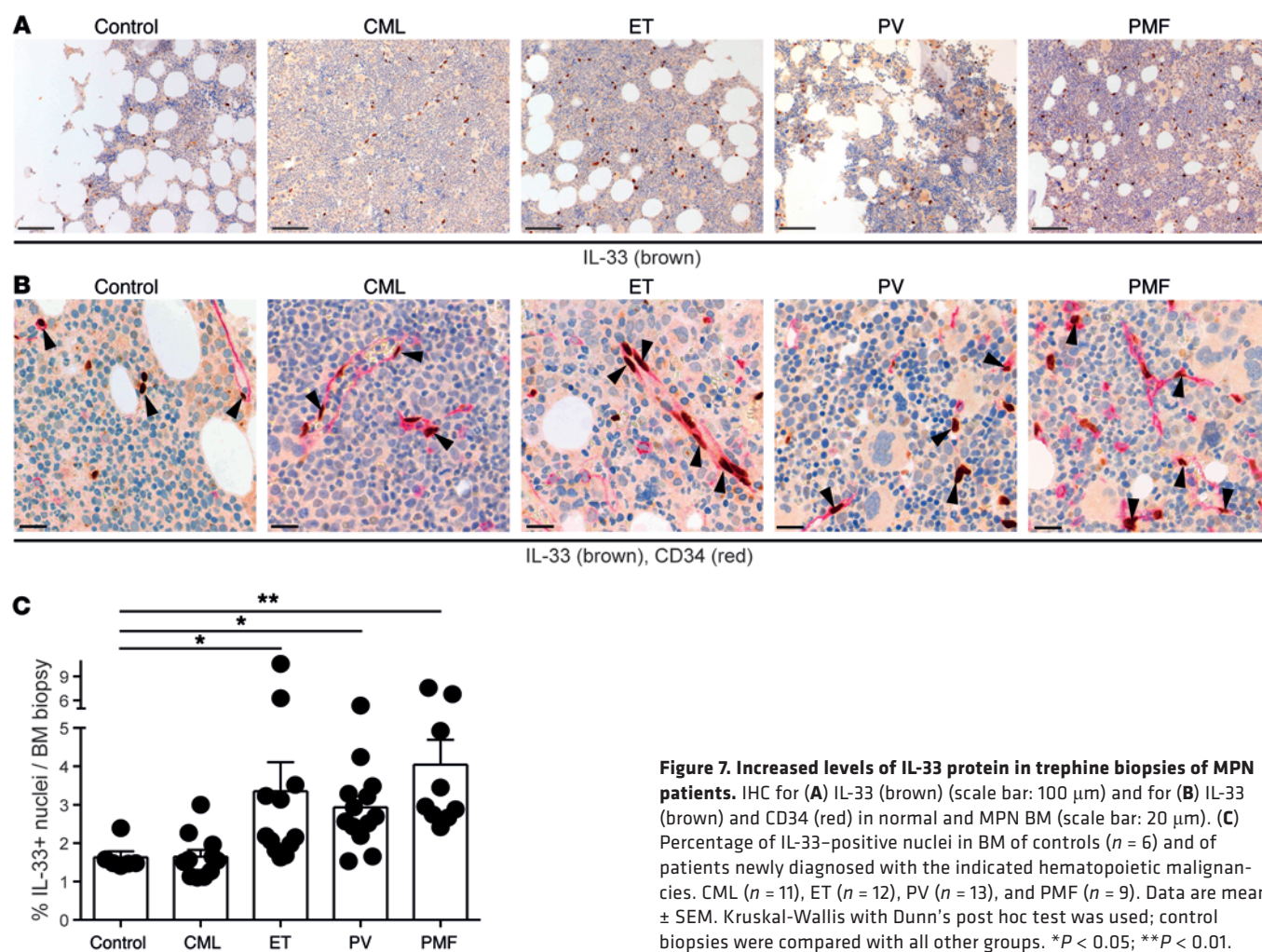
Although we can merely speculate about the initial source of IL-33 to induce MPN-like disease in *styx* mice, the BM stroma emerges as a valid local provider of this cytokine, since IL-33 is expressed in the niche. Exposure to fungus allergens leads to the ATP-dependent release of IL-33 in the airways of challenged mice (33). However, no mechanism has been proposed so far as to whether and how active IL-33 may be released or secreted from healthy, non-stressed cells in vivo. Nevertheless, low amounts of the active form of IL-33 can be detected in the serum of healthy human donors (34) and in 2 of the 8 naive WT mice we tested. Serum IL-33 can likely transit through the BM. Notably, *St2*<sup>-/-</sup> mice displayed higher levels of serum IL-33 than WT controls ( $122 \pm 42$  versus  $3 \pm 1$  pg/ml,  $P < 0.01$ ). This suggests that IL-33 is released and circulates even under steady-state conditions and that ST2 regulates its availability. Thus, it is conceivable that steady-state levels of IL-33 may trigger a self-amplifying inflammatory milieu favorable to tumor development in genetically susceptible hosts.

