HHS Public Access

Author manuscript

*J Immunol*. Author manuscript; available in PMC 2015 May 30.

Published in final edited form as:

*J Immunol*. 2011 December 1; 187(11): 5795–5804. doi:10.4049/jimmunol.1102242.

# Lineage$^{-}$Sca1$^{+}$c-Kit$^{-}$CD25$^{+}$ Cells Are IL-33–Responsive Type 2 Innate Cells in the Mouse Bone Marrow

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## Abstract

IL-33 promotes type 2 immune responses, both protective and pathogenic. Recently, targets of IL-33, including several newly discovered type 2 innate cells, have been characterized in the periphery. In this study, we report that bone marrow cells from wild-type C57BL/6 mice responded with IL-5 and IL-13 production when cultured with IL-33. IL-33 cultures of bone marrow cells from *Rag1 KO* and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice also responded similarly; hence, eliminating the possible contributions of T, B, and mast cells. Rather, intracellular staining revealed that the IL-5– and IL-13–positive cells display a marker profile consistent with the Lineage$^{-}$Sca-1$^{+}$c-Kit$^{-}$CD25$^{+}$ (LSK$^{-}$CD25$^{+}$) cells, a bone marrow cell population of previously unknown function. Freshly isolated LSK$^{-}$CD25$^{+}$ cells uniformly express ST2, the IL-33 receptor. In addition, culture of sorted LSK$^{-}$CD25$^{+}$ cells showed that they indeed produce IL-5 and IL-13 when cultured with IL-33 plus IL-2 and IL-33 plus IL-7. Furthermore, i.p. injections of IL-33 or IL-25 into mice induced LSK$^{-}$CD25$^{+}$ cells to expand, in both size and frequency, and to upregulate ST2 and $\alpha_4\beta_7$ integrin, a mucosal homing marker. Thus, we identify the enigmatic bone marrow LSK$^{-}$CD25$^{+}$ cells as IL-33 responsive, both in vitro and in vivo, with attributes similar to other type 2 innate cells described in peripheral tissues.

Interleukin-33, the most recent addition to the IL-1 family, has gained much interest in recent years because of its ability to promote type 2 immunity and its role in various human diseases with underlying type 2 phenotype (1, 2). Identified initially as a nuclear factor found broadly expressed in endothelial and epithelial cells (3–5), IL-33 was subsequently shown to be released from these cells upon cellular damage and to function as an alarmin, an endogenous danger signal of cell necrosis and tissue damage (6–8). IL-33 alerts the immune system by binding to its receptor, a heterodimer of ST2 and IL-1R accessory protein (1, 9), found on immune cells including mast cells, eosinophils, basophils, and Th2 cells, resulting in polarization toward a type 2 immune response (1, 10–17). Consistent with this, IL-33 helps mediate the expulsion of helminth infection in mouse models but has a pathogenic role in models of asthma, atopic dermatitis, and other allergic diseases (2).



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The online version of this article contains supplemental material.

**Disclosures**

The authors have no financial conflicts of interest.

Recent publications have identified novel cellular targets of IL-33 (18–21). Natural helper cells, nuocytes, multipotent progenitor $^{typ2}$ (MPP$^{typ2}$), and innate type 2 helper (Ih2) cells are newly discovered innate cells associated with production of large amounts of IL-5 and IL-13 in response to IL-33 and/or IL-25 (another type 2 immunity promoting cytokine) stimulation. These type 2 innate cells share common characteristics including lack of lineage marker expression and expression of the progenitor markers c-Kit and/or Sca-1. In addition, these cells are found predominantly in peripheral lymphoid organs, particularly ones associated with the mucosa, and help promote helminth expulsion in mouse models. Despite these similarities, the developmental relationship between these type 2 innate cells and their origin are unknown. Although the bone marrow represents the most likely source for these type 2 innate cells, to date, no IL-33–responsive bone marrow cell has been identified.

Several lines of evidence indicate, however, that there may be IL-33–responsive cells in the bone marrow. Prior studies found IL-33 mRNA in both mouse and human osteoblasts (22–24), and immunohistochemistry of mouse bone sections revealed IL-33 protein staining in osteoblasts and several other cell types in the bone (23). In addition, culture of bone marrow cells with IL-33 resulted in the production of several cytokines, including type 2 cytokines (23, 25). These observations led us to hypothesize the existence of IL-33–responsive, type 2 cytokine-producing bone marrow cells. In this article, we identify LSK$^{-}$CD25$^{+}$ cells, previously described as a “mystery population” of unknown function (26, 27), as a novel target of IL-33 in the mouse bone marrow and report that they produce IL-5 and IL-13.

## Materials and Methods

### Mice

Male, 8- to 11-wk-old C57BL/6, *Rag-1 KO*, *IL-7Rα KO*, *IL-2Rγ KO*, *Kit*$^{W\text{-}sh/W\text{-}sh}$, and *Foxn1*$^{nu/nu}$ mice, all on a C57BL/6 background, were purchased from The Jackson Laboratory and housed in the animal facilities at the Mayo Clinic (Rochester, MN). All experiments were approved and conducted according to guidelines set by the Mayo Clinic Institutional Animal Care and Use Committee.

### Media and cytokines

Cell culture media used in all experiments consists of RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals), 1× penicillin-streptomycin-glutamine (Life Technologies), and 1×2-ME (Life Technologies). Gentamicin (Life Technologies) was added to culture media at 50 μg/ml when culturing sorted cells. When indicated, recombinant mouse IL-33 (catalog number 3626-ML-010/CF, R&D Systems or catalog number 34-8332, eBioscience), IL-2 (catalog number 1150-ML/CF, R&D Systems), and/or IL-7 (catalog number 407-ML/CF, R&D Systems) was added to culture media at 10 ng/ml.

### Culture of bone marrow cells

Bone marrow cells were flushed from the femurs and tibias of mice with cold culture media using a 23-gauge needle and syringe under sterile conditions. Cells were treated with ammonium chloride-potassium lysis buffer (Invitrogen) to lyse RBCs. Remaining nucleated

bone marrow cells were cultured in culture media at 0.5 million cells/ml in 24- or 6-well plates. Culture medium was supplemented with the appropriate cytokine(s) depending on conditions in each experiment. Sorted $LSK^{-}CD25^{-}$ or $LSK^{-}CD25^{+}$ cells were cultured in culture media or culture media supplemented with cytokine(s) in 96-well round-bottom plates at 1000–3000 cells/well with the same number of cells per well within an experiment. Cell culture supernatants from bulk bone marrow cultures or sorted cells cultures were harvested daily or on day 5 of culture for ELISA.

### Measurement of cytokine production

IFN-γ in culture supernatant was measured using OptEIA–Mouse IFN-γ ELISA Set (BD Biosciences). IL-4, IL-5, and IL-13 production was measured using DuoSet ELISA Development Kit for each individual cytokines (R&D Systems). Multiplex analysis was performed using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad).

### Abs for flow cytometry

Lineage Ab mixture included the following: fluorochrome-conjugated anti-mouse B220 (RA3-6B2), CD3ε (145-2C11), CD4 (RM4-5), CD8α(53-6.7), CD11b (M1/70), CD11c (N418), DX5 (DX5) or NK1.1 (PK136), Gr-1 (RB6-8C5), and $Ter^{119}$ (TER119) Abs purchased from BD Biosciences or BioLegend. Fluorochrome-conjugated anti-mouse CD25 (PC61.5), CD44 (IM7), CD45.2 (104), c-Kit (ACK2), FcεRIα(MAR-1), Flt3 (A2F10), IL-13 (eBio13A), or Sca-1 (D7) was purchased from eBioscience. Fluorochrome-conjugated anti-mouse $\alpha_4\beta_7$ intergrin (DATK32), IL-5 (TRF5), Siglec-F (E50-2440), or Thy 1.2 (53-2.1) was purchased from BD Biosciences. Fluorochrome-conjugated anti-mouse IL-7Rα(A7R34) and anti-mouse ST2 (DJ8) were purchased from BioLegend and MD Biosciences, respectively.

