Identification of Innate IL-5–Producing Cells and Their Role in Lung Eosinophil Regulation and Antitumor Immunity

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IL-5 is involved in a number of immune responses such as helminth infection and allergy. IL-5 also plays roles in innate immunity by maintaining B-1 B cells and mucosal IgA production. However, the identity of IL-5–producing cells has not been unambiguously characterized. In this report, we describe the generation of an IL-5 reporter mouse and identify IL-5–producing non-T lymphoid cells that reside in the intestine, peritoneal cavity, and lungs in naive mice. They share many characteristics with natural helper cells, nuocytes, and Ih2 cells, including surface Ags and responsiveness to cytokines. However, these phenotypes do not completely overlap with any particular one of these cell types. Innate non-T IL-5–producing cells localized most abundantly in the lung and proliferated and upregulated IL-5 production in response to IL-25 and IL-33. IL-33 was more effective than IL-25. These cells contribute to maintaining sufficient numbers of lung eosinophils and are important for eosinophil recruitment mediated by IL-25 and IL-33. Given that eosinophils are shown to possess antitumor activity, we studied lung tumor metastasis and showed that innate IL-5–producing cells were increased in response to tumor invasion, and their regulation of eosinophils is critical to suppress tumor metastasis. Genetic blockade or neutralization of IL-5 impaired eosinophil recruitment into the lung and resulted in increased tumor metastasis. Conversely, exogenous IL-5 treatment resulted in suppressed tumor metastasis and augmented eosinophil infiltration. These newly identified innate IL-5–producing cells thus play a role in tumor surveillance through lung eosinophils and may contribute to development of novel immunotherapies for cancer. The Journal of Immunology, 2012, 188: 703–713.

The innate immune system responds rapidly to invading pathogens, recognizing pathogen-associated molecular patterns, and subsequently promotes Ag-specific adaptive immune responses to the pathogens. It provides a variety of defenses against not only exog-
enous pathogens, but also endogenous, life-threatening tumors. In addition to a number of reports describing the roles of Th1 cytokines, such as IL-12 and IFN-γ, in Ag-specific tumor immunity mediated by CD8⁺ CTL, there has been accumulating evidence that Th2 cells are also involved in tumor rejection by employing activation of innate immune cells such as macrophages and eosinophils (22–28). One of the intriguing functions of IL-4, a well-characterized Th2 cytokine, is to induce eosinophil influx into tumor-growing sites (25, 26) and prevent angiogenesis (27). Interestingly, exogenous treatment of tumor-bearing mice with IL-5 led to prolonged survival of the mice (29). In this case, IL-5–dependent augmentation of eosinophil infiltration into tumor sites was necessary for tumor surveillance (30).

In this study, we describe the generation of IL-5/Venus knockin (KI) mice and used these mice to identify innate IL-5–producing cells in the small and large intestines, peritoneal cavity, and the lung. The innate IL-5–producing cells possess several similar characteristics with natural helper cells, nuocytes, and Ih2 cells (18, 19, 21). We found unique tissue localization of innate IL-5–producing cells in the lungs of untreated mice. We describe in this study innate IL-5–producing cells that regulate eosinophil infiltration into the lung and appear to prevent tumor metastasis. In addition to their potential roles in regulating Th2 immune responses, innate IL-5–producing cells may contribute to antitumor immunity via lung eosinophils, indicating a role in tumor surveillance.

Materials and Methods

Generation of IL-5/Venus KI mice

Embryonic stem (ES) cells derived from the 129S4/SvJae mouse strain were electroporated with the linearized targeting vector (Fig. 1A), including the HSV thymidine kinase. ES cells transfected with the targeting vector were screened with G418 and gancyclovir, selected, and separately expanded to obtain genomic DNA. Two of the ES cells were chosen to inject into C57BL/6 blastocysts, and the resulting male mice were crossed to C57BL/6 female mice. Offspring derived from ES cells carrying the IL-5/Venus KI allele were identified by their coat color and backcrossed to C57BL/6 mice at least 10 generations. During the period of backcrossing, mice were mated with CAg-cre transgenic mice (31) to delete the neomycin resistance gene from the germline. Those mice were intercrossed to obtain mice mated with CAG-cre transgenic mice (31) to delete the neomycin resistance gene from the germline. Those mice were intercrossed to obtain mice expressing IL-5⁺/-, IL-5⁺/+Venus (IL-5⁺/Venus), and IL-5⁻/⁻Venus genotypes. The primers used for genotyping are as follows: IL-5 forward, 5'-CTCTTCTTCTTTGCTGAAAGGCCCAG-3'; IL-5 reverse, 5'-GAGTAGGGCAGAAGGCTC ATCG-3'; and Venus reverse, 5'-CTCGATATGTTGGCGC ATCTT-3'.

