Interleukin-5 Facilitates Lung Metastasis by Modulating the Immune Microenvironment

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Abstract

Although the lung is the most common metastatic site for cancer cells, biologic mechanisms regulating lung metastasis are not fully understood. Using heterotopic and intravenous injection models of lung metastasis in mice, we found that IL5, a cytokine involved in allergic and infectious diseases, facilitates metastatic colonization through recruitment of sentinel eosinophils and regulation of other inflammatory/immune cells in the microenvironment of the distal lung. Genetic IL5 deficiency offered marked protection of the lungs from metastasis of different types of tumor cells, including lung cancer, melanoma, and colon cancer. IL5 neutralization protected subjects from metastasis, whereas IL5 reconstitution or adoptive transfer of eosinophils into IL5-deficient mice exerted prometastatic effects. However, IL5 deficiency did not affect the growth of the primary tumor or the size of metastatic lesions. Mechanistic investigations revealed that eosinophils produce CCL22, which recruits regulatory T cells to the lungs. During early stages of metastasis, Treg created a protumorigenic microenvironment, potentially by suppressing IFNγ-producing natural killer cells and M1-polarized macrophages. Together, our results establish a network of allergic inflammatory circuitry that can be co-opted by metastatic cancer cells to facilitate lung colonization, suggesting interventions to target this pathway may offer therapeutic benefits to prevent or treat lung metastasis.

Introduction

Most cancer deaths occur due to metastatic disease and its complications, and the lungs are the most common metastatic site for a variety of cancers (1). Metastasis is a complex multistep process, which includes detachment of malignant cells from the primary tumor, intravasation and circulation of malignant cells in the bloodstream, extravasation in the target organs, degradation of extracellular matrix, and colonization in the target organ (2). Although the majority of malignant cells never reach the invasion and colonization stages due to immune surveillance, some cells develop the ability to escape immune control and invade distant organs, including the lungs. To avoid the host immune system, cancer cells can “educate” immune cells to support metastases within the pulmonary microenvironment through secretion of cytokines. However, specific cellular and molecular mechanisms by which immune/inflammatory cells support invasion, survival, and proliferation of malignant cells are incompletely understood.

Cytokines are essential in sculpting the tumor microenvironment and have been implicated in regulation of tumor growth and progression. To this end, the role of Th2 cytokines is increasingly appreciated (3). IL5 is a Th2 cytokine produced mainly by lymphocytes and to a lesser degree by mast cells, type II innate lymphoid (ILC2) cells, and eosinophils (4, 5). IL5 was originally shown to stimulate antibody production by activated B cells (6), but more recently has become known as a major factor in eosinophil differentiation and proliferation. IL5 appears to have a role in cancer biology, but existing evidence is contradictory. We previously reported that IL5 does not alter tumor formation after treatment with the carcinogen urethane (9); however, we found that IL5 enhances adenocarcinoma-induced malignant pleural effusion, a hallmark of progressive disease (10). In contrast, other studies have found that IL5 facilitates immune surveillance against carcinogen-induced fibrosarcoma and melanoma (11, 12). In humans, elevated serum IL5 levels and excessive eosinophilia have been detected in patients with advanced stage non–small cell lung cancer (13). These data led us to investigate a possible contribution of IL5 to tumor cell metastasis in the lungs. By using a variety of preclinical models of metastasis, we found that IL5 is a pivotal factor in determining metastatic colonization of the lungs.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Mouse models

All animal care and experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee (Nashville, TN). Sex-, weight-, and age-matched 8- to 10-week-old wild-type C57BL/6 mice, and IL5-deficient mice (C57BL/6-Il5−/−), hereafter called IL5KO mice (9), c-kit mutant mice (B6.Cg-Kit−/−/H2bNih/nHaesBsmGlij), hereafter called c-kit−/−, ref. 14) were used. For intravenous (i.v.) metastasis models, Lewis lung adenocarcinoma (LLC; 1.5 × 106), B16-F10 mouse melanoma (2.5 × 105; ATCC), or MC38 colon adenocarcinoma (0.5 × 105) cells (from Dr. D. Lee Gorden, Vanderbilt University, Nashville, Tennessee) were injected into the tail vein (15). Mice were euthanized at day 14 after injection of tumor cells. For flank tumor generation, LLC cells (2.5 × 105) were injected subcutaneously (s.q) and surface tumors were counted as previously described (16). Tumor diameters were determined using digital calipers (Fisher Scientific).