Upon disease progression in SHIP-deficient animals, infiltrating immune cells progressively cause tissue damage to the

lung and intestine — organs that contain relatively high numbers of IL-33-expressing cells (Supplemental Figure 9; ref. 17). This immunopathology likely leads to rising concentrations of IL-33 in the serum, as detected in *styx* mutants. The evidence that altered hematopoiesis develops spontaneously in our model is still reconcilable with our initial hypothesis proposing that external stimuli may promote MPN development. IL-33 released during infection with pathogens (19, 20) may indeed further precipitate uncontrolled myeloproliferation. Also, opportunistic infections may be the cause of the accelerated disease observed in *styx* mice deficient in *Tlr4*, *Unc93b1*, or *Il18* (Figure 1, A and B, Supplemental Figure 2A, and Figure 1C). Accordingly, the irradiation-induced increase in proinflammatory cytokines such as IL-6 and G-CSF, but also IL-33 (35, 36), likely accounts for the accelerated disease kinetics observed in chimeric mice reconstituted with *styx* BM compared with nonmanipulated *styx* mutants (Figures 1 and 3). Irradiation-induced systemic inflammation may also mask the relative contribution of radio-resistant cells to MPN-like disease in our model. This may explain why the *styx*→WT and *styx*→*St2*<sup>-/-</sup> sets of chimeras develop MPN-like disease with similar kinetics (Figure 3); however, our data show that BM stromal cells can engage ST2, for example to promote colony formation in vitro (Figure 4E). Increased availability of IL-33 in the BM may similarly support disease pathogenesis in BCR-ABL1-negative MPN patients.

Critically, we observed a different ST2 expression pattern in mice compared with humans. In the BM of *styx* and WT mice, ST2 expression was restricted to non-hematopoietic stromal and committed hematopoietic cells. ST2 protein was undetectable on murine HSCs and myeloid progenitors, and addition of IL-33





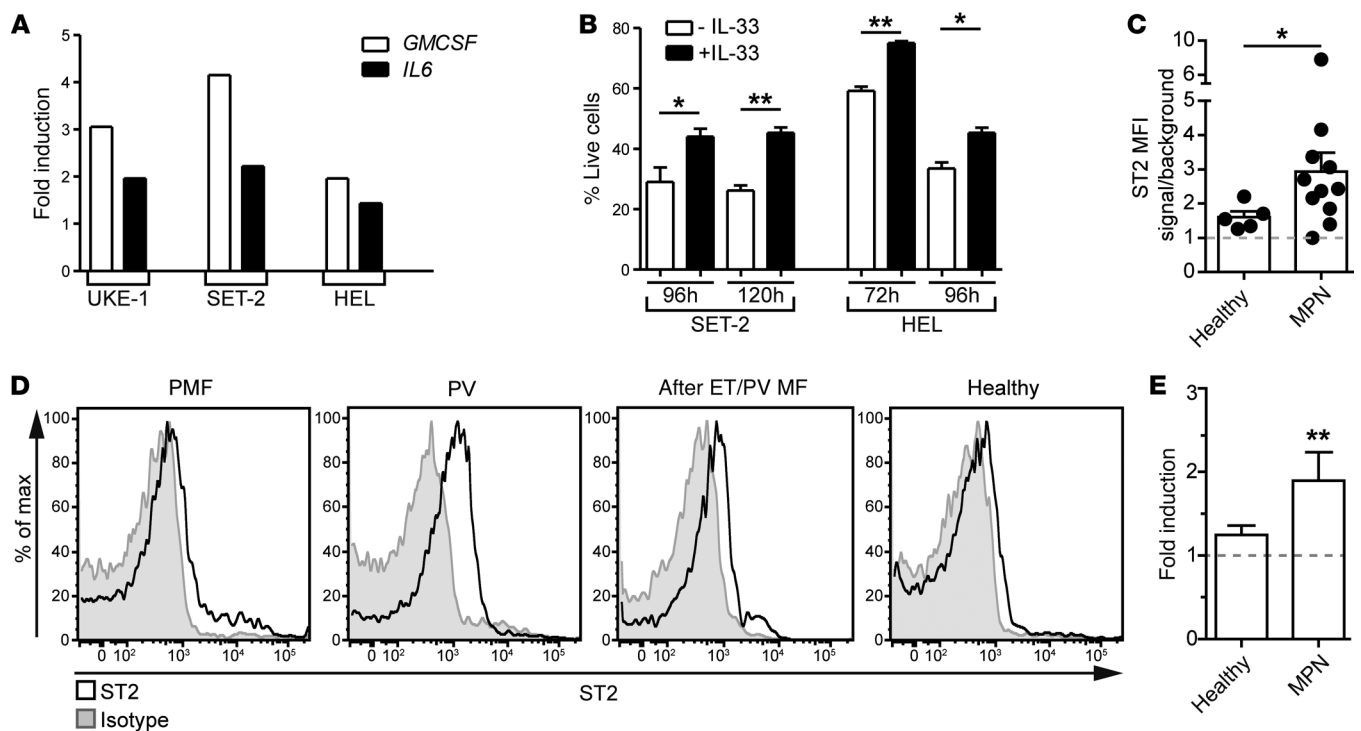
**Figure 7. Increased levels of IL-33 protein in trephine biopsies of MPN patients.** IHC for (A) IL-33 (brown) (scale bar: 100  $\mu$ m) and for (B) IL-33 (brown) and CD34 (red) in normal and MPN BM (scale bar: 20  $\mu$ m). (C) Percentage of IL-33-positive nuclei in BM of controls ( $n = 6$ ) and of patients newly diagnosed with the indicated hematopoietic malignancies. CML ( $n = 11$ ), ET ( $n = 12$ ), PV ( $n = 13$ ), and PMF ( $n = 9$ ). Data are mean  $\pm$  SEM. Kruskal-Wallis with Dunn's post hoc test was used; control biopsies were compared with all other groups. \* $P < 0.05$ ; \*\* $P < 0.01$ .