Mice

C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan) and Japan SLC (Hamamatsu, Japan). All mice used in this study were maintained without specific pathogen-free conditions in the animal facility of the University of Toyama. All mice used for research were 7–10 wk of age. For systemic IL-25 and IL-33 (R&D Systems, Minneapolis, MN) administration, mice were i.p. injected with 400 ng each cytokine daily for 3 d and euthanized 6 h after the last administration. For local treatment, 20 µl PBS or an indicated cytokine (200 ng) was delivered directly into the bronchus with a flat-tipped 23-gauge needle (Terumo, Tokyo, Japan). rIL-5 was obtained according to procedures as previously described (32, 33). To neutralize IL-5, a single bolus of 500 µg anti–IL-5 mAb (NC17) (34, 35) or a control rat IgG Ab (Sigma-Aldrich, St. Louis, MO) was i.p. administered just before the injection of tumor cells. The indicated number of tumor cells was i.v. injected into the tail vein. All experiments were performed according to the guidelines for the care and treatment of experimental animals at the University of Toyama.

Cell culture for Th1 or Th2 differentiation

RPMI 1640 supplemented with 10% FCS, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin was used. Purified splenic CD4⁺ T cells were cultured in the presence of rIL-2 (10 U/ml), rIL-4 (5 ng/ml), anti-IFN-γ (10 µg/ml), anti–IL-12 (10 µg/ml), and anti-CD28 (1 µg/ml) for Th2 differentiation and rIL-2 (10 U/ml), rIL-12 (10 ng/ml), anti–IL-4 (5 µg/ml), and anti-CD28 (1 µg/ml) for Th1 differentiation in a culture plate coated with anti-CD3e (20 µg/ml). Cells were then restimulated with plate-bound anti-CD3e (20 µg/ml) or PMA (10 ng/ml) and ionomycin (1 µM) for 12 h in the presence of GolgiStop (BD Biosciences, San Jose, CA). Intracellular staining for IL-5 and IFN-γ was performed using a fixation/permeabilization kit (eBioscience, San Diego, CA). rIL-2, rIL-4, and rIL-12 were purchased from R&D Systems, and anti-mouse IL-12 (C17.8), IFN-γ (XM1.2), CD28 (37.51), and IL-4 (11B11) Abs were purchased from BD Biosciences.

Tumor cells and cell culture

A lung metastatic melanoma cell line, B16F10, was kindly provided by Dr. S. Wakazawa (Hokuriku University, Kanazawa, Japan). B16F10 cells were maintained in RPMI 1640 supplemented with 10% FCS, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Abs and reagents for flow cytometry

Abs used for flow cytometry were anti-mouse B220 (RA3-6B2), c-Kit (2B8), CD3e (145-2C11), CD4 (RM-45), CD8a (53-7.3), CD8x (53-6.7), CD11c (HL3), CD19 (1D3), CD23 (B3B4), CD24 (PC6I), CD27 (LG.3A10), CD43 (S7), CD44 (IM7), CD45 (30-F11), CD69 (H1.2F3), CD49b (DX5), IFN-γ (XM1.2), IL-4 (11B11), IL-13 (eBio13A5), IL-5 (TRFK3), IL-7 (R73), F4/80 (BM8), FcεR1 (MAR-1), Gr-1 (RB6-8C5), Mac-1 (M1/70), NK1.1 (PK1.36), Sca-1 (E13-161.7), T1/ST2 (D18), TCRβ (H57-597), TCRγ (GL3), TER-119, and Thy-1.2 (30-H12) were purchased from BD Biosciences, eBioscience, Invitrogen (Carlsbad, CA), and MD Bioscience (St. Paul, MN). FcγRs were blocked with anti-mouse FcγR (2.4G2). Flow cytometry was performed on a FACScan Canto II (BD Biosciences). Dead cells were gated out by 7-aminomethinamine D staining (BD Biosciences). FlowJo (Tree Star, Ashland, OR) was used for analysis.