### Flow cytometry and cell Sorting

Cells were stained with fluorochrome-conjugated Abs in PBS containing 0.5% BSA (staining buffer) on ice for 30 min. Nonspecific binding of Abs was blocked with preincubation in staining buffer containing 5% normal rat IgG and 5% normal mouse IgG (Invitrogen) and/or Fc Block (2.4G2; BD Biosciences). After staining, cells were resuspended in staining buffer containing DAPI for exclusion of dead cells. Flow cytometry data were collected on an LSR II flow cytometer running the Diva Software (BD Biosciences) and analyzed with FlowJo version 9.2 (Tree Star). Doublets were excluded by plotting forward scatter width versus height and side scatter width versus height, and dead cells were excluded by gating on $DAPI^{-}$ cells.

For intracellular cytokine staining, BD GolgiPlug (contains brefeldin A) was added to culture media at 1 μl/1 ml on day 4 of culture. Cells were harvested 12 h later on day 5 and labeled with the Violet Live/Dead Fixable Dead Cell Stain Kit (Invitrogen) following the manufacturer's instructions. Intracellular cytokine staining was performed using BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. Data were collected and analyzed as described above. Live cells were gated as violet low cells.

For sorting of LSK$^{-}$CD25$^{-}$ and LSK$^{-}$CD25$^{+}$ cells, bone marrow cells were first depleted of lineage-positive cells using Lineage Cell Depletion Kit, mouse (Miltenyi Biotec), according to the manufacturer's instructions. Lineage-depleted cells were then stained with fluorochrome-conjugated Abs as described above. LSK$^{-}$CD25$^{-}$ and LSK$^{-}$CD25$^{+}$ cells were sorted using FACSAria I running the Diva software (BD Biosciences). Doublets were excluded by plotting forward scatter width versus height and side scatter width versus height.

### Cell imaging

Sorted LSK$^{-}$CD25$^{+}$ cells pre- and postculture with IL-33 plus IL-2 or IL-33 plus IL-7 were stained with Wright–Giemsa staining by the Hematopathology Laboratory at the Mayo Clinic (Rochester, MN). Stained cells were visualized with a Zeiss Axiophot microscope under ×1000 magnification (Plan-Neofluar, ×100/1.3 oil objective) at 25°C. Photos were taken with an AxioCam HRc (Model 412-312) with the AxioVision Release 4.2 acquisition software and processed on Canvas 8.

### Statistical analysis

All values reported in bar graphs are means ± SEM as calculated by Microsoft Excel. Statistical significance was determined using the unpaired *t* test with Welch correction as calculated by GraphPad InStat.

## Results

### Bone marrow cells producing IL-5 and IL-13 in response to IL-33 are not T, B, or mast cells

To determine whether there are cells in the bone marrow that can respond to IL-33 in a type 2 fashion, we harvested bone marrow cells from wild-type (WT) C57BL/6 mice and cultured them with IL-33. In addition, because IL-7 and IL-7R signaling have been shown to promote the survival, expansion, and/or differentiation of some type 2 innate cells (18, 19), we also cultured bone marrow cells with IL-7 or IL-33 plus IL-7. A kinetic study showed that bone marrow cells produced IL-5 and IL-13, but not IL-4 or IFN-γ, when cultured with IL-33 (Fig. 1*A*). Production of IL-5 and IL-13 was enhanced upon addition of IL-7 (Fig. 1*A*). None of the assayed cytokines was detectable in the supernatant of bone marrow cells cultured in media only or media with IL-7 alone (Fig. 1*A*).

Previous reports have shown that T cells, specifically Th2 cells, and mast cells could produce IL-5 and IL-13, along with several other cytokines, in response to IL-33 (1, 10, 15, 16). To address the roles of T cells and mast cells, we used bone marrow cells from *Rag1 KO* mice and mast cell-deficient mice (*Kit*$^{W\text{-}sh/W\text{-}sh}$). As shown in Fig. 1*B* and 1*C*, T cells, B cells, and mast cells were not responsible for IL-33–induced IL-5 and IL-13 production. However, bone marrow cells from *IL-7Rα KO* mice, which also lack T and B cells, did not produce IL-5 or IL-13 when cultured with IL-33 or IL-33 plus IL-7 (Fig. 1*B*). Collectively, these results show that the IL-5– and IL-13–producing bone marrow cells are not T, B, or mast cells but require IL-7R signaling for their differentiation and/or survival.

## IL-5– and IL-13–producing bone marrow cells display hematopoietic and progenitor markers

To identify the type 2 cytokine-producing cells, we stained bone marrow cells cultured with IL-33 plus IL-7 for intracellular IL-5 along with various surface lineage markers. As shown in Fig. 2*A*, IL-5$^+$ cells were negative for lineage markers (B220, CD3ε, CD4, CD8α, NK1.1, CD11b, CD11c, Gr-1, and TER-119) but expressed a progenitor cell-associated marker, Sca-1, and the IL-2Rα-chain CD25. Further characterization of the IL-5$^+$ cells showed that they did not express c-Kit, FcεRIα, or Siglec-F, the former two being markers found on mast cells and the latter on eosinophils (Fig. 2*B*). They, however, did express the pan-hematopoietic marker CD45 and the adhesion molecule CD44 (Fig. 2*B*). Last, these IL-5–producing cells also made IL-13 (Fig. 2*C*). Collectively, these results show that the IL-5– and IL-13–producing cells are hematopoietic and that, based on their lineage negativity and Sca-1 positivity, they have a surface marker profile characteristic of progenitor cells.

## LSK$^-$CD25$^+$ cells as potential IL-33–responsive, IL-5– and IL-13–producing cells

To identify the IL-5– and IL-13–producing cells in their resting state prior to IL-33 stimulation, we isolated bone marrow cells from a WT C57BL/6 mouse and tested for expression of ST2, the receptor for IL-33, in lineage-negative cell populations. After excluding Lin$^+$ cells (expressing B220, CD3ε, CD4, CD8α, DX5, CD11b, CD11c, Gr-1, or TER-119), we divided the Lin$^-$ cells into four populations, based on their expression of Sca-1 and c-Kit (Fig. 3*A*). Lin$^-$Sca-1$^+$c-Kit$^+$ cells were ST2$^-$, whereas the Lin$^-$Sca-1$^-$c-Kit$^+$ and the Lin$^-$Sca-1$^-$c-Kit$^-$ populations showed low-level ST2 staining (Fig. 3*B*). A distinctly ST2$^+$ population was found among the Lin$^-$Sca-1$^+$c-Kit$^-$ (LSK$^-$) cells (Fig. 3*B*).

LSK$^-$ cells were first identified by Randall and Weismann (26) and were dubbed a “mystery population” because of their inability to reconstitute irradiated hosts and to respond to stimulation from various cytokines. Later, Kumar et al. (27) divided LSK$^-$ cells in two populations, based on their CD25 expression, and showed that LSK$^-$CD25$^-$ cells have lymphoid differentiation potential. However, the function of LSK$^-$CD25$^+$ cells still remained unknown (27). Following this precedent, we used CD25 to further characterize the LSK$^-$ cells and found that the LSK$^-$CD25$^+$ cells, but not LSK$^-$CD25$^-$ cells, are uniformly ST2$^+$ (Fig. 3*C*). LSK$^-$ CD25$^+$ cells, which made up 0.054 ± 0.003% of the total nucleated bone marrow cells from 8-wk-old C57BL/6 mice (mean, $n$ = 10), also express IL-7Rα, Thy1, CD44, and CD45 but are mutually exclusive with LSK$^-$Flt3$^+$ cells (Fig. 3*C*). These results suggest that LSK$^-$CD25$^+$ cells are potentially IL-33 responsive.