Reconstitution or neutralization of IL5 in vivo

To reconstitute IL5 in IL5KO mice, 50 mg/mL IL5 (R&D) in 100 mL PBS or PBS alone was delivered to mice by intraperitoneal (i.p.) injection every other day for 4 days before i.v. injection of LLC cells or every other day starting the day of tumor cell injection until harvest at day 14 (17). For IL5 neutralization studies, mice received weekly i.v. injections of mouse IL5 mAb (Clone TRFK5, R&D; 1 mg/kg) or isotype control (IgG) antibodies (Biolegend) by i.p. injection 2 days before i.v. inoculation of LLC or bone marrow-derived eosinophils (BMDEos).

Measurement of IL5 protein

Concentration of IL5 protein in mouse lung, blood, and bone marrow was determined using ELISA (R&D).

Cell proliferation

After seeding equal numbers of LLC cells into 96-well plates, the CellTiter-Glo assay (Promega) was used to measure viable cells. Cell proliferation was determined using ELISA (R&D). CellTiter-Glo (Promega) was used to measure viable cells.

Reconstitution or neutralization of IL5

To reconstitute IL5 in IL5KO mice, 30 ng/mL IL5 (R&D) in 100 mL PBS or PBS alone was delivered to mice by intraperitoneal (i.p.) injection every other day for 4 days before i.v. injection of LLC cells or every other day starting the day of tumor cell injection until harvest at day 14 (17). For IL5 neutralization studies, mice received weekly i.v. injections of mouse IL5 mAb (Clone TRFK5, R&D; 1 mg/kg) or isotype control (IgG) antibodies (Biolegend) by i.p. injection 2 days before i.v. inoculation of LLC or bone marrow-derived eosinophils (BMDEos).

Measurement of IL5 protein

Concentration of IL5 protein in mouse lung, blood, and bone marrow was determined using ELISA (R&D).

Cell proliferation

After seeding equal numbers of LLC cells into 96-well plates, the CellTiter-Glo assay (Promega) was used to measure viable cells during different intervals of culture in the presence of rmIL5 (10 ng/mL, R&D) or IL5 antibodies (5 ng/mL, R&D).

Fluorescent microscopy and IHC

For studies to detect LLC cells in the lungs, cells were stained with CellTracker Red CMTPX (Molecular Probes) before i.v. injection and lungs were harvested 24 hours later. Using Metamorph software, LLC cells were counted on four randomly selected fields of lung parenchyma (×20 magnification) and divided by the total area of lung tissue measured using Metamorph software. For detection of eosinophils, 5-μm lung sections were immunostained with rat anti-mouse MBP-1 monoclonal antibody (from Dr. James Lee, Mayo Clinic, Rochester, MN) and detected using diaminobenzidine-peroxidase reagents and methyl green counterstain (18). MBP-1+ eosinophils were counted on 10 randomly selected fields of lung parenchyma at ×20 magnification.

Flow cytometry

Isolation of lung cells for flow cytometry was performed using a previously described protocol (19). Cells were stained with the following antibodies: CD4 - FITC, CD25 - APC, MHC-II - biotin (e-Bioscience), CD45 - APC-Cy7, Foxp3 - PE, CD19 - PerCP-Cy5.5, CD11c - Brilliant Violet 605, Siglec-F - Brilliant violet 421, CD49b - Brilliant Violet 421, CD206 - APC (Biologend), CD3 - PE-Cy7, CD8 - Alexa Fluor 700, IFNγ - APC (BD Biosciences), CCR3 - APC (R&D), CD68 - FITC (AbD Serotec), and F4/80 - PE (Life Technologies). Dead cells were excluded using Live/Dead Fixable Blue Dead Cell Stain Kit (Life Technologies). For determining production of IFNγ, lung cells were stimulated with PMA (1 ng/mL) and ionomycin (1 μmol/L) for 6 hours in the presence of Golgi-stop (BD Biosciences) at 37°C, 5% CO2. After staining for surface markers, cells were permeabilized and processed for intracellular staining using anti-IFNγ or anti-IL4 antibodies. Flow cytometry was performed using BD LSR II flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

Adaptive transfer of eosinophils

Bone marrow-derived eosinophils (BMDEos) were obtained as described (20) and 5 × 106 cells were injected via the tail vein 1 hour before injection of LLC cells.