Preparation of lung cells

Lungs were perfused with 5 ml ice-cold PBS and harvested, and all lobes were minced in 1 ml RPMI 1640 with 10% FCS in the presence of 1 ng/ml collagenase A (Roche Diagnostics, Basel, Switzerland) and 100 µg/ml DNase I (Sigma-Aldrich). After incubation for 30 min at 37°C, minced lungs were washed on 700-µm nylon mesh. The resulting cell suspension was then subjected to flow cytometric analysis or culture with indicated cytokines (10 ng/ml). For detection of IL-5 in culture supernatants, a mouse IL-5 ELISA kit (Thermo Fisher Scientific, Waltham, MA) was used.

Preparation of lamina propria cells

To obtain lamina propria (LP) cells, the small intestine was harvested, and Peyer’s patches (PPs) were removed. The resulting small intestine was then reversely used as a polyethylene tube and cut into five to seven pieces. The large intestine was harvested and cecal patches, and colonic patches were removed. The large intestine was then excised, opened longitudinally, and cut into three pieces. The small and large intestines were washed twice with 40 ml Ca²⁺− and Mg²⁺−free HBSS (Sigma-Aldrich) supplemented with 5% FCS, 1 mM DTT, and 5 mM EDTA and incubated at 37°C for 40 min with shaking at 150 rpm. Tissues were minced and incubated with RPMI 1640 (Invitrogen) supplemented with 5% FCS. For the small intestine, 1 mg/ml collagenase type I (Sigma-Aldrich) was added, and 2 mg/ml collagenase was added for the large intestine. The tissues were then incubated at 37°C for 40–60 min with stirring with 100 ng/ml DNase I (Roche Diagnostics). Collected cells were placed on the boundary between 40/57% concentrations of Percoll (GE Healthcare, Piscataway, NJ) solution and centrifuged at 1800 rpm, 20°C for 20 min. After centrifugation, the collected cells were washed and used as LP lymphocytes.

Immunofluorescence histology and immunohistochemistry

To detect Venus⁺ cells, fixed lungs were embedded in OCT compound (Sakura Finetec Japan, Tokyo, Japan) and frozen at −80°C. Frozen samples were cut into 30-µm slices using a cryostat (Leica Microsystems, Wetzlar, Germany). Sections were incubated with TSA blocking reagent (PerkinElmer, Boston, MA) for 30 min, washed, and incubated with polyclonal rabbit anti-GFP Ab (MBL, Nagoya, Japan) overnight at 4°C. Subsequently, sections were washed and incubated with Alexa 568-conjugated goat anti-rabbit IgG (Invitrogen), FITC-conjugated hamster anti-mouse CD3e (145-2C11) and, for nuclei staining, Hoechst33342 (Invitrogen) overnight at 4°C. Finally, samples were washed and mounted in Fluorount (BDS, Pleasanton, CA). Individual fluorescent images were acquired using a TCS SP5 confocal microscope and its imaging system (Leica Microsystems) and trimmed and overlaid with Photoshop software (Adobe Systems, San Jose, CA). For detection of eosinophils, lungs from mice injected with melanoma cells or PBS were fixed with 4% paraformaldehyde and embedded in par-
These samples were cut into 4-μm slices using a microtome (Sakura Finetec Japan). EG2, recognizing human eosinophilic cationic protein, was used for staining eosinophils. After deparaffinization, sections were soaked in target retrieval solution (DakoCytomation, Glostrup, Denmark) and treated in a microwave oven for 15 min (500 W). Sections were then rinsed for 2 min and soaked in 3% H2O2 methanol solution for 5 min and 5% BSA for 1 min. Subsequently, sections were incubated with M.O.M mouse Ig blocking reagent for 1 h, followed by EG2 Ab (Kabi Pharmacia, Uppsala, Sweden) application and intermittent microwave treatment for 30 min (250 W, 4 s on, 3 s off). After washing, a peroxidase-conjugated envision kit for mouse (DakoCytomation) was applied and subjected to microwave intermittently for 30 min. Following washing, sections were immersed in DAB solution (Sigma-Aldrich) with H2O2. Melanoma cells were detected by Fontana-Masson staining. Images were acquired using a D-33E digital camera (Olympus, Tokyo, Japan).

RNA preparation for real-time quantitative RT-PCR
Total RNA from the indicated tissues or sorted cells was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) and prepared for real-time PCR using the Mx3000P Real Time QPCR System (Stratagene, La Jolla, CA). TaqMan primers and probes were purchased from Applied Biosystems (Foster City, CA): for hypoxanthine phosphoribosyltransferase, Mm00446968_m1; for IL-2, Mm9999922_m1; for IL-5, Mm00439646_m1; for IL-7, Mm00434291_m1; for IL-13, Mm99999190_m1; for IL-25, Mm00499822_m1; and for IL-33, Mm00505403_m1.