to these cells did not modulate their capacity to form colonies. However, human CD34<sup>+</sup> stem/progenitor cells from controls and MPN patients were able to engage IL-33/ST2 signaling to produce growth factors and proinflammatory cytokines, an observation previously made by others (31). Also, a recent study reported that ST2 is upregulated on CD34<sup>+</sup> stem/progenitor cells from CML patients (37). Therefore, it remains to be addressed whether these differences in ST2 expression patterns rely on species-dependent divergences and whether ST2 can be also engaged on human BM stromal cells. This is relevant because humoral factors secreted by BM stromal cells may also support MPN pathogenesis by impairing compound-mediated JAK2 inhibition (11).

Our data indicate an indirect effect of the IL-33/ST2 pathway for the development of MPN-like disease in the *styx* model, via the production of soluble factors. Several of the cytokines released by ST2<sup>+</sup> BM cells have previously been shown to support dysregulated myeloproliferation in *Inpp5d* KO mice (23) and also in other murine models of MPN (38). These IL-33-induced cytokines likely also promoted the aberrant expansion of *JAK2*<sup>V617F</sup> hematopoietic cells in BM recipient mice. However, IL-33-induced cytokines appear to be redundant for MPN development. For instance, blockade of IL-6 signaling only partially corrects the aberrant hematopoiesis in SHIP-deficient (28) or transgenic BCR-ABL1 mice (4).

IL-33 has a dual function, both as an inhibitor of transcription when residing in the nucleus (39) or as a proinflammatory cytokine upon release (16). Our results suggest that in its extracellular function and through ST2 binding, IL-33 promotes dysregulated hematopoiesis in the *styx* and transgenic *JAK2*<sup>V617F</sup> models, as well as cytokine production in stimulated MPN cells. Interestingly, plasma levels of IL-33 are not altered in CML patients (37) and are even reduced in patients suffering from PV or ET (34). Although availability of secreted IL-33 in the niche has yet to be demonstrated, we clearly show that nuclear IL-33 is expressed to a greater extent in the BM of newly diagnosed BCR-ABL1-negative MPN patients compared with controls. Since MPN stem/progenitor cells display hypersensitivity to cytokines and growth factors, similarly to SHIP-deficient HSCs and myeloid progenitor cells (2, 23), it is conceivable that even low amounts of secreted IL-33 within the niche may suffice to initiate ST2-dependent inflammation that supports MPNs.

While the soluble form of ST2 appears to be a predictive marker for clinical stage and outcome in CML (37), no such data exist on the role for IL-33/ST2 signaling in the progression of BCR-ABL1-negative MPNs. In particular, its potential contribution to the progression to end-stage myelofibrosis is unclear. IL-33 has a known direct proangiogenic effect on ECs (40), and increased microvessel den-



**Figure 8. IL-33/ST2 signaling promotes MPN development.** (A) Indicated  $JAK2^{V617F}$  cell lines were incubated for 24 hours  $\pm$  IL-33, and expression levels were measured for the transcripts of the indicated genes. Mean expression levels of technical duplicates are represented as fold induction and have been normalized to conditions without IL-33. (B) The MPN cell lines SET-2 and HEL were cultured  $\pm$  IL-33, and percentage of live cells (annexin V<sup>-</sup> DAPI<sup>-</sup>) was assessed at the indicated time points. Results show means  $\pm$  SEM. (C) Relative median fluorescence intensity (MFI) of ST2 expression on blood CD34<sup>+</sup> stem/progenitor cells from MPNs (PMF [ $n = 3$ ], PV [ $n = 5$ ], and after ET/PV myelofibrosis [ $n = 3$ ]) and control ( $n = 5$ ) donors. (D) Representative histograms showing ST2 expression on blood CD34<sup>+</sup> stem/progenitor cells from the indicated donors. (E) Colony formation assay (CFA) from MACS-purified blood CD34<sup>+</sup> stem/progenitor cells from controls and MPN patients cultured in standard CFC medium  $\pm$  IL-33 for 2 weeks. Data are mean  $\pm$  SEM and show fold changes in colony numbers after normalization to conditions without IL-33. Healthy ( $n = 5$ ) and 10 MPN donors were used. (A) One representative of 2 independent experiments, and (B) pooled values from 3 individual experiments are shown. (C and D) Pooled data from 2 independent experiments are shown. (B) Standard Student's  $t$  test, (C) Standard Student's  $t$  test with Welch's correction, and (D) Wilcoxon signed-rank test were used. \* $P < 0.05$ ; \*\* $P < 0.01$ .