On the basis of the results in Fig. 1, we hypothesized that LSK$^-$ CD25$^+$ cells are present in WT C57BL/6, *Rag1 KO*, and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice but are absent or drastically reduced in numbers in *IL-7Rα KO*. Analysis of bone marrow cells from these mice revealed LSK$^-$cells in all four types of mice (Fig. 4*A*). Further gating on these cells based on CD25 expression revealed that, although LSK$^-$CD25$^+$ cells are present in WT C57BL/6, *Rag1 KO*, and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, they are reduced in frequency and number in *IL-7Rα KO* mice (Fig. 4*A*, 4*C*). In addition to being absent in *IL-7Rα KO* mice, several of the previously identified type 2 innate cells were also reported to be absent in *IL-2Rcγ KO* mice but present in nude, athymic mice (18–21). Similarly, we found that LSK$^-$CD25$^+$ cells are also reduced in

frequency and number in *IL-2Rcγ KO* mice but present in nude, athymic mice (Fig. 4*A*–*C*). Collectively, these results show that the presence of LSK$^-$CD25$^+$ cells correlates with the ability of the bone marrow to make IL-5 and IL-13 when cultured with IL-33 and that the development and/or survival of LSK$^-$CD25$^+$ cells is *Rag1* and thymus independent. Furthermore, the development of LSK$^-$ CD25$^+$ cells is likely dependent on signals from receptors that use the IL-2Rcγ, such as the IL-7R.

In summary, our data not only show that LSK$^-$CD25$^+$ cells have the potential to respond to IL-33 but also suggest that they are the IL-5– and IL-13–producing cells in our IL-33 cultures.

## Sorted LSK$^-$CD25$^+$ cells produce IL-5 and IL-13 in vitro

To test the IL-33 responsiveness of LSK$^-$CD25$^+$ cells directly, we sorted LSK$^-$CD25$^-$ cells (as controls) and LSK$^-$CD25$^+$ cells (Fig. 5*A*) and cultured them in media only or media with IL-33. Both populations were short lived under these culture conditions (data not shown) and did not produce any of the assayed cytokines after 5 d of culture (Fig. 5*B*). Because the frequency of LSK$^-$CD25$^+$ cells was drastically reduced in *IL-2Rcγ KO* and *IL-7Rα KO* mice, and because they express both CD25 and IL-7Rα, we suspected that IL-2 and/or IL-7 might provide necessary signals for survival and/or optimal function of isolated LSK$^-$CD25$^+$ cells. As shown in Fig. 5*B*, LSK$^-$CD25$^+$ cells produced IL-5 and IL-13 after 5 d culture in media containing IL-33 plus IL-2 or IL-33 plus IL-7. In contrast, IL-4 and IFN-γ were not detectable when LSK$^-$CD25$^+$ cells were cultured with IL-33 plus IL-2 or IL-33 plus IL-7 (Fig. 5*B*). Examination of culture supernatants in a multiplex assay of 23 cytokines confirmed that IL-5 and IL-13 were the predominant cytokines produced, but low levels of IL-6, GM-CSF, and MIP-1α were also detected (data not shown). LSK$^-$CD25$^+$ cells cultured in media containing only IL-2 or IL-7 and LSK$^-$CD25$^-$ cells cultured in all test conditions did not produce IL-4, IL-5, IL-13, or IFN-γ (Fig. 5*B*).

Morphologically, freshly isolated LSK$^-$CD25$^+$ cells are homogeneously lymphocyte-like with circular nuclei and scanty cytoplasm (Fig. 5*C*). In contrast, after 5 d of culture in IL-33 plus IL-2 or IL-33 plus IL-7, the cells became much larger and more heterogeneous (Fig. 5*C*). They appeared to have irregularly shaped nuclei and extensive cytoplasms filled with vacuoles (Fig. 5*C*). Hence, the LSK$^-$CD25$^+$ cells underwent extensive morphological changes after stimulation with IL-33 plus IL-2 or IL-33 plus IL-7. Taken together, these findings suggest that LSK$^-$CD25$^+$ cells respond to IL-33 plus IL-2 and IL-33 plus IL-7 by producing IL-5 and IL-13 and by changing their size and morphology.

## LSK$^-$CD25$^+$ cells respond to IL-25 and IL-33 in vivo

The type 2 innate cells in the periphery respond to IL-33 and/or IL-25 in vivo (18–21). Hence, we next sought to examine the in vivo effect of IL-33 and IL-25 on LSK$^-$CD25$^+$ cells in the bone marrow. We therefore injected PBS, IL-25, or IL-33 i.p. into C57BL/6 mice daily for 4 consecutive days and harvested bone marrow from these mice on day 5 for analysis by flow cytometry. We did not observe any significant changes in total bone marrow cellularity (Supplemental Fig. 1*A*), and we did not detect any global changes in the frequency and number of cells in the Lin$^-$ and Lin$^+$ gates (Supplemental Fig. 1*B*). Upon

inspection of the $Lin^-$ cells, we also detected no significant change in either frequency or number of $Lin^-Sca\text{-}1^-c\text{-}Kit^+$, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$, and $Lin^-Sca\text{-}1^-c\text{-}Kit^-$ cells (Supplemental Fig. 1*C*). In contrast, $LSK^-$ cells in IL-33–treated mice significantly increased in both frequency and number compared with $LSK^-$ cells in mice treated with PBS ($p < 0.05$; Supplemental Fig. 1*C*). Further analysis showed that, unlike $LSK^-CD25^-$ cells, the frequency and cell number of $LSK^-CD25^+$ cells significantly increased when mice were treated with IL-25 or IL-33 compared with PBS treatment ($p < 0.05$; Fig. 6*A*).

Honing in on $LSK^-CD25^+$ cells, we further characterized these cells in terms of expression level of surface markers and size across treatment groups. In mice treated with IL-25 or IL-33, $LSK^-CD25^+$ cells significantly upregulated their expression of CD25 ($p < 0.05$) and ST2 ($p < 0.01$) but not IL-7Rα(Fig. 6*B*, 6*C*). In addition, we found that $LSK^-CD25^+$ cells express $\alpha_4\beta_7$ integrin and that its expression was also significantly upregulated on $LSK^-CD25^+$ cells after treatment with IL-25 or IL-33 ($p < 0.001$; Fig. 6*B*, 6*C*). Last, and consistent with our previous morphological analysis, $LSK^-CD25^+$ cells increased in their forward and side scatter after treatment with both cytokines (Fig. 6*D*), while still remaining negative for the mast cell and eosinophil markers, FcεRIα, and Siglec-F, respectively (Supplemental Fig. 2).

We next wanted to determine, among all the cells in the bone marrow, which cells responded to IL-33 or IL-25 by upregulating ST2. As shown in Fig. 7, in a lineage versus ST2 plot of total bone marrow cells, there was a population of $Lin^-$ cells that most clearly upregulated ST2 in response to stimulation by IL-25 and especially by IL-33. Gating on this population, we found that these cells were in fact the $LSK^-CD25^+$ cells (Fig. 7).

Collectively, these results show that $LSK^-CD25^+$ cells are a unique population in the bone marrow that respond to in vivo IL-25 or IL-33 stimulation by increasing in frequency, number, and size and by upregulating the expression of CD25, ST2, and $\alpha_4\beta_7$ integrin.