Statistical analysis
The p values were calculated using the two-tailed Student t test.

**FIGURE 1.** Generation of IL-5/Venus KI mice. A, Schematic diagram of the generation of IL-5/Venus KI mice. A Venus gene with a neomycin resistance gene cassette flanked by loxp genes was introduced at the translation initiation site in exon 1 of the IL-5 gene locus. B, Probe design for Southern blotting to detect homologous recombination in the IL-5 gene locus. The probe used for Southern blotting was positioned at the beginning of exon 4, out of the arm of the targeting vector. C, Southern blot analysis of ES cells. Extracted genomic DNA was AffII-digested, yielding a 10-kbp fragment from the WT allele, 13 kbp from the Venus-targeted allele (IL-5+/V), including a neomycin resistant cassette, and 11 kbp from the Cre-treated allele (IL-5+/V). D, IL-5 and Venus expression in cultured Th2 cells. CD4+ splenocytes were purified from the indicated genotypes and maintained in Th2-skewing cultures. Following stimulation with PMA and ionomycin or plate-bound anti-CD3ε Ab, IL-5 and Venus expression were analyzed by flow cytometry. E, Expression of IL-5 and Venus in cultured Th1 cells. IFN-γ and Venus expression were analyzed in cells from IL-5+/V mice stimulated with plate-bound anti-CD3ε Ab. F, Cell size (forward scatter [FSC]) analysis of B-1 and B-2 B cells in the peritoneal cavity from IL-5+/V and IL-5+/V mice (n = 3). B-1 and B-2 B cells were characterized as CD19+CD43+CD23- and CD19+CD43+CD23+, respectively. Data are representative of duplicate cultures of two independent experiments (D, E) and three independent experiments (F).
Results

Generation of IL-5/Venus KI mice

To monitor IL-5 production in vivo, the Venus gene (36) was inserted at the translation initiation site in exon 1 of the IL-5 gene locus by homologous recombination (Fig. 1A). Successful homologous recombination was confirmed by Southern blot analysis (Fig. 1B, 1C). CD4+ splenocytes from IL-5+/+, IL-5+/V, and IL-5/V/V mice were cultured under Th2-skewing conditions, and the expression of IL-5 along with Venus was measured (Fig. 1D). As expected, no IL-5 but strong Venus expression was measured (Fig. 1D). B-1 B cells in IL-5+/+ mice were smaller in size, as observed in IL-5Rα knockout (KO) mice (11), than those in IL-5+/+ mice, confirming deletion of IL-5 in homozygous IL-5/Venus KI mice (Fig. 1F).

Identification and characterization of Venus+ cells

We next examined the localization of Venus+ cells in immune-related tissues and organs. Venus+ cells were barely detectable in the bone marrow, spleen, lymph nodes, MLNs, and PPs. In contrast, Venus+ lymphoid cells were found in LP from the small and large intestines, peritoneal cavities, and the lungs of unimmunized naive mice (Fig. 2A). Detectable levels of Venus were not observed in monocyte/macrophages, eosinophils, or mast cells (Supplemental Fig. 1). The Venus+ lymphoid cells consisted of at least three different populations: c-Kit+, CD3ε+, and double-negative (c-Kit−CD3ε−) cells. In these tissues, c-Kit+ cells were the majority of Venus+ IL-5 producers. Calculation of the absolute cell numbers revealed that c-Kit+Venus+ cells localized most abundantly in the lung (Fig. 2B). The majority of Venus+ cells in various tissues were lineage negative (B220, CD3ε, CD4, CD8α, CD5, TCRβ, CD11b, CD19, CD21, and CD23).