sity has been associated with fibrosis in  $JAK2^{V617F}$  mutation-bearing MPN patients (41). Furthermore, abnormal expression and activity of a number of proinflammatory cytokines are linked to myelofibrosis (10), and our results indicate that IL-33 promotes the secretion of various cytokines in the BM microenvironment. Therefore, it may be relevant in the future to address whether therapeutic blockade of the IL-33/ST2 axis may prevent myelofibrosis or halt its progression.

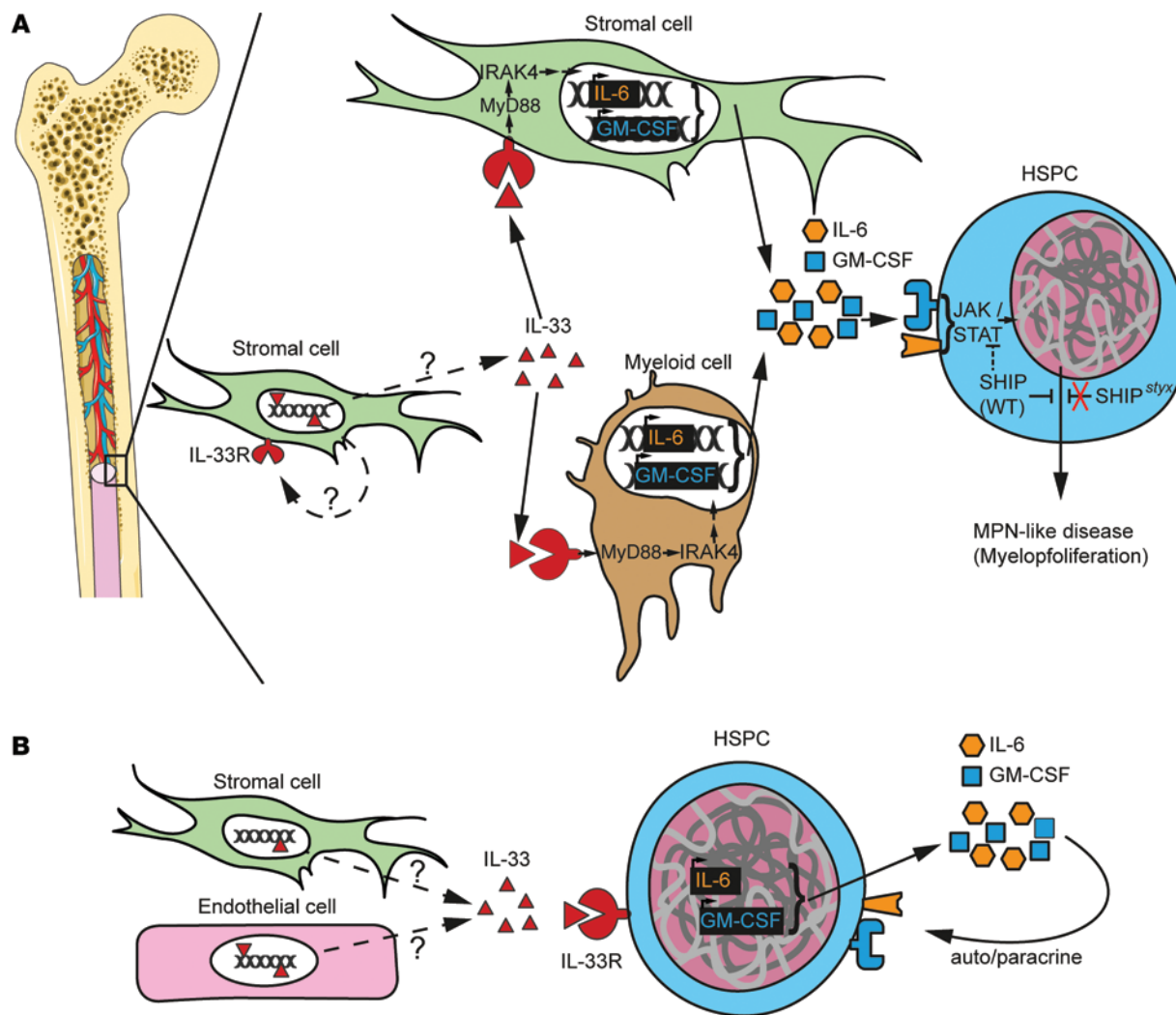
In summary, our data provide evidence for a role of IL-33/ST2 signaling for dysregulated hematopoiesis in mouse models of MPN. They also indicate that IL-33 expression is specifically increased in the BM of BCR-ABL1-negative MPN patients. Our results warrant further investigation on the contribution of the IL-33/ST2 axis to disease pathogenesis in MPN patients.