## Discussion

Previous reports have identified cellular targets of IL-33 in the periphery, including Th2 cells, mast cells, and the recently discovered type 2 innate cells (18–21). In this article, we identify an IL-5– and IL-13–producing target of IL-33 in the mouse bone marrow. We initially observed that an IL-33 culture of bulk bone marrow cells produced IL-5 and IL-13. T cells, B cells, and mast cells were not responsible for production of these type 2 cytokines because these cytokines were also detected in IL-33 cultures of bone marrow cells from *Rag1 KO* and $Kit^{W\text{-}sh/W\text{-}sh}$ mice. However, cytokine production disappeared in IL-33 culture of *IL-7R KO* mice bone marrow cells. Through intracellular staining, we observed that the producers of these cytokines share a surface marker profile reminiscent of the $LSK^-CD25^+$ cells. Characterization of $LSK^-CD25^+$ cells revealed that they are $ST2^+$ and that they are present in *Rag1 KO* and $Kit^{W\text{-}sh/W\text{-}sh}$ mice but not in *IL-7R KO* mice. To test directly for $LSK^-CD25^+$ cells' ability to respond to IL-33, we sorted $LSK^-CD25^+$ cells, and when they were cultured with IL-33 plus IL-2 or IL-33 plus IL-7, they produced robust amounts of IL-5 and IL-13. Furthermore, i.p. injections of IL-33 or IL-25 resulted in increased frequency of $LSK^-CD25^+$ cells in the bone marrow, upregulation of ST2 and several other

markers by these cells, and changes in their forward and side scatter characteristics. Thus, we conclusively showed, both in vitro and in vivo, that LSK$^-$CD25$^+$ cells are bone marrow targets of IL-33.

Since Randall and Weissman (26) first identified LSK$^-$ cells as a "mystery population," there has been extensive effort to characterize and to assign functions to these cells. However, none has elicited a functional response specifically from LSK$^-$CD25$^+$ cells. Although Kumar et al. (27) showed that LSK$^-$CD25$^-$ cells had lymphoid differentiation potential, they did not observe such potential from LSK$^-$CD25$^+$ cells. However, they did note that, unlike LSK$^-$CD25$^-$ cells, LSK$^-$CD25$^+$ cells increase in frequency in *Rag1 KO* mice and older mice and are uniformly Flt3$^-$ (27). Harman et al. (28) and Fossati et al. (29) also described sub-populations of LSK$^-$ cells that have lymphoid and B cell potential, respectively, but the cells studied in both reports were mostly Flt3$^+$; hence, unlikely to be LSK$^-$CD25$^+$ cells. Recently, Trow-bridge et al. (30) reported that LSK$^-$ cells can differentiate into multiple lineages when transferred into newborn mice liver after Wnt3a stimulation, and Elkabets et al. (31) showed that Sca-1$^+$c-Kit$^-$ bone marrow cells stimulated by a primary tumor can promote outgrowth of secondary tumors. It is unclear, however, as to which subpopulations of the heterogeneous LSK$^-$ cells these properties could be ascribed to. Hence, our study is unique in showing direct stimulation of LSK$^-$CD25$^+$ cells and in clearly ascribing functional responses specifically to these cells.

Comparing these functional responses and other characteristics of bone marrow LSK$^-$CD25$^+$ cells to the ones of type 2 innate cells found in the periphery (18–21) inevitably leads to the question: are LSK$^-$CD25$^+$ cells the same cells as the type 2 innate cells described in previous reports? Indeed, LSK$^-$CD25$^+$ cells share many similarities with these previously identified type 2 innate cells, including a similar surface marker profile, responsiveness to IL-33 and/or IL-25, and the ability to secrete IL-5 and IL-13. Nevertheless, LSK$^-$CD25$^+$ cells have distinctive characteristics that set them apart. Most noticeably, LSK$^-$CD25$^+$ cells are found in the bone marrow, whereas the previously identified type 2 innate cells are found predominantly in the peripheral tissues and lymphoid organs. However, nuocyte-like and Ih2-like cells were also reported in the bone marrow (19, 21). Yet, these cells are unlikely to be LSK$^-$CD25$^+$ cells because both nuocytes and Ih2 cells express varying level of c-Kit, and Ih-2 cells are Sca-1$^-$. In addition, LSK$^-$CD25$^+$ cells differ from natural helper cells and MPP$^{typ2}$ cells in that the later two are c-Kit$^+$ (18, 20). Nonetheless, considering LSK$^-$CD25$^+$ cells' morphological change post–IL-33 stimulation, they may be similar to MPP$^{typ2}$ cells in their ability to undergo further differentiation. However, unlike MPP$^{type2}$ cells, which differentiate into mast cells, basophils, and macrophages (20), LSK$^-$CD25$^+$ cells did not acquire any of the lineage markers we tested and remained LSK$^-$,CD25$^+$ after IL-33 stimulation. In addition, differentiation assays performed specifically on LSK$^-$CD25$^+$ cells by Kumar et al. (27) strongly suggest that they do not have multilineage differentiation potential. Hence, their differentiation is more reminiscent of a mature, resting cell differentiating into an effector cell. Collectively, we propose that there are sufficient similarities to group LSK$^-$CD25$^+$ cells together with the other type 2 innate cells, but the differences argue that LSK$^-$CD25$^+$ cells are a distinct type of type 2 innate cells from all the other ones described thus far.

Hence, we next ask: what is the relationship between $LSK^{-}$ $CD25^{+}$ cells and the other type 2 innate cells? To this, we consider two possibilities. First, because of their location in the bone marrow, $LSK^{-}CD25^{+}$ cells might be the source of the other type 2 innate cells in the periphery. The ability of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells, common lymphoid progenitors, and $LSK^{-}CD25^{-}$ cells to give raise to $LSK^{-}CD25^{+}$-like cells and the resting, unresponsive phenotype of $LSK^{-}CD25^{+}$ cells in prior reports have led others to suggest that $LSK^{-}CD25^{+}$ cells are a new type of mature lymphoid cell (26, 27). We agree with this and raise the possibility that some of these cells may exit the bone marrow to seed the periphery, where they locally acquire peripheral type 2 innate cell phenotypes. In addition, some may also be retained in the bone marrow. Upon receiving appropriate signals, such as IL-33 and/or IL-25 released during a helminth infection, they upregulate homing markers, including $\alpha_4\beta_7$ integrin, and gain potential to migrate to the infected intestine, where they mediate worm expulsion as peripheral type 2 innate cells. Thus, $LSK^{-}CD25^{+}$ cells may represent the missing cell type that links peripheral type 2 innate cells to established hematopoiesis in the bone marrow.

Alternatively, $LSK^{-}CD25^{+}$ cells may be specialized type 2 innate cells with a specific function in the bone marrow. There is a growing body of literature that argues for a systemic nature of type 2 immune responses with the bone marrow being receptive to signals from the periphery and a source of monocyte precursors, eosinophils, and other effector cells (32–35). $LSK^{-}CD25^{+}$ cells may serve as a bone marrow sensor for these peripheral signals and create a favorable environment for production of cells important to type 2 immunity. Once activated, $LSK^{-}CD25^{+}$ cells produce IL-5, IL-13, and GM-CSF, which may promote eosinophil development, inhibit osteoclast development, and promote myeloid precursor and dendritic cell differentiation, respectively, in the bone marrow (23, 25, 36). In line with this possibility, we observed an increased number of eosinophils in the bone marrow of mice treated with i.p. injections of IL-25 and IL-33 (data not shown). Both possibilities, either function in the periphery or function in the bone marrow, are, of course, not mutually exclusive.

In summary, we have established that $LSK^{-}CD25^{+}$ cells are IL-33–responsive type 2 innate cells in the mouse bone marrow. This finding extends our understanding of these previously enigmatic cells by showing that they can be directly stimulated, resulting in functional responses. Furthermore, the similarities we have found between bone marrow $LSK^{-}CD25^{+}$ cells and peripheral type 2 innate cells suggest a possible relationship between these cells. Thus, this finding will also permit delineation of this relationship and of the potential role of $LSK^{-}CD25^{+}$ cells in shaping the bone marrow microenvironment.

## Acknowledgments

This work was supported by the College of Medicine, Mayo Graduate School, and the Mayo Clinic.