Real-time PCR

Total RNA was isolated from lung tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s specifications. DNase-treated samples were subjected to qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems). PCR primers used were: IFNγ forward: 5’-TCTCGGATGACTCTAATTGGG-3’, reverse: 5’-CTCCGAGTACGTCATATTGAC-3’; IL4 forward: 5’-TCTCAGAGCAAGCAGAAGAC-3’, reverse: 5’-TGAGCTCTCATTGAGAACCT-3’; CCL17 forward: 5’-TGGTCTTGGGAGACTTTCTG-3’, reverse: 5’-CATCCCCGACACACTTAC-3’; CCL20 forward: 5’-TTTGGGGATGGAATTGGACAC-3’, reverse: 5’-TGCAAGTCCATTGAGAACCT-3’; CCL17 forward: 5’-TGCAAGTCCATTGAGAACCT-3’, reverse: 5’-TGCAAGTCCATTGAGAACCT-3’; CCL17 forward: 5’-TGCAAGTCCATTGAGAACCT-3’, reverse: 5’-TGCAAGTCCATTGAGAACCT-3’; CCL17 forward: 5’-TGCAAGTCCATTGAGAACCT-3’, reverse: 5’-TGCAAGTCCATTGAGAACCT-3’. Relative mRNA expression in each sample was normalized to GAPDH and presented using the comparative C method (20).

In vivo CCL2 and regulatory T-cell depletion

For CCL2 depletion, mice received two consecutive daily i.p. injections with anti-CCL2 antibodies (100 μg, R&D) or IgG2A isotype control antibodies (Biologend) completed 24 hours before i.v. injection of LLC cells. For depletion of regulatory T cells (Treg), mice received 500 μg of monoclonal anti-CD25 antibodies (clone PC61; ATCC) or IgG1 isotype control antibodies (Biologend) by i.p. injection 2 days before i.v. injection of LLC cells.

Statistical analysis

Data were analyzed using the GraphPad Prism 5.0 software (GraphPad Software Inc.) and values are presented as mean ± SEM. Pair-wise comparisons were made using t tests. For experiments conducted over several time points or with multiple comparisons, a two-way ANOVA with a Bonferroni post-test was used to determine differences among groups. *P < 0.05 was considered statistically significant.
Results

IL5 promotes metastasis to the lungs

To investigate whether IL5 affects lung metastasis, we utilized a heterotopic model in which we injected LLC cells (2.5 × 10⁵ cells/mouse) subcutaneously into the flank of WT and IL5KO mice and measured primary tumor growth and metastatic lesions in the lungs 28 days later. Although IL5 deficiency did not alter the growth of primary tumors (Fig. 1A), it almost completely abrogated pulmonary metastasis from these primary tumors (Fig. 1B). To test whether protection from lung metastasis could be reproduced by neutralization of IL5, we treated WT mice weekly with anti-IL5 antibodies (IL5 mAb, 1 mg/kg) or isotype control (IgG) antibodies at days 0, 7, 14 after s.q. injections of LLCs and analyzed lung metastasis. Consistent with results from IL5KO mice, antibody-mediated depletion of IL5 reduced lung metastasis of LLC cells in the flank tumor model (Fig. 1C), but did not alter growth of the primary subcutaneous tumors (Fig. 1D). We next investigated whether reconstitution of IL5 in IL5KO mice would increase LLC lung metastasis. For these studies, IL5KO mice received s.q. injection of LLC cells followed by i.p. injection of rmIL5 (50 ng) or vehicle (PBS) every other day starting on the day of tumor cell injection. As shown in Fig. 1E, reconstitution of IL5 in IL5KO mice significantly increased the number of pulmonary metastases in this model. Together, these findings show that IL5 plays an important role in supporting pulmonary metastasis.

To confirm our findings in the heterotopic model and begin to investigate the mechanisms by which IL5 impacts lung metastasis, we delivered LLC cells to the pulmonary circulation via injection into the lateral tail vein. For these studies, we injected LLC cells (1.5 × 10⁵ cells/mouse) into WT and IL5KO mice and measured lung metastases 14 days later. As with the heterotopic model, IL5 deficiency almost completely abrogated metastatic lesions in the lungs (Fig. 2A and B); however, no effect on the size of metastatic lesions was identified (Fig. 2C). Similarly, antibody-mediated IL5 depletion in WT mice significantly reduced lung metastases after intravenous delivery of LLC cells (Fig. 2D). In contrast, reconstitution of IL5 in IL5KO mice significantly increased the number of metastatic lesions in the intravenous lung metastasis model (Fig. 2E). Together with studies in the heterotopic lung metastasis model, these studies suggest that IL5 functions to regulate metastasis in the distal organ (lung) after hematogenous dissemination of tumor cells.