## Methods

**Patients.** PBMCs from CML or  $JAK2^{V617F}$ -positive patients at diagnosis — or from donors who underwent peripheral blood stem and progenitor cell mobilization and collection for reasons other than hematological neoplasms — were obtained from the Department of Hematology of the University Hospitals of Bern and Zurich, Switzerland, and were enriched for CD34<sup>+</sup> stem/progenitor cells by MACS. Samples from BM biopsies were retrieved from the archives of the Institute of Pathology, University

of Bern, Switzerland; control samples were obtained from non-leukemic individuals showing minimal reactive changes. All CML patients were positive for the BCR-ABL1 transcript.

**Animals.** All strains used were on a C57BL/6J background. C57BL/6J mice bred at The Scripps Research Institute (TSRI) were injected with ENU, as described (42). The  $Inpp5d^{styx/styx}$  (*styx* mice),  $Myd88^{doc}$ ,  $Irak4^{otiose}$ ,  $Tlr2^{languid}$ ,  $Tlr4^{lps}$ ,  $Tnf^{ranR1}$ , and  $Unc93b1^{3d}$  strains are described at the Southwestern Medical Center Mutagenetix database (<http://mutagenetix.utsouthwestern.edu>). B6.129S7- $Il1r1^{tm1mx}/J$ , B6.129P2- $Il18^{tm1Aki}/J$ , and B6.SJL- $Ptprca$   $Pep3b/Boy$  mice were obtained from The Jackson Laboratory.  $Il33^{-/-}$  mice (43) were obtained through the RIKEN Center for Developmental Biology (Acc. No. CDB0631K; <http://www.cdb.riken.jp/arg/mutant%20mice%20list.html>).  $Il1r1^{-/-}$  ( $St2^{-/-}$ ) mice have been described (44). The *styx* mice were rederived to germ-free status by 2-cell embryo transfer and were maintained in flexible film isolators in the Clean Mouse Facility, University of Bern, Switzerland. BM cells from transgenic  $JAK2^{V617F}$  ( $Scl$ - $Cre$   $FFI$ ; ref. 45) mice were provided by Radek C. Skoda (University Hospital Basel, Basel, Switzerland). For survival analyses, data were collected during the entire course of the study and pooled for graphic representation. Unless indicated in the corresponding figure, animals used in this study were 6–12 weeks old and age/sex-matched.



**Figure 9. Models for the development of MPN in the BM microenvironment.** (A) Model for SHIP-deficient mice. Stromal cell-derived IL-33 is released under stress or steady-state conditions through a pathway that is yet to be determined. IL-33 engages its receptor ST2 on stromal cells or, preferentially, on a committed population of myeloid cells. Signal transduction via the ST2→MyD88→IRAK4 axis promotes the secretion of several cytokines and growth factors promoting myoproliferation – among others GM-CSF and IL-6 – that bind to their respective receptors and activate the JAK2/STAT5 pathway (49). SHIP is a negative regulator in this process, and impaired SHIP-mediated inhibition results in MPN-like disease. (B) IL-33 released by stromal cells in the human BM engages ST2 receptor on CD34<sup>+</sup> HSPCs. Activation of cell signaling downstream of ST2 leads to the production of autocrine cytokines and growth factors that support proliferation.

**Flow cytometry.** BM cells were resuspended and filtered through 70  $\mu$ m cell strainers (Falcon Technologies BD) to obtain single-cell suspensions. Following rbc lysis, FcR blockade, and lineage depletion, HSCs and myeloid progenitors were stained as described previously (26), using the antibodies indicated in Supplemental Table 2. Live/dead cell discrimination was performed using DAPI (BioLegend) and annexin V (BioLegend). The gating strategy used to discriminate HSC and myeloid progenitor cell populations is shown in Supplemental Figure 3, A and B.

Blood-derived, MACS-purified CD34<sup>+</sup> human stem/progenitor cells were carefully thawed in HBSS containing 10% human serum. After centrifugation, cells were resuspended in IMDM and analyzed for colony formation or ST2 surface expression. The gating strategy used to assess ST2 expression on primary human CD34<sup>+</sup> stem/progenitor cells is shown in Supplemental Figure 10.

**Colony formation assay.** Purified, lin<sup>-</sup> murine BM cells were cultured at a starting density of  $2 \times 10^3$  to  $5 \times 10^3$  for 7 days in MethoCult-based medium (StemCell Technologies Inc.) supplemented with 15% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 100  $\mu$ M 2-mercaptoethanol, and 20% BIT (50 mg/ml BSA in IMDM, 1.44 U/ml rh-insulin, 250 ng/ml human holo transferrin). The following cytokines were always included: 50 ng/ml rm SCF, 10 ng/ml, 50 ng/ml rm Flt3-ligand, and 10 ng/ml rm IL-3. The following cytokines were added when specified: 10 ng/ml rh IL-6, 10 ng/ml, rm GM-CSF, and 100 ng/ml rm IL-33 (all from ProSpec). Colonies were counted using a DMIL inverted microscope (Leica Microsystems) mounted with an Intensilight C-HGFI unit (Nikon). Alternatively,  $1 \times 10^3$  primary blood CD34<sup>+</sup> stem/progenitor cells from MPN patients or healthy donors were plated in MethoCult H4435 Enriched medium (StemCell Technologies Inc.) with or without 100 ng/ml rh IL-33 (ProSpec). Colonies were counted after 14 days.