We thank Dr. Tobias Peikert for thoughtful discussions and careful reading of the manuscript. We also thank Michael J. Hansen, Koji Iijima, Gail M. Kephart, and Diane L. Squillace for technical assistance. Last, we thank Terri L. Felmlee for editing and formatting the manuscript.

## Abbreviations used in this article

| | |
|---|---|
| **Ih2 cells** | innate type 2 helper cells |
| **LSK$^-$** | Lineage$^-$Sca-1$^+$c-Kit$^-$ |
| **LSK$^-$CD25$^-$** | Lineage$^-$Sca-1$^+$c-Kit$^-$CD25$^-$ |
| **LSK$^-$CD25$^+$** | Lineage$^-$Sca-1$^+$c-Kit$^-$CD25$^+$ |
| **MPP$^{typ2}$** | multipotent progenitor$^{typ2}$ |
| **WT** | wild-type |

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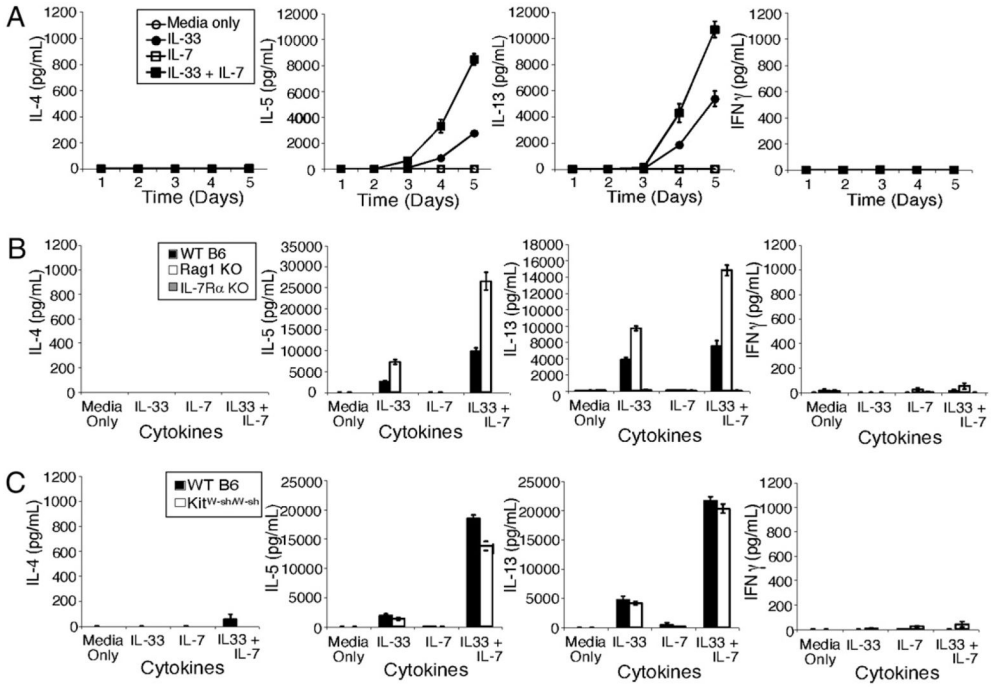


**FIGURE 1.**

Cytokine production from mouse bone marrow cells in response to IL-33 and IL-7. *A*, Bone marrow cells were harvested from C57BL/6 mice and cultured in media only or media supplemented with 10 ng/ml IL-33, 10 ng/ml IL-7, or 10 ng/ml IL-33 + 10 ng/ml IL-7. Culture supernatants were sampled daily, and IL-4, IL-5, IL-13, and IFN-γ levels were measured by ELISA. Error bars represent the SEM calculated from triplicate wells. *B*, Bone marrow cells from C57BL/6, *Rag-1*$^{-/-}$, and *IL-7Rα* $^{-/-}$ mice were cultured as in *A*, and day 5 supernatants were analyzed by ELISA. Error bars represent the SEM calculated from 9 wells in three independent experiments. *C*, Bone marrow cells from *C57BL/6* and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were cultured as in *A*, and day 5 supernatants were analyzed by ELISA. Error bars represent the SEM calculated from 6 wells in two independent experiments.

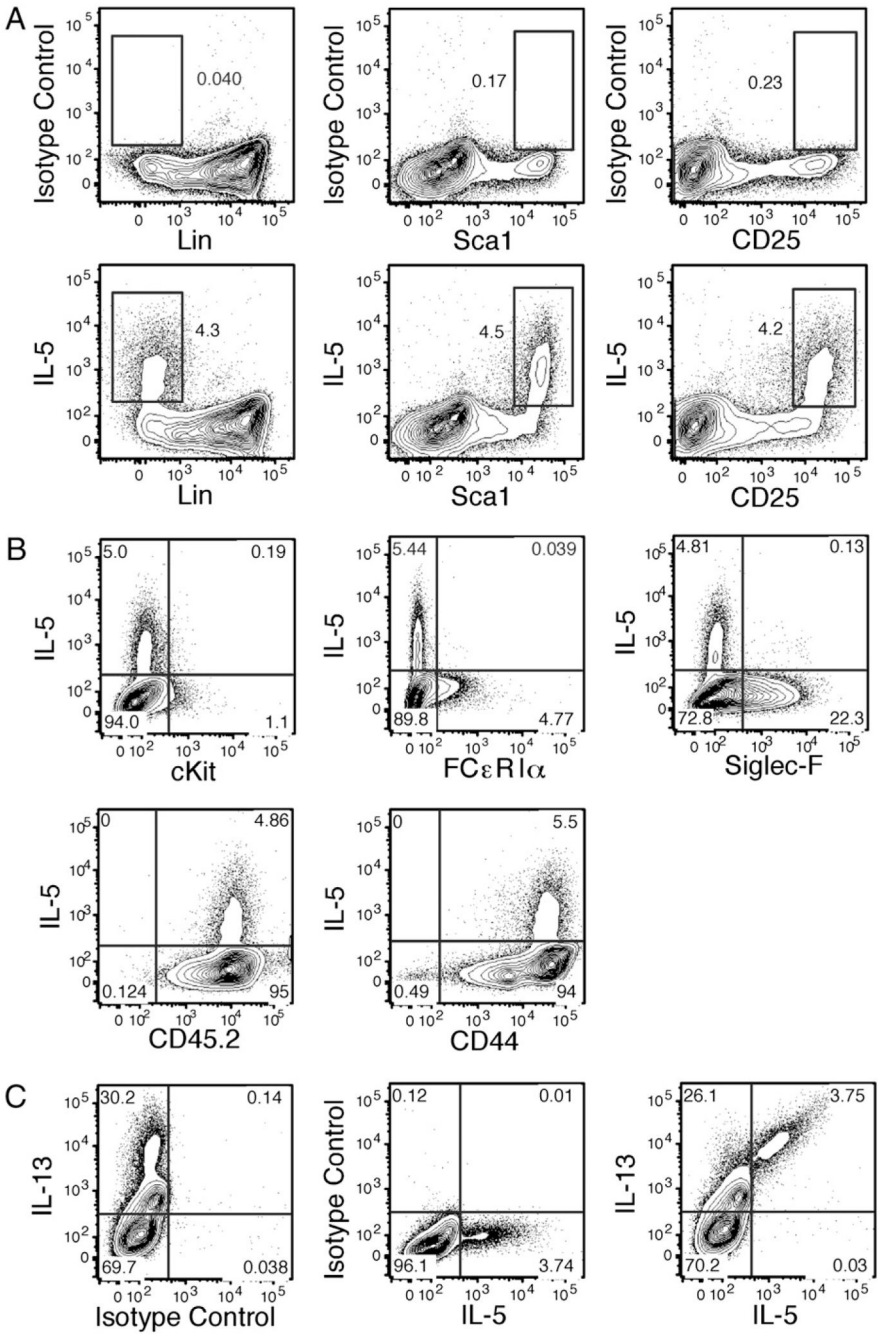


**FIGURE 2.**

IL-5– and IL-13–producing cells in an in vitro culture of mouse bone marrow cells in media containing IL-33 + IL-7 are positive for progenitor and hematopoietic markers. Bone marrow cells from C57BL/6 mice were harvested as in Fig. 1 and were cultured in media supplemented with 10 ng/ml IL-33 + 10 ng/ml IL-7. BD GolgiPlug was added to culture media 12 h before intracellular cytokine staining and subsequent analysis by flow cytometry. Cells were stained for intracellular IL-5, or with corresponding isotype control Ab, and for surface expression of lineage markers (B220, CD3ε, CD4, CD8α, NK1.1, CD11b, CD11c,

Gr-1, TER-119), Sca-1, and CD25 (*A*); or c-Kit, FcεRIα, SiglecF, CD45.2, or CD44 (*B*); or intracellular IL-13 (*C*). Gates in *B* and *C* were set using data from stains with corresponding isotype control Abs. All data shown are representative of at least two independent experiments.