To determine whether prometastatic effects of IL5 were broadly applicable to other tumor types, we injected B16-F10 melanoma (2.5 × 10⁵) or MC38 colon adenocarcinoma (0.5 × 10⁵) cells i.v. via the lateral tail vein into WT and IL5KO mice and measured lung metastases 14 days later. As with LLC cells, IL5KO mice developed significantly fewer lung metastases than WT mice after injection of these tumor cell types (Fig. 2F and G). As with LLC cells, the mean size of lung metastatic foci was not different between WT and IL5KO mice (Fig. 2H and I). In combination, these studies indicate that IL5 plays an important role in lung metastasis of a variety in different tumor cell types and models without affecting growth of the primary or metastatic lesions.

To determine whether IL5 production is increased in the setting of lung metastasis, we analyzed IL5 protein expression in the lungs, blood, spleen, and bone marrow of WT mice after i.v. injection of LLC cells. We found a transient increase in IL5 in lung and bone marrow at 24 hours after i.v. LLC cell injection, but no change in IL5 levels was observed in blood and spleen (Fig. 3A). Because IL5 levels were low and increased only at a very early time point, we postulated that IL5 might affect the early stages of metastasis. Therefore, we tested whether reconstitution of IL5 before LLC cell injection would augment pulmonary metastasis. To this end, IL5KO mice received rmIL5 every other day for 4 days before i.v. injection of LLC cells. At day 14 after LLC injection, IL5KO mice pretreated with rmIL5 displayed significantly increased number of pulmonary metastases compared with IL5KO mice injected with LLC cells and similar to the number of metastases in IL5KO mice injected with IL5 every other day for
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Figure 2.
Deficiency of IL5 reduces lung metastases after i.v. delivery of a variety of different tumor cells. A–C, representative images of lungs with metastatic foci (arrows; A), number of lung metastases (B), and size of lung metastases (C) at day 14 after i.v. injection of LLC cells into WT and IL5KO mice (1.5 × 10^7 cells/mouse; n = 17–19 mice per group; *, P < 0.05). D, number of lung metastases in WT mice at day 14 after i.v. injection of LLC cells followed by i.p. injection with IL5 antibodies (IL5 mAb, 1 mg/kg) or control antibodies (IgG) on days 0 and 7 (n = 13 mice per group; *, P < 0.05). E, number of lung metastases at day 14 after i.v. injection of LLC cells into WT and IL5KO mice treated every other day with i.p. injection of PBS–vehicle (veh) or rmIL5 (50 ng/mouse, n = 7–12 mice per group starting the day of LLC injection; *, P < 0.05 compared with the IL5KO–veh group). F–I, the number of lung metastases (F and G) and size of lung metastases (H and I) in WT and IL5KO mice at day 14 after i.v. injection of B16–F10 melanoma cells (2.5 × 10^7/mouse, n = 8 mice per group) or MC38 colon cancer cells (0.5 × 10^7/mouse, n = 5–7 mice per group); *, P < 0.05.

The findings suggest that IL5 is most critical during early stages of lung metastasis. In the i.v. LLC model, the majority of tumor cells are cleared from the lung vasculature by 24 hours, whereas a small number of surviving cells extravasate and begin the process of establishing a metastatic niche (15). We next investigated whether IL5 affects these initial steps of vascular clearance or extravasation of tumor cells into the lungs. For these studies, LLC cells were labeled with Cell Tracker Red CMPTX in vitro and were subsequently delivered by i.v. injection to WT and IL5KO mice. Lungs were then harvested and examined 24 hours later for dye-containing LLC cells in the lung parenchyma. At this point, similar numbers of LLC cells were identified in the lungs of WT and IL5KO mice (Fig. 3C and D). These findings indicate that IL5 does not affect the initial steps of metastasis (i.e., intravascular tumor cell survival and extravasation into the lungs), but more likely regulates development of the early metastatic niche to allow survival and growth of tumor cells that invade the lung interstitium.