**Coculture experiment.** Regarding preparation of BM stromal responders, BM stromal cell (BMSC) cultures were set up from epiphyses from 5 mice per group, as described previously (46). CD45<sup>+</sup> BMSCs were MACS-purified after 3 weeks of expansion, and ST2 expression was confirmed by flow cytometry (data not shown). Then,  $5 \times 10^4$  purified BMSC were stimulated with 100 ng/ml rm IL-33 for 24 hours. In preparation of supernatant from BM hematopoietic responders,  $5 \times 10^4$  FACS-purified CD11b<sup>int</sup> Ly6G<sup>-</sup> Ly6C<sup>-</sup> hematopoietic BM cells pooled from 5 mice per group were stimulated with or without 100 ng/ml rm IL-33. Supernatant was collected after 24 hours and stored frozen. Next,  $5 \times 10^5$  lin<sup>-</sup> MACS-purified BM cells pooled from 6 mice per group were added to IL-33-stimulated BMSCs for coculture or supplemented with supernatant from IL-33-stimulated hematopoietic cells. After 48 hours of culture,  $5 \times 10^3$  lin<sup>-</sup> cells were seeded for colony formation assay as described above.

**CBC.** Blood was obtained through retro orbital bleeding of anesthetized mice and collected in Microvette (Sarstedt) tubes. CBC was assessed on a Vet abc<sup>TM</sup> Animal Blood Counter (Scil Animal Care Company GmbH).

**Chimeric experiments.** Mice were irradiated twice with an individual dose of 650 cGy with 4 hours recovery time between. Mice were then adoptively transferred i.v. with  $1 \times 10^7$  cells from whole BM of donor mice.

**Scoring of Disease Activity Index (DAI).** DAI was determined twice every week using a score index approved by the local authorities and based on previous reports (47, 48). Score was determined according to specific criteria. Behavior scores were as follows: apathy (2.8); hiding, not active, aggressive upon touch (1.8); hiding, calm, active upon touch (0.5); and active, attentive (0). Breathing scores were as follows: impaired, heavy breathing (2.8); and normal breathing (0). Body conditioning scores were as follows: emaciated (1.8); under-conditioned (0.5); and well-conditioned (0). Exercise and posture scores were as follows: paralysis (2.8); bent posture, strong bristling of hairs, motoric disorder (1.8); uncoordinated upon touch, bent posture, mild bristling of hairs (0.5); and normal (0).

**Histology and IHC.** Mouse and human tissues were fixed in 4% formaldehyde for 24 hours prior to dehydration and paraffin embedding. Sections were deparaffinized and stained with hematoxylin and eosin (H&E) or PAP under standard conditions. Antigen retrieval for IHC was performed with treatment in a 1 mM Tris/1 mM EDTA pH 9.0 solution for 18 minutes or in Bond Epitope Retrieval Solution 2 (Leica Biosystems) for 30 min at 95°C, for murine (IL-33) or human tissue (IL-33 and CD34), respectively. After an endogenous peroxidase block, primary goat anti-mouse IL-33, goat anti-human IL-33 (both from R&D Systems), and mouse anti-human CD34 (Dako) antibodies were used at a dilution of 1:100, 1:400, and 1:200, respectively. A secondary rabbit anti-goat antibody (Dako) or rabbit anti-rat antibody (Leica Biosystems) (1:100), followed by a tertiary horseradish peroxidase-linked (HRP-linked) anti-rabbit antibody (Dako or Leica Biosystems), was used undiluted to detect positive cells.

IHC of tissues from *Il33*<sup>-/-</sup> mice was performed to exclude unspecific staining of the murine IL-33 antibody used in this study. As another negative control for antibody specificity, we incubated the goat anti-mouse IL-33 antibody with rm IL-33 prior to the staining of tissue from *Il33*<sup>+/+</sup> mice. We also stained tissue from *Il33*<sup>+/+</sup> mice with a goat anti-rabbit isotype control antibody (Dako). Slides were quantified with Aperio Group LLC. Settings used for analysis were adjusted and controlled by a board certified pathologist in a blinded fashion.

**Quantitative PCR assay.** Mouse primary BM cells were MACS-purified with a murine CD45 micro-bead assay (Miltenyi Biotec) according to the manufacturer's protocol. RNA from human cell lines or primary MACS-purified CD34<sup>+</sup> stem/progenitor cells from donors was purified using TRI-reagent (Sigma-Aldrich). RNA was transcribed into cDNA using M-MLV reverse transcriptase (Promega). FastStart SYBR Green Master (Roche Diagnostics) was used to detect the target genes *GCSF*, *GMCSF*, *IL6*, and *GAPDH* (QIAGEN). All reactions were run in a StepOnePlus Real-Time PCR System (Invitrogen). Expression levels of genes were normalized to *GAPDH* mRNA, and IL-33-stimulated versus unstimulated groups were compared applying the  $2^{-\Delta\Delta CT}$  method.