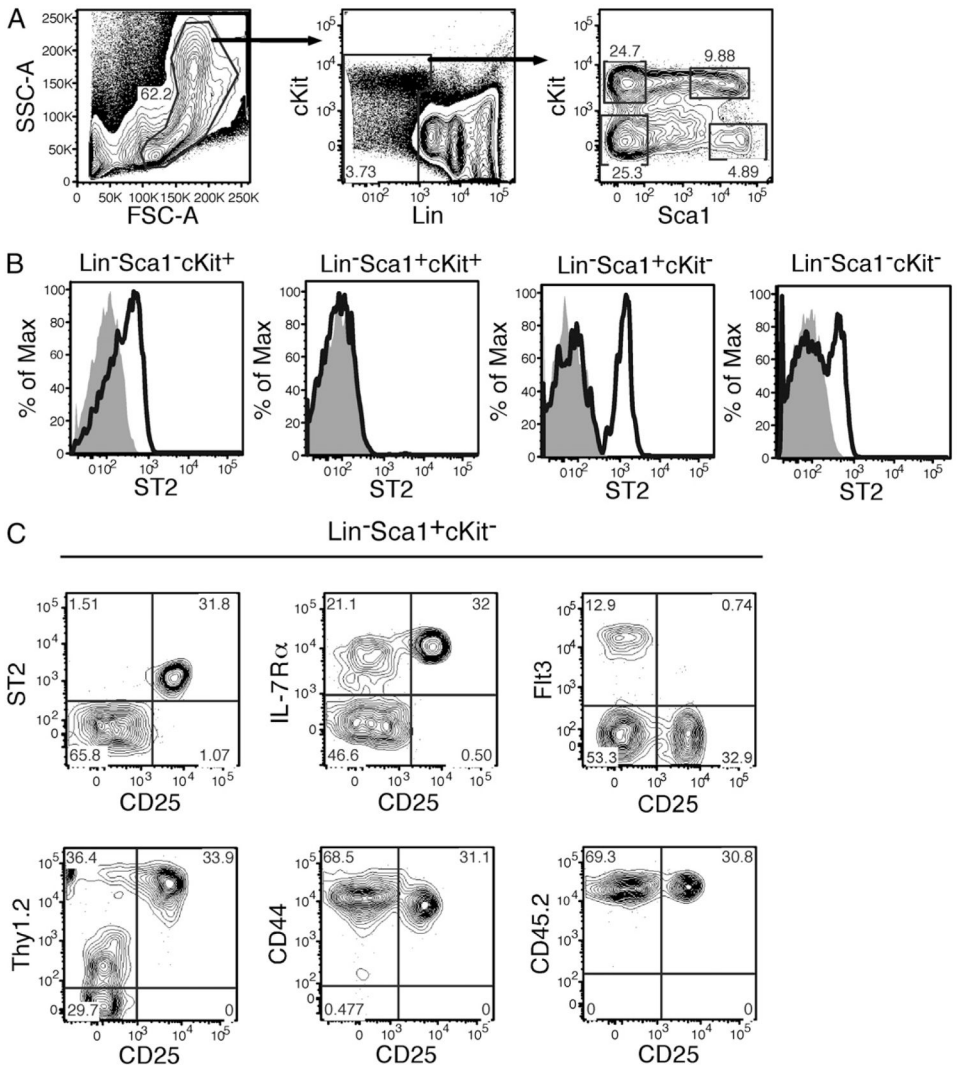


**FIGURE 3.**

ST2 expression on lineage low populations in the bone marrow. Bone marrow cells from 8-wk-old C57BL/6 mice were analyzed by flow cytometry. *A*, Gating strategy. Lineage low cells (low to no expression of B220, CD3ε, CD4, CD8α, DX5, CD11b, CD11c, Gr-1, and TER-119) were subdivided into four populations, based on their expression of Sca-1 and c-Kit. *B*, Expression of ST2 on the various lineage low subpopulations. Open histograms show anti-ST2 staining, and gray shaded histograms show isotype control Ab staining. Plots representative of stains from three different mice. *C*, ST2 and other cell surface markers expression on $CD25^{-}$ and $CD25^{+}LSK^{-}$ cells. Quadrants were drawn on the basis of isotype control Ab stains. All data shown are representative of stains from two to three mice.