FIGURE 2. Identification and characterization of innate IL-5-producing cells. A, Representative flow cytometric analysis of lymphocytes in LP of the small and large intestines (S-LP and L-LP, respectively), peritoneal cavities (PC), and lungs from IL-5+/+ mice (n = 5). Upper panels show percentages of Venus+ cells gated on CD45+ lymphocytes and cell size (forward light scatter [FSC]). Lower panels show expression of c-Kit and CD3ε on the gated Venus+ cells. B, Total cell numbers of categorized Venus+ cells in S-LP, L-LP, PC, and lungs from IL-5+/+ mice (n = 5) determined by the indicated expression pattern of surface Ags. Cell numbers were calculated based on cells in the lung digest. Data are shown as mean ± SD. C, Expression pattern of the indicated surface Ags on the majority of Venus+ cells (CD3ε+ cells for LP and c-Kit+ cells for PC and lungs) in IL-5+/+ mice (n = 4). For lineage markers, Venus+ (gray) and Venus− (open) lymphoid cells are shown in histograms, and CD3ε was excluded from the lineage mixture for LP. Expression of the other surface Ags is shown in gray with isotype control (open). Data are representative of at least three independent experiments. DN, double-negative.
CD11c, CD19, DX5, FcεRIα, Gr-1, NK1.1, TCRγδ, and TER-119) and expressed Sca-1, Thy1.2, CD25, CD27, CD44, CD69, IL-7Rα, and T1/ST2, a subunit of IL-33R (Fig. 2C). This is reminiscent of natural helper cells as well as of nuocytes (18, 19). Thy1, CD25, CD27, and T1/ST2 were heterogeneously expressed among Venus⁺ cells found in the indicated organs.

**FIGURE 3.** In vitro analysis of innate IL-5-producing cells. A, Representative flow cytometric analysis of cultured lung cells from IL-5⁺/⁺ mice (n = 3). Following stimulation with PMA and ionomycin and culture for 12 h, expression of c-Kit and CD3ε on Venus⁺ cells in CD45⁺ lymphocytes was analyzed by flow cytometry. Venus⁺ cells were gated on CD45⁺ lymphocytes (upper panel). B, Analysis of Th2 cytokines in the cultured Venus⁺ cells from IL-5⁺/⁺ (upper panels) or IL-5⁻/⁻ (lower panels) mice (n = 3). C, Representative flow cytometric analysis of lung CD45⁺ lymphocytes from IL-5⁺/⁺ or IL-5⁻/⁻ mice (n = 3) cultured for 4 d in the presence of indicated cytokines. D, Total cell numbers of categorized Venus⁺ cells, determined by the indicated expression pattern of surface Ags, calculated based on C. E, ELISA analysis of culture supernatant obtained from cultures of lung cells from IL-5⁺/⁺ or IL-5⁻/⁻ mice. Data are shown as mean ± SD and are representative of duplicates and at least two independent experiments. Asterisks indicate statistical significance (***p < 0.01).
In vitro analysis of innate IL-5–producing cells in the lung

The existence of relatively abundant c-Kit+ IL-5–producing cells (Fig. 2B) compared with intestine led us to assess expression of Th2 cytokines and Venus in the lung innate lymphoid cells. Cells prepared from lung were cultured with or without PMA plus ionomycin. The majority of the Venus+ cells were c-Kit+CD3ε2 cells representing non-T innate cells (Fig. 3A). The expression of IL-5, IL-4, and IL-13 among the Venus+ cells was assessed by intracellular staining (Fig. 3B). There were IL-5+Venus−, IL-5+Venus+, and IL-5−Venus−, indicating that IL-5 loci are biallelically expressed in lung innate IL-5–producing cells (as seen in cultured Th cells). This also indicated that Venus+ lung innate cells are indeed producing IL-5 protein. Concerning other Th2 cytokines, approximately one-third of Venus+ cells also expressed IL-13. Thus, there was a population of cells competent to produce IL-5 (Venus+) but not IL-13, Venus+ cells rarely expressed IL-4.

Because innate IL-5–producing cells expressed receptors for IL-2, IL-7, and IL-33 (Fig. 2C) and IL-25 and IL-33 are well known to induce Th2 responses, we cultured lung cells in the presence of IL-2, IL-7, IL-25, or IL-33 and examined proliferation and IL-5 production. IL-33 treatment increased the number, size, and Venus expression levels of Venus+ cells (Fig. 3C, 3D). The majority of Venus+ cells maintained a c-Kit+CD3ε2 phenotype. Essentially the same results were obtained with the stimulation with IL-25, but to a lesser extent compared with IL-33. Reflecting the

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increased Venus intensity, the amounts of IL-5 secreted in cultured supernatants upon stimulation with IL-33 were significantly augmented (Fig. 3E). The lung cells from IL-5<sup>V/V</sup> mice carrying both intact IL-5 alleles produced more IL-5 in response to IL-33 than those from IL-5<sup>V/V</sup> mice.