We next asked whether IL5 directly modulates survival or proliferation of tumor cells. LLC cells in culture expressed neither IL5 nor IL5 receptors (data not shown). We then conducted in vitro experiments in which LLC cells were incubated in the presence of PBS (control), neutralizing anti-IL5 mAb, or rmIL5. Serial assessment revealed no differences in cell number between treatment groups at any time point (Fig. 3E), suggesting that IL5 facilitates pulmonary metastasis indirectly, by influencing cells in the local lung microenvironment, rather than through directly affecting tumor cells.

IL5 facilitates pulmonary metastasis by regulating eosinophils in the lungs

We postulated that immune/inflammatory cells regulated by IL5 might facilitate pulmonary metastasis. Because IL5 promotes recruitment and expansion of tissue eosinophils (7, 8), we analyzed lungs from WT and IL5KO mice for infiltration with eosinophils. We immunostained lung sections from WT and IL5KO mice at days 0, 1, and 3 after i.v. injection of LLC cells. We identified eosinophils as cells expressing eosinophil-specific anti–MBP-1 antibodies (18). As shown in Fig. 4A and B, we detected MBP-1–positive eosinophils in lung metastases of WT mice, while very few MBP-1–positive cells were detected in lungs from IL5KO mice. To determine whether IL5 deficiency results in decreased infiltration of lungs with eosinophils at early time points after injection of LLC cells, we harvested lungs from WT and IL5KO mice on days 0, 1, and 3 after i.v. injection of LLC cells and performed IHC using anti–MBP-1 antibodies. Similar to the results at day 14, MBP-1–positive eosinophils were markedly reduced in the lungs from IL5KO mice compared with WT mice (Fig. 4C and D). To confirm these findings, we performed flow-cytometric analysis of lung eosinophils from WT and IL5KO mice at days 0, 1, and 3 after i.v. injection of LLC cells. We identified eosinophils as cells expressing CD45, Siglec-F, and F4/80, but not CD11c (21–23). These eosinophils expressed high levels of CCR3, low levels of Gr1, and were negative for CD68 (Supplementary Fig. S1) as previously reported (24). As shown in Fig. 4E, lungs of IL5KO mice contained very few eosinophils before or after LLC cell injection and WT lungs contained more than 10-fold greater eosinophils than IL5KO mice at each time point (Fig. 4F). Together, these studies demonstrate that IL5 is important for the recruitment and maintenance of pulmonary eosinophils during metastatic colonization of the lungs.

The entire 14 days (Fig. 3B).
To test whether eosinophils play an important role in lung metastasis, we differentiated normal eosinophils from bone marrow progenitor cells of WT mice using published protocols (20) and performed adoptive transfer of these cells to WT and IL5KO mice. The purity of cultured BMDEos was confirmed to be >98% by analysis of cell morphology (Fig. 4G). WT and IL5KO mice received BMDEos (5 × 10^5) by i.v. injection one hour before i.v. injection of LLC. We chose this timing for injection of BMDEos because of the relatively short in vivo half-life of these cells (20). Adoptive transfer of BMDEos resulted in a large (3-fold) increase in lung metastases in IL5KO mice, whereas WT mice showed a trend toward increased numbers of pulmonary metastases (P = 0.07; Fig. 4H). On the basis of these results, we concluded that eosinophils are important for IL5-mediated promotion of pulmonary metastasis.

Because mast cells have been previously reported to produce IL5 (5), we next evaluated whether these cells support lung metastasis by influencing recruitment of eosinophils to the lungs. We used c-kit−/−mutant mice (c-kit^W-sh mice), which have been shown to be deficient in mast cells (14). Littermate WT mice were used as controls. First, we analyzed MBP-1-positive eosinophils in lung sections from c-kit^W-sh mice and WT controls at 24 hours after i.v. injection of LLCs and found that eosinophils were markedly reduced in the lungs of c-kit^W-sh mice (Fig. 4I). In addition, we detected significantly fewer lung metastases in c-kit^W-sh mice compared with WT mice after i.v. injection of LLC cells (Fig. 4J). These studies suggest that c-kit+ cells regulate the number of eosinophils in the lungs, possibly through local production of IL5.

**Eosinophil-secreted CCL22 facilitates metastasis through local recruitment of regulatory T cells**

In addition to exerting effects on eosinophils, IL5 can affect lymphocyte fate (25) and the role of T and B lymphocytes in modulation of tumor growth and metastasis is increasingly appreciated (26). We therefore performed flow cytometry to analyze lymphocyte subsets in the lungs of WT and IL5KO mice before and after i.v. injection of LLC cells. Compared with untreated mice, we detected increased total CD4+ lymphocytes and NK cells in the lungs of both WT and IL5KO mice at 24 hours after LLC cell injection, but no differences were found between WT and IL5KO mice (Supplementary Fig. S2). In contrast, CD8+ lymphocytes and B lymphocytes were not increased in the lungs after LLC cell injection in either WT or IL5KO mice (Supplementary Fig. S2).