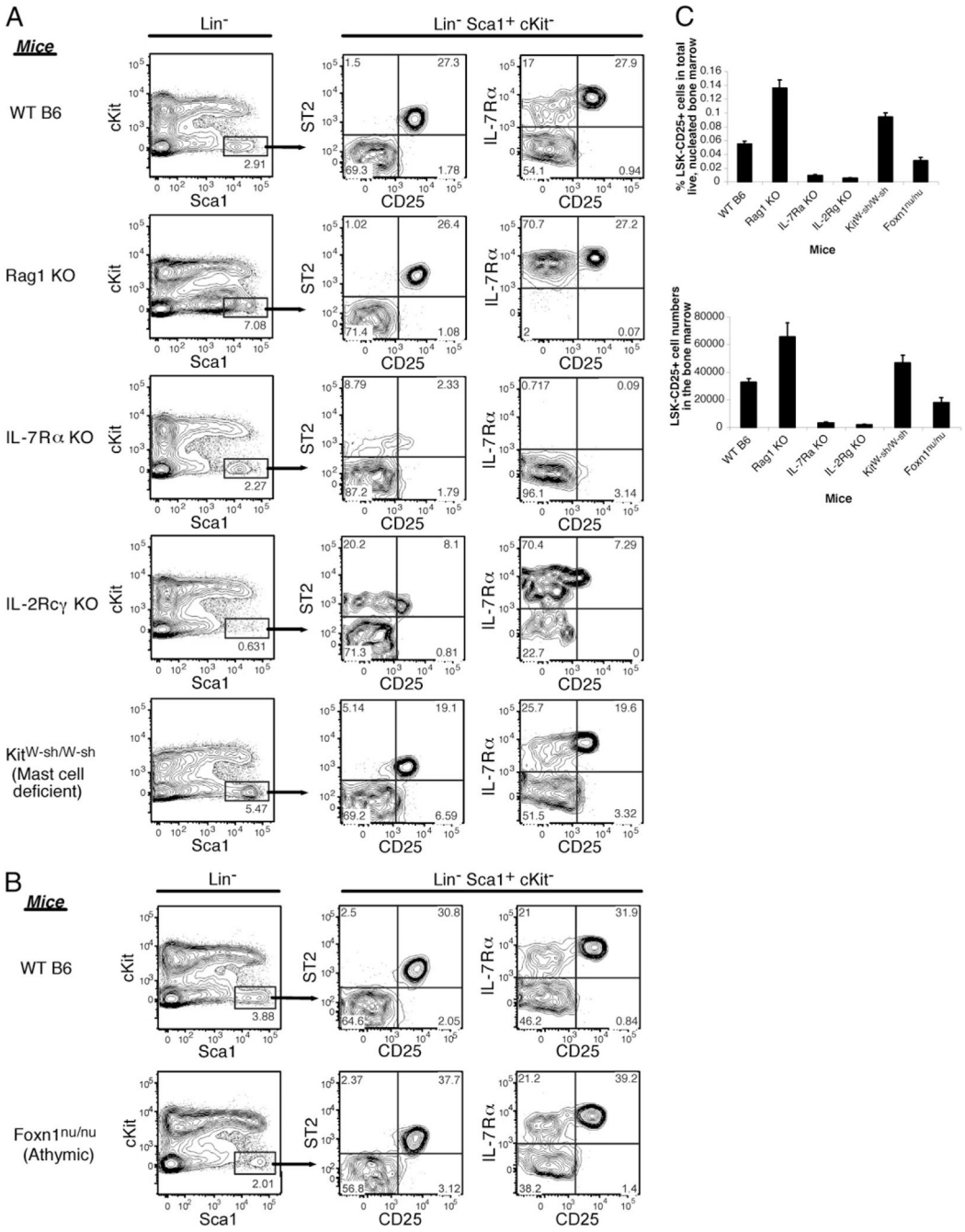


**FIGURE 4.**

LSK⁻CD25⁺ cells in mutant and knockout mice. *A*, Bone marrow cells were harvested from 8- to 11-wk-old WT C57BL/6, *Rag-1 KO*, *IL-7Rα KO*, *IL-2Rγ KO*, and *Kit*$^{W\text{-}sh/W\text{-}sh}$ or from 8- to 9-wk-old WT C57BL/6 and *Foxn1*$^{nu/nu}$ mice (*B*). Cells were stained as in Fig. 3 for LSK⁻CD25⁺ cells and for their expression of ST2 and IL-7Rα. Plots representative of two to three mice per mouse type. *C*, The frequency and absolute cell number of LSK⁻CD25⁺ cells in the bone marrow from two femurs and two tibias of each mouse. Error bars represent the SEM calculated from two to three mice per mouse type.

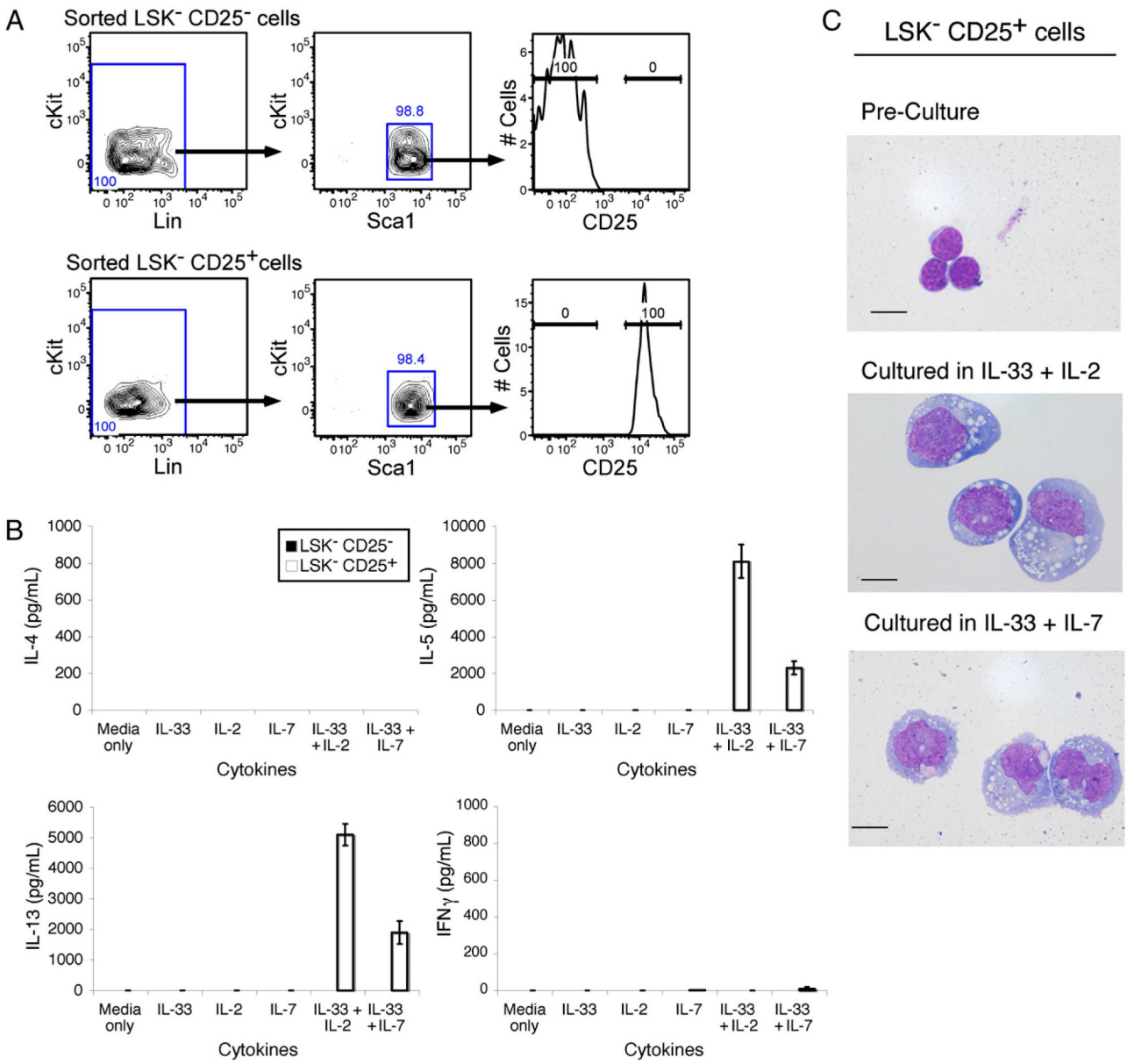


**FIGURE 5.**

Sorted LSK$^{-}$CD25$^{+}$ cells cultured in IL-33 + IL-2 and IL-33 + IL-7 produce IL-5 and IL-13 and lose their lymphoid morphology. Bone marrow cells from C57BL/6 mouse were lineage depleted using the Lineage Cell Depletion Kit (mouse) from Miltenyi Biotec. Lineage-depleted cells were stained and sorted for LSK$^{-}$CD25$^{-}$ and LSK$^{-}$CD25$^{+}$ cells. *A*, Purity of sorted cells. *B*, Sorted cells were cultured in media only or media supplemented with 10 ng/ml IL-33, 10 ng/ml IL-2, 10 ng/ml IL-7, 10 ng/ml IL-33 plus 10 ng/ml IL-2, or 10 ng/ml IL-33 plus 10 ng/ml IL-7. Culture supernatants were harvested on day 5 of culture, and IL-4, IL-5, IL-13, and IFNγ productions were assayed by ELISA. Error bars represent the SEM calculated from 3 wells per condition. *C*, Wright–Giemsa stained LSK$^{-}$CD25$^{+}$ cells preculture and after 5 d culture in media supplemented with IL-33 plus IL-2 or IL-33 plus

IL-7. Photos were taken with ×1000 magnification. Scale bars, 10 μm. All data shown are representative of three independent experiments.