**Human MPN cell lines.** The human CML cell lines JURL-MK1, KU812, LAMA-84, and NALM-1 were provided by Patrick Ziegler (University Hospital Aachen, Aachen, Germany). The human *JAK2*<sup>V617F</sup> cells lines HEL 92.1.7 (HEL), SET-2, and UKE-1 were provided by Radek C. Skoda (University Hospital Basel, Basel, Switzerland).

**Cytokine measurement.** MACS-purified primary cells were stimulated with 100 ng/ml rm IL-33 for 24 hours ex vivo. Cells were centrifuged for 5 minutes at 500g, and supernatant was removed. Cell culture supernatant and serum cytokines were analyzed by Multiplexing LASER Bead Technology (Eve Technologies).

**Statistics.** Two-tailed standard Student's *t* test with or without Welch's correction and Wilcoxon signed-rank tests were used as indicated for single comparisons. For datasets where multiple comparisons were performed, one-way ANOVA was performed with Bonferroni post hoc test. For survival curves, log-rank (Mantel-Cox) test was used. For all statistical analyses, *P* values of less than 0.05 were considered significant.

**Study approval.** Patients and donors gave written informed consent, and the analyses of samples were approved by the cantonal Ethics Committee from Bern (Ethics Committee Bern, Bern, Switzerland) and Zurich (Ethics Committee Zurich, Zurich, Switzerland).

All animal experiments were performed in accordance with institutional and federal regulations governing animal care and use, and were approved by TSRI Institutional Animal Care and Use Committee (La Jolla, California, USA) and the Cantonal Veterinary Office of Bern (Bern, Switzerland).