Compared with IL5KO mice, we detected increased numbers of CD4+ /CD25+/Foxp3- Tregs in the lungs of WT mice at baseline and 24 hours after LLC cell injection (Fig. 5A and B). To determine whether Tregs play a role in metastasis of LLC cells to the lungs, we depleted these cells using monoclonal anti-CD25 antibodies. We treated WT mice with anti-CD25 (500 μg/mouse) or isotype control IgG 2 days before i.v. injection of LLC cells and assessed lung metastases after 14 days. As indicated in Fig. 5C and D, Treg depletion significantly decreased pulmonary metastases.

To explain the reduction of Tregs found in IL5KO mice, we wondered whether IL5 could affect local generation of Tregs from naïve T cells through TGFβ- and/or IL10-dependent mechanisms (27). We therefore measured TGFβ and IL10 mRNA expression in lung tissue by qRT-PCR at 24 hours after i.v. injection of LLC cells.
and found no differences between WT and IL5KO mice (Fig. 6A and B). Next, we evaluated whether IL5 affects recruitment of Tregs to the lungs during metastasis. We measured chemokines that could support Tregs immigration into the lungs, including C-C motif chemokine ligands (CCL) 17, 20, and 22 (28). Although we did not observe significant differences in CCL17 or CCL20 mRNA expression in lung tissue, CCL22 expression was markedly reduced in IL5KO mice compared with WT mice (Fig. 6C–E).

Because both Tregs and eosinophils were reduced in lungs of IL5KO mice, we analyzed CCL22 expression by eosinophils. In vitro, mRNA expression of CCL22 by WT BMDEos was increased >30-fold after 4 hours of incubation with LLC-conditioned culture media (Fig. 6F). Consistent with these findings, eosinophils isolated from lungs of WT mice at 24 hours after i.v. LLC injection expressed higher mRNA levels of CCL22 in comparison with lung eosinophils obtained from untreated WT mice (Fig. 6G). In contrast, no differences in CCL22 mRNA expression were detected in lung macrophages from these mice following i.v. LLC cell injection (data not shown).

Subsequently, we investigated whether CCL22 contributes to lung recruitment of Tregs after injection of tumor cells. WT mice received two consecutive daily injections with anti-CCL22 antibody (100 μg/mouse) or isotype-control IgG completed 24 hours before intravenous delivery of LLC cells. We found that neutralization of CCL22 significantly reduced lung Tregs at 24 hours after LLC cell injection (Fig. 6H). To determine whether CCL22 regulates lung metastasis, we injected WT mice with CCL22 or IgG-control antibodies before i.v. injection of LLC cells (as described above) and analyzed lung metastases 14 days later. As shown in Fig. 6I and J, mice pretreated with CCL22-neutralizing antibodies had significantly fewer pulmonary metastases compared with isotype-treated controls. Collectively, these results indicate that IL5 promotes lung metastasis in part through CCL22-dependent recruitment of Tregs.
findings show that eosinophils produce CCL22, which facilitates lung metastasis through recruitment of Tregs to the lung microenvironment.

Tregs alter the lung microenvironment during metastasis by suppressing IFNγ production by NK cells and altering macrophage polarization

To investigate the mechanisms by which Tregs affect lung metastasis, we tested whether Tregs alter polarization of effector lymphocytes and macrophages after delivery of LLC cells to the lungs. We measured mRNA expression of the Th1/Th2 cytokines IFNγ and IL4 in the lungs of LLC cell-injected WT and IL5KO mice and found increased expression of the Th1 cytokine IFNγ in lungs of IL5KO mice, but not in WT mice. In contrast, no differences in IL4 expression were evident between groups (Fig. 7A and B). To determine the cell type(s) responsible for increased IFNγ expression in IL5KO mice, we performed flow cytometry to identify IFNγ expressing CD4+ T lymphocytes, CD8+ T lymphocytes, and NK cells. As shown in Fig. 7C and D, the proportion of CD4+ and CD8+ T lymphocytes expressing IFNγ after LLC cell injection was similar in WT and IL5KO mice; however, IFNγ-expressing NK cells were significantly increased in IL5KO mice (Fig. 7E). Consistent with mRNA expression results, we found no differences in the percentage of lung lymphocytes producing IL4 in WT and IL5KO mice at 24 hours after i.v. LLC injection (data not shown). To test whether CCL22-mediated recruitment of Tregs during metastasis suppresses NK cell activation, we treated WT mice with two consecutive daily injections of anti-CCL22 antibodies (100 μg/mouse) or isotype-control IgG followed by i.v. injection of LLC cells. At 24 hours after LLC cell injection, we found significantly increased numbers of NK cells expressing IFNγ in mice treated with anti-CCL22 antibodies (Fig. 7F). These results suggest that Tregs may support tumor cell survival through suppression of IFNγ production by NK cells.