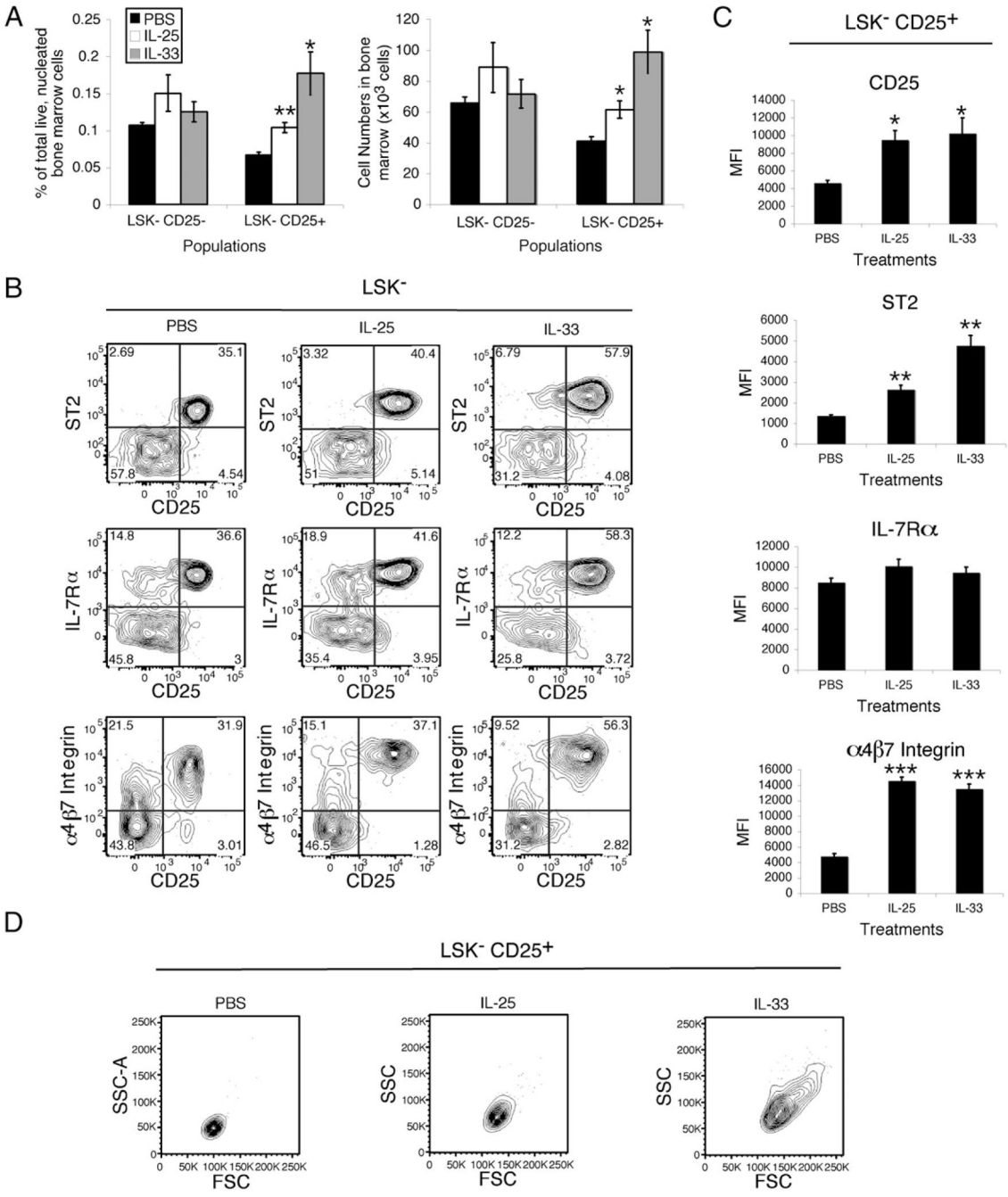


**FIGURE 6.**

LSK$^{-}$CD25$^{+}$ cells are responsive to IL-25 and IL-33 stimulation in vivo. PBS, 400 ng IL-25, or 400 ng IL-33 in PBS was injected i.p. into 8-wk-old WT C57BL/6 mice daily for 4 consecutive days. On day 5, nucleated bone marrow cells were harvested and stained for flow cytometry as in Fig. 3. *A*, Mean frequency and absolute cell number of LSK$^{-}$CD25$^{-}$ and LSK$^{-}$CD25$^{+}$ cells per mouse in each treatment group. Error bars represent the SEM from four mice per treatment group (*$p < 0.05$, **$p < 0.01$ compared with PBS treatment by unpaired *t* test with Welch correction). *B*, ST2, IL-7Rα, and $\alpha_4\beta_7$ integrin expression on

$LSK^{-}CD25^{-}$ and $LSK^{-}CD25^{+}$ cells across treatments. *C*, Mean fluorescence intensity (MFI) of CD25, ST2, IL-7Rα, and $\alpha_4\beta_7$ integrin expression on $LSK^{-}CD25^{+}$ cells across treatments. Error bars represent the SEM calculated from four mice per treatment group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with PBS treatment by unpaired *t* test with Welch correction). *D*, Forward scatter and side scatter of $LSK^{-}CD25^{+}$ cells across treatments. All data shown in *B* and *D* are representative of data from four mice per treatment group in two independent experiments.

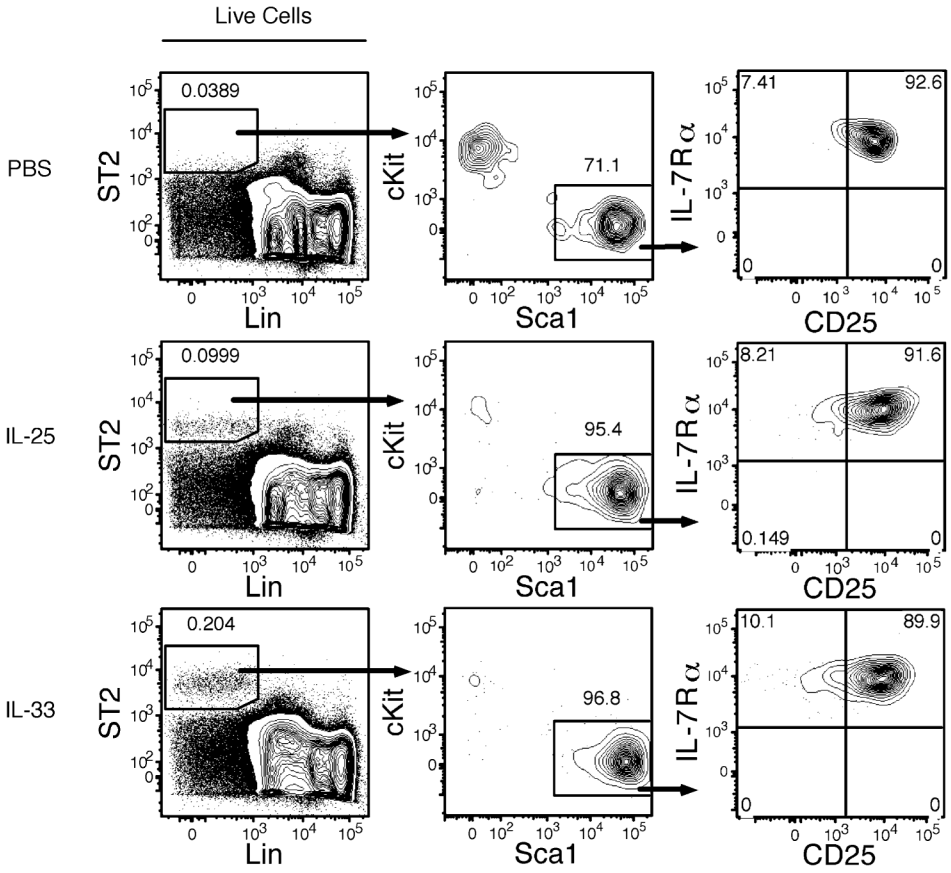


**FIGURE 7.**

ST2 high cells in the bone marrow of mice treated with IL-25 or IL-33 are $LSK^{-}CD25^{+}$ cells. Total live bone marrow cells from Fig. 6 were analyzed for ST2 expression. Subgating on the ST2 high population shows that they are mostly $LSK^{-}$ $CD25^{+}$ cells. Plots are representative of data from four mice per treatment group in two independent experiments.