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1. Wadleigh M, Tefferi A. Classification and diagnosis of myeloproliferative neoplasms according to the 2008 World Health Organization criteria. *Int J Hematol*. 2010;91(2):174–179.
2. Jedidi A, et al. Selective reduction of JAK2V617F-dependent cell growth by siRNA/shRNA and its reversal by cytokines. *Blood*. 2009;114(9):1842–1851.
3. Jiang X, Lopez A, Holyoake T, Eaves A, Eaves C. Autocrine production and action of IL-3 and granulocyte colony-stimulating factor in chronic myeloid leukemia. *Proc Natl Acad Sci U S A*. 1999;96(22):12804–12809.
4. Reynaud D, et al. IL-6 controls leukemic multipotent progenitor cell fate and contributes to chronic myelogenous leukemia development. *Cancer Cell*. 2011;20(5):661–673.
5. Gallipoli P, et al. Autocrine TNF- $\alpha$  production supports CML stem and progenitor cell survival and enhances their proliferation. *Blood*. 2013;122(19):3335–3339.
6. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*. 2011;121(1):396–409.
7. Bhagwat N, Levine RL, Koppikar P. Sensitivity and resistance of JAK2 inhibitors to myeloproliferative neoplasms. *Int J Hematol*. 2013;97(6):695–702.
8. Skov V, et al. Molecular profiling of peripheral blood cells from patients with polycythemia vera and related neoplasms: identification of deregulated genes of significance for inflammation and immune surveillance. *Leuk Res*. 2012;36(11):1387–1392.
9. Fleischman AG, et al. TNF $\alpha$  facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. *Blood*. 2011;118(24):6392–6398.
10. Hasselbalch HC. The role of cytokines in the initiation and progression of myelofibrosis. *Cytokine Growth Factor Rev*. 2013;24(2):133–145.
11. Manshouri T, et al. Bone marrow stroma-secreted cytokines protect JAK2(V617F)-mutated cells from the effects of a JAK2 inhibitor. *Cancer Res*. 2011;71(11):3831–3840.
12. Kristinsson SY, Bjorkholm M, Hultcrantz M, Derolf AR, Landgren O, Goldin LR. Chronic immune stimulation might act as a trigger for the development of acute myeloid leukemia or myelodysplastic syndromes. *J Clin Oncol*. 2011;29(21):2897–2903.
13. Anderson LA, Pfeiffer RM, Landgren O, Gadalla S, Berndt SI, Engels EA. Risks of myeloid malignancies in patients with autoimmune conditions. *Br J Cancer*. 2009;100(5):822–828.
14. Titmarsh GJ, McMullin MF, McShane CM, Clarke M, Engels EA, Anderson LA. Community-acquired infections and their association with myeloid malignancies. *Cancer Epidemiol*. 2014;38(1):56–61.
15. Fleischman AG, et al. Exaggerated response to toll-like receptor agonist contributes to excessive TNF production in myeloproliferative neoplasm. *Blood*. 2013;122(21):4097.
16. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol*. 2010;10(2):103–110.
17. Pichery M, et al. Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel IL-33-LacZ gene trap reporter strain. *J Immunol*. 2012;188(7):3488–3495.
18. Pastorelli L, et al. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc Natl Acad Sci U S A*. 2010;107(17):8017–8022.
19. Bonilla WV, et al. The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses. *Science*. 2012;335(6071):984–989.
20. Byers DE, et al. Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. *J Clin Invest*. 2013;123(9):3967–3982.
21. Croker BA, et al. Inflammation and autoimmunity caused by a SHP1 mutation depend on IL-1, MyD88, and a microbial trigger. *Proc Natl Acad Sci U S A*. 2008;105(39):15028–15033.
22. Rivas MN, et al. MyD88 is critically involved in immune tolerance breakdown at environmental interfaces of Foxp3-deficient mice. *J Clin Invest*. 2012;122(5):1933–1947.
23. Helgason CD, et al. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev*. 1998;12(11):1610–1620.
24. Liu Q, et al. SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes Dev*. 1999;13(7):786–791.
25. Wang JW, et al. Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. *Science*. 2002;295(5562):2094–2097.
26. Wilson A, et al. Dormant and self-renewing hematopoietic stem cells and their niches. *Ann N Y Acad Sci*. 2007;1106:64–75.
27. Pinho S, et al. PDGFR $\alpha$  and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med*. 2013;210(7):1351–1367.
28. Maeda K, et al. IL-6 blocks a discrete early step in lymphopoiesis. *Blood*. 2005;106(3):879–885.
29. Hazen AL, Smith MJ, Despons C, Winter O, Moser K, Kerr WG. SHIP is required for a functional hematopoietic stem cell niche. *Blood*. 2009;113(13):2924–2933.
30. Kubovcakova L, et al. Differential effects of hydroxyurea and INC424 on mutant allele burden and myeloproliferative phenotype in a JAK2-V617F polycythemia vera mouse model. *Blood*. 2013;121(7):1188–1199.
31. Allakhverdi Z, et al. CD34<sup>+</sup> hemopoietic progenitor cells are potent effectors of allergic inflammation. *J Allergy Clin Immunol*. 2009;123(2):472–478.
32. Meehansan J, Tsuda H, Komine M, Tominaga S, Ohtsuki M. Regulation of IL-33 expression by IFN- $\gamma$  and tumor necrosis factor- $\alpha$  in normal human epidermal keratinocytes. *J Invest Dermatol*. 2012;132(11):2593–2600.
33. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J Immunol*. 2011;186(7):4375–4387.
34. Gangemi S, et al. Decreased plasma levels of IL-33 could contribute to the altered function of Th2 lymphocytes in patients with polycythemia vera and essential thrombocythemia. *Cancer Invest*. 2013;31(3):212–213.
35. Singh VK, Fatanmi OO, Singh PK, Whitnall MH. Role of radiation-induced granulocyte colony-stimulating factor in recovery from whole body gamma-irradiation. *Cytokine*. 2012;58(3):406–414.
36. Ha CT, et al. Circulating interleukin-18 as a biomarker of total-body radiation exposure in mice, minipigs, and nonhuman primates (NHP). *PLoS One*. 2014;9(10):e109249.
37. Levescot A, et al. BCR-ABL-induced deregulation of the IL-33/ST2 pathway in CD34(+) progenitors from chronic myeloid leukemia patients. *Cancer Res*. 2014;74(10):2669–2676.
38. Jiang X, et al. Primitive interleukin 3 null hematopoietic cells transduced with BCR-ABL show accelerated loss after culture of factor-independence in vitro and leukemogenic activity in vivo. *Blood*. 2002;100(10):3731–3740.
39. Ali S, et al. The dual function cytokine IL-33 interacts with the transcription factor NF- $\kappa$ B to dampen NF- $\kappa$ B-stimulated gene transcription. *J Immunol*. 2011;187(4):1609–1616.
40. Choi YS, et al. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood*. 2009;114(14):3117–3126.
41. Medinger M, et al. Angiogenesis and vascular endothelial growth factor- $\beta$  receptor expression in myeloproliferative neoplasms: correlation with clinical parameters and JAK2-V617F mutational status. *Br J Haematol*. 2009;146(2):150–157.
42. Hoebe K, et al. Identification of Lps2 as a key transducer of MyD88-independent TIR signaling. *Nature*. 2003;424(6950):743–748.
43. Oboki K, et al. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A*. 2010;107(43):18581–18586.
44. Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med*. 2000;191(6):1069–1076.
45. Tiedt R, et al. Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood*. 2008;111(8):3931–3940.
46. Cheng CC, et al. Isolation and characterization of novel murine epiphysis derived mesenchymal stem cells. *PLoS One*. 2012;7(4):e36085.
47. Ullman-Cullere MH, Foltz CJ. Body condition scoring: a rapid and accurate method for assessing health status in mice. *Lab Anim Sci*. 1999;49(3):319–323.
48. Paster EV, Villines KA, Hickman DL. Endpoints for mouse abdominal tumor models: refinement of current criteria. *Comp Med*. 2009;59(3):234–241.
49. Xiao W, Hong H, Kawakami Y, Lowell CA, Kawakami T. Regulation of myeloproliferation and M2 macrophage programming in mice by Lyn/Hck, SHIP, and Stat5. *J Clin Invest*. 2008;118(3):924–934.