Because IFNγ is a well-known stimulus for antitumor M1 macrophage polarization, we also analyzed expression of MHC II (M1 marker) and CD206 (mannose receptor, M2 marker) by lung macrophages at 24 hours after i.v. LLC cell injection. Using flow cytometry, we found that more lung macrophages from IL5KO mice expressed MHC II and fewer expressed CD206 compared with WT mice, indicating a shift toward M1 macrophage polarization in IL5KO mice (Fig. 7G–I). Together, these findings suggest that Tregs alter the lung microenvironment during lung metastasis by suppressing IFNγ production by NK cells, thereby limiting M1 macrophage polarization.

Discussion

The ability of tumor cells to metastasize to distant organs is highly dependent on their capacity for survival during interactions with host cells in the metastatic microenvironment (2). Accumulating evidence suggests a role for cytokines in the orchestration of prosurvival or antitumor responses by local immunity at the target site. However, specific mechanisms by which cytokines support...
metastasis of tumor cells to the lungs remain incompletely understood. Here, we demonstrated that IL5 is important for survival of metastatic cancer cells in the lungs. Using genetic and antibody-mediated IL5 depletion models, we found reduced metastasis of LLC cells to the lungs from WT or IL5KO mice at 24 hours after i.v. injection of LLC cells (1.5 × 10⁶ cells/mouse; *, P < 0.05). F and G, expression of CCL22 by BMDEos from WT mice alone or after 4 hours of incubation in media supplemented with 40% media from LLC cells (F; *, P < 0.05), or expression of CCL22 by lung CD45<sup>+</sup>/CD11c<sup>−</sup>/F4/80<sup>−</sup>/Siglec-F<sup>−</sup> eosinophils that were FACs sorted from WT mice prior (pre-LLC) or at 24 hours after iv injection of LLC cells (post-LLC; G). For the analysis of expression of CCL22 by lung eosinophils, cells from 6 mice per groups were pooled together. H, the number of Tregs in lungs of WT mice pretreated with isotype control antibodies (IgG) or anti-CCL22 antibodies (100 μg/mouse) before injection of LLC cells (1.5 × 10⁷/mouse) and analyzed by flow cytometry at 24 hours after LLC cell injection (n = 3–4 mice per group; *, P < 0.05). I and J, representative photographs of lungs (I) and number of lung metastases (J) in WT mice pretreated with isotype control antibodies (IgG) or anti-CCL22 antibodies (100 μg/mouse) at 48 and 24 hours before i.v. injection of LLC cells (1.5 × 10⁷/mouse). Lungs were harvested at day 14 after LLC injection (n = 9 mice per group; *, P < 0.05).
been linked to higher rates of development of distant metastasis and poor prognosis (29). Similarly, we reported that host-derived IL5 is critical for formation of malignant pleural effusions induced by adenocarcinoma cells (10). In the current study, we used a variety of cancer cell lines and models to elucidate an important role for IL5 in regulating a network of cellular interactions that sculpt the early metastatic niche in the lungs. Our findings are in direct opposition to a study by Ikutani and colleagues that utilized another IL5 deficient mouse model and found that IL5 suppressed metastasis of B16F10 melanoma cells to the lungs (12). Although explanation of these discrepant findings is not clear, the preponderance of evidence now indicates that IL5 is a prometastatic factor.