**Innate IL-5–producing cells in the lung respond to IL-25 and IL-33, leading to inducing eosinophil infiltration in vivo**

As we demonstrated by in vitro analysis that innate IL-5–producing cells responded to IL-25 and IL-33 (Fig. 3C), we examined whether innate IL-5–producing cells in the lung of IL-5<sup>V/V</sup> mice could be expanded by passive administration of rIL-25 and rIL-33 in vivo. IL-25 treatment resulted in an ∼2-fold increase of c-Kit<sup>+</sup> Venus<sup>+</sup> cells, whereas IL-33 dramatically increased the number of these cells, ∼10-fold, with larger cell size and higher Venus intensity (Fig. 4A, 4B). Expansion of Venus<sup>+</sup> cells was not seen in non-lymphoid cell fractions. Immunofluorescent histological analysis also showed increased Venus<sup>+</sup> cells in response to IL-33 and that many of them resided around bronchioles and bronchia (Fig. 4C). Most Venus<sup>+</sup> cells did not express CD3ε.

The effect of in vivo administration of IL-25 and IL-33 on IL-5 production in the intestines was also analyzed (Supplemental Fig. 2). In the small intestine, a larger number of Venus<sup>+</sup> cells were observed in rIL-25– and rIL-33–treated mice than in rIL-33–treated mice, whereas IL-33 treatment was more effective to induce Venus<sup>+</sup> cells than IL-25 treatment in the large intestine (Supplemental Fig. 2A). This was further confirmed by flow cytometric analysis of LP cells from the large intestine (Supplemental Fig. 2B). We then evaluated the IL-5 and IL-13 mRNA expression in the small and large intestines in response to IL-25 and IL-33 by quantitative RT-PCR analysis (Supplemental Fig. 2C, 2D). IL-13–producing cells respond almost equally to IL-25 and IL-33 in the intestine or MLNs (19, 21). To compare the expression patterns of IL-5 and IL-13, we analyzed IL-13 mRNA expression (Supplemental Fig. 2D). Intriguingly, IL-13 mRNA expression was enhanced by IL-25 more significantly than by IL-33, both in the small and large intestines. We also examined the effect of IL-25 and IL-33 on IL-5 production in MLNs (Supplemental Fig. 2E) and PPs (Supplemental Fig. 2F). IL-25 and IL-33 also induced Venus<sup>+</sup> cells in MLNs and, to a lesser degree, in PPs. In both cases, IL-33 was more effective than IL-25.

It has been shown that in vivo treatment of IL-25 and IL-33 in mice attracts eosinophils into local tissues. When we treated wild-type (WT) with rIL-25 and rIL-33 locally, there was clear eosinophil infiltration in the lung, whereas the effect of IL-25 and IL-33 was not observed in IL-5<sup>V/V</sup> mice (Fig. 5A). Treatment of WT mice by IL-25 and IL-33 resulted in a significant increase of eosinophils in the lung, ∼3-fold in cell number in comparison with PBS-injected controls, which was not observed in IL-5<sup>V/V</sup> mice (Fig. 5B). Notably, numbers of eosinophil in the lungs of naive IL-5<sup>V/V</sup> mice were significantly decreased compared with those from WT mice. These data clearly demonstrate roles of IL-25 and IL-33 in the regulation of innate IL-5–producing cells and subsequent accumulation and migration of eosinophils into the lung.

**Mice deficient in IL-5 signaling or treated with IL-5–neutralizing Abs exhibit increased lung tumor metastasis and impaired eosinophil regulation**

One of the functions of eosinophils primed by Th2 cytokines is tumor surveillance and clearance (25, 30). Given that deficiency of IL-5 causes impaired eosinophil recruitment to the lung (Fig. 5), we used an experimental model of lung tumor metastasis using a melanoma cell line (B16F10). Melanoma cells were i.v. injected into IL-5<sup>+/+</sup>, IL-5<sup>V/V</sup>, or IL-5<sup>V/V</sup> mice, and tumor nodules were counted (Fig. 6A). The number of nodules in IL-5<sup>V/V</sup> mice was significantly increased compared with that in IL-5<sup>+/+</sup> and IL-5<sup>V/V</sup> mice (Fig. 6B). Increased tumor metastasis was also observed in IL-5R<sup>a</sup> KO mice (Supplemental Fig. 3A–C). In IL-5<sup>V/V</sup> and IL-5R<sup>a</sup> KO mice, IL-5 signaling is constitutively defective. To investigate the effect of secreted IL-5 after tumor injection, IL-5 was neutralized with anti–IL-5 mAb. A bolus injection of anti–IL-5 mAb resulted in increased numbers of tumor nodules (Fig. 6C). Importantly, neutralization of IL-5 completely blocked the recruitment of eosinophils into the lung (Fig. 6D). We also evaluated the migration of eosinophils into the lungs of WT and IL-5R<sup>a</sup> KO mice on days 1, 3, 5, and 7 after tumor inoculation (Supplemental Fig. 3D). There was a notable difference in the migration of eosinophils into the lung between WT and IL-5R<sup>a</sup> KO mice.

**Exogenous IL-5 treatment induces an elevated level of eosinophilia and prolonged eosinophil migration toward tumor cells**

To further evaluate the cooperative relationship between IL-5 and eosinophils in antimetastatic activity in the lung, we induced lung eosinophilia by administering rIL-5. This treatment successfully recruited eosinophils into the lung (Fig. 7A, 7B). As IL-5 overexpression resulted in increased tumor-infiltrating eosinophils (30), the location of eosinophils in relation to tumor metastases was analyzed by H&E staining (Supplemental Fig. 3E) and immunohistochemistry (Fig. 7C). Metastasized melanoma cells developed clusters of cells that localized to the capillary vessels of alveolar walls (Supplemental Fig. 3E). Although eosinophils were not reliably detectable in tissues stained with H&E, combination

**FIGURE 6.** Increased tumor metastasis in mice deficient in IL-5 signaling and treated with IL-5–neutralizing Abs. A, Tumor nodules on day 14 in IL-5<sup>+/+</sup>, IL-5<sup>V/V</sup>, and IL-5<sup>V/V</sup> mice (n = 19, 13, and 17, respectively) injected with B16F10 melanoma (2 × 10<sup>5</sup> cells). B, Representative images of tumor metastasis from A. C, Tumor nodules in mice treated i.p. with IL-5–neutralizing mAbs (NC17) or control IgG (n = 8/group) 7 d after injection of B16F10 melanoma (2 × 10<sup>5</sup>) cells. Data are shown as mean ± SD and are representative of two independent experiments. Asterisks indicate statistical significance (*p < 0.05).
staining with Fontana-Masson followed by immunohistological analysis clearly demonstrated eosinophils around melanoma cell clusters. Eosinophils visible in a high-power field (HPF), centered on melanoma cells, were collected and counted. Quantitation revealed that the numbers of migrating eosinophil toward tumor cells were significantly greater in the rIL-5–treated mice at 48 h post-tumor injection than in IL-5–nontreated controls (Fig. 7C, 7D). We infer from these results that IL-5 is able to prolong eosinophil migration toward tumor cells in the lung tissue.

Invading tumor cells enhance local IL-5 production from innate IL-5–producing cells

As eosinophil infiltration in the lung was increased after tumor inoculation (Fig. 6D), IL-5 production may be upregulated by melanoma cell infiltration into the lung. To more directly measure the role of IL-5 in preventing tumor metastases, we analyzed IL-5 mRNA expression in the sorted lung CD45+Mac-1− fraction from WT mice at 6 and 24 h post-tumor injections (Fig. 8A). Compared to the PBS control group, IL-5 mRNA was significantly upregulated ∼3-fold at 6 h and 15-fold at 24 h after the melanoma injection. This IL-5 upregulation was not observed in other sites (Fig. 8B). These results suggest that upregulation of IL-5 expression in the lung contributes to induce eosinophil infiltration, suppression of metastasis, or progression of tumor cells in the lung. In support of this idea, administration of rIL-5 into the bronchus suppressed progression or metastasis of melanoma cells (Fig. 8C).

The source of IL-5 was primarily c-Kit+Venus+ lymphoid cells (Fig. 8D), and they showed an ∼2.5-fold increase on day 3 after tumor injection (Fig. 8E). This appeared to be well correlated with the numerical increase observed in eosinophils post-tumor inoculation (Fig. 6D). Supplemental Fig. 3D. As innate IL-5–producing cells in the lung responded to IL-25 and IL-33 (Fig. 4), the mRNA expression of IL-25 and IL-33 was analyzed periodically after melanoma inoculation (Fig. 8F). The mRNA expression of IL-2 and IL-7 was also assessed. IL-33 expression was transiently upregulated ∼2-fold at 6 h post-tumor injection and declined to control level at 24 h, whereas IL-25 expression was not detected in the lung. The mRNA expression of IL-2 and IL-7 was not upregulated significantly. These results imply that upregulation of IL-5 mRNA expression in the lung of melanoma-bearing mice may be caused by the transient increase in IL-33 expression.