The effects of IL5 on primary tumors remain to be fully elucidated. In these studies, we found that IL5 did not affect growth of primary tumors or metastatic lesions. These findings are consistent with our previous study showing that IL5 does not alter lung tumor formation after treatment with the carcinogen urethane (9). In contrast, IL5 was shown to increase immune surveillance in a carcinogen-induced fibrosarcoma model (11). Also, IL5 expression has been detected in some non–small cell lung cancer cell lines (30) and primary non–small cell lung tumors (13) and Lee and colleagues showed that IL5 could directly promote migration and invasion of bladder cancer cells through activation of MAPK and Jak-Stat signaling pathways (31). Together, these findings suggest that the effects of IL5 on primary tumors may be dependent on cellular origin and microenvironmental factors.

We detected greater numbers of sentinel eosinophils in the lungs of WT mice compared with IL5KO mice under basal conditions and during lung metastasis. Like IL5, eosinophils have been reported to be increased in some malignancies but the role of these cells in cancer remains controversial. A number of studies have shown antitumor effects of eosinophils in primary
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colorectal, gastrointestinal, head and neck cancers, and melanoma (12, 32–36). Although it has been reported that higher numbers of tumor infiltrating eosinophils correlate with better prognosis in NSCLC (37), several studies have indicated that accumulation of eosinophils is associated with aggressiveness of human NSCLC and indicative of poor prognosis (13, 38, 39). In addition, the presence of eosinophils in oral squamous cell carcinoma is associated with poor prognosis (40, 41). In the current study, we found that adoptive transfer of eosinophils increases pulmonary metastasis in IL5KO mice, indicating a prometastatic role for pulmonary eosinophils. In addition, we found that c-kit+ cells support lung localization of eosinophils during metastasis. c-kit–deficient mice, which have reduced numbers of mast cells (14), had markedly reduced lung eosinophils and developed fewer lung metastases. In addition to mast cells, ILC2 cells are also known to express c-kit could be a source of IL5 in the lungs (4). Therefore, further studies are required to determine specific c-kit+ cell subsets contributing to promotion of IL5-mediated lung metastasis. Cumulatively, available data suggest that eosinophils support metastasis of tumor cells to the lungs but the effects of eosinophils, like IL5, on primary tumors may be context dependent.

We found that tumor cells induce eosinophils to express markedly elevated levels of CCL22, which is a well-known chemokine for recruitment of Tregs (28), a subset of CD4+ T lymphocytes that support survival of tumor cells by suppressing the antitumor immune response. Accumulating evidence suggests a strong connection between accumulation of Tregs in tumors and poor clinical outcome (42, 43). In the context of chronic airway inflammation, we previously reported that Tregs support primary lung tumorigenesis (44); however, the role of Tregs in lung metastasis has not been well characterized. Previous studies have linked Tregs to breast cancer metastasis in the lungs through CCR4, the receptor for chemokines CCL17 and CCL22 (45, 46). Although we did not observe changes in CCL17 mRNA expression in our studies, we found reduced CCL22 expression and fewer Tregs in the lungs of IL5KO mice after LLC cell injection. In conjunction with these findings, we identified increased expression of the antitumor cytokine IFNγ by NK cells in IL5KO mice, supporting the concept that Tregs are recruited to the lungs via eosinophil-derived CCL22 and suppress NK cell activation to facilitate survival of metastatic tumor cells.

Despite recent progress in deciphering the mechanisms that support lung tumor growth and progression, the efficacy of existing therapeutic strategies remains limited. Metastasis to the lungs accounts for the majority of deaths from lung cancer (47). Since the "Seed and Soil" hypothesis was first proposed (48), an ever-expanding body of evidence supports a critical role for the local microenvironment in supporting colonization of the lungs by metastatic tumor cells. Therefore, understanding microenvironment factors supporting pulmonary metastasis would be beneficial for the development of novel cancer treatment strategies. Our current study indicates that IL5 is involved in facilitating colonization of the lungs with tumor cells and suggests that IL5 neutralizing therapy could be beneficial for the prevention of pulmonary metastases. Humanized monoclonal antibodies against IL5 (including Mepolizumab, GlaxoSmithKline) have been extensively studied in patients with asthma and eosinophil-related disorders and are well tolerated (49). Thus, this approach could be rapidly translated into studies designed to prevent metastasis of primary lung cancers and other cancers that frequently develop pulmonary metastasis.

In conclusion, our studies identify a novel immune/inflammatory network that supports metastasis of tumor cells to the lungs through coordinated interactions between a variety of host cell types, including eosinophils, Tregs, NK cells, and macrophages. Together, these studies define several important therapeutic targets with the potential for translation into clinical studies designed to prevent metastasis to the lungs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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