Discussion

We have generated IL-5/Venus KI mice that enabled us to identify innate IL-5–producing cells residing in the small and large intestines, peritoneal cavities, and the lung. We have shown that T cells are not the major IL-5 producers in the steady state as well as under the conditions promoted by IL-25 and IL-33. Innate IL-5–producing cells share several characteristics with natural helper cells, nuocytes, MPPtype2, and Ih2 cells, such as tissue localization, surface markers, namely, lineage-negative c-Kit+Sca-1+, and responsiveness to IL-25 and/or IL-33. In contrast, there are several phenotypic and regulatory differences as well. Innate IL-5–producing cells are phenotypically different from MPPtype2 cells that do not express IL-7Ra, T1/ST2, Thy1.2, and CD44. As previously suggested for other innate cells (37), innate IL-5–producing cells are possibly differentiated from MPPtype2 cells. In vitro experiments showed that there were innate lymphoid cells that produced IL-5, IL-13, or both of these cytokines, raising a possibility that IL-5– and IL-13–producing cells might be regulated differently. Although it is difficult to perform a numerical comparison among the innate cells due to different reporter genes used, innate IL-5–producing cells seem to be more abundant than IL-13–producing cells in the lung and peritoneal cavities in unprimed mice (21). In addition, IL-25 is better at inducing IL-13 production in the small and large intestines, compared with IL-33, which showed a larger effect in the lung and peritoneal cavities in unprimed mice (21). These data support the idea that innate IL-5– and IL-13–producing...
cells may differ, although a significant fraction of them have the
ability to produce both cytokines. Innate IL-5–producing cells are
phenotypically and functionally closer to natural helper cells be-
cause natural helper cells express the surface Ags examined in this
study and produce Th2 cytokines naturally, and both were reported
to maintain B-1 B cells in the peritoneal cavity (18). As demon-
strated in innate IL-5–producing cells by the current study, natural
helper cells possibly express Th2 cytokines heterogeneously. In the
current study, we have not examined the function of innate IL-5–
producing cells in the same experimental model used to study other
newly identified innate cell populations. It is therefore not clear
whether all of them including innate IL-5–producing cells are
identical in vivo in the case of parasite infection or immune
responses such as allergies. Further work is required to more pre-
cisely define and categorize these novel innate cells.

To investigate the role(s) of IL-5 in the lung, we used an ex-
perimental tumor metastasis model because eosinophils are potent
effectors for tumor rejection and accumulating evidence has
established that IL-5 is essential for eosinophil development. We
demonstrated that deficiency of IL-5 signaling and neutral-
ization of IL-5 resulted in increased lung tumor metastasis, very
likely due to a shortage of lung eosinophils and impaired eosinophil
recruitment in response to invading tumors. IL-5/Venus KI mice
demonstrated that enhanced IL-5 production mediated by tumor

FIGURE 8. Invading tumors upregulate IL-5 production locally from innate IL-5–producing cells. A, IL-5 mRNA expression in the lungs of tumor-injected mice. mRNA from lung CD45+Mac-1+ cells sorted from WT mice (n = 4/group) was measured for IL-5 mRNA expression at 6 and 24 h after injection of B16F10 (B16) cells (1 × 10⁶). B, IL-5 mRNA expression in immune-related tissues and organs in tumor-injected mice. mRNA from the indicated tissues and organs of WT mice (n = 3 to 4/group) was measured for IL-5 expression at 24 h after tumor injection (1 × 10⁶ melanoma cells). C, Tumor metastasis in mice treated with rIL-5 locally. PBS or rIL-5 (1000 U) was injected directly into the bronchus of WT mice (n = 6/group) for 3 d, and tumor nodules were counted on day 7 after tumor injection (2 × 10⁷ melanoma cells). D and E, Temporal analysis of Venus+ cell number from IL-5+/V mice (n = 4–6/group) after tumor injection (1 × 10⁶ melanoma cells). Flow cytometric analysis (D) and numbers of c-Kit+ Venus+ cells (E) are shown. F, mRNA expression of the indicated cytokines in lungs from WT mice (n = 4 per group) at 6 and 24 h after tumor injection (1 × 10⁶ melanoma cells). Data are shown as mean ± SD and are representative of two or three independent experiments. Asterisks indicate statistical significance (*p < 0.05, **p < 0.01).
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Disclosures

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References


