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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, biological mechanisms regulating lung metastasis are not fully understood. Using heterotopic and intravenous injection models of lung metastasis in mice, we found that IL-5, 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 IL-5 deficiency offered marked protection of the lungs from metastasis of different types of tumor cells, including lung cancer, melanoma and colon cancer. IL-5 neutralization protected subjects from metastasis, whereas IL-5 reconstitution or adoptive transfer of eosinophils into IL-5 deficient mice exerted pro-metastatic effects. However, IL-5 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 (Treg) to the lungs. During early stages of metastasis Treg created a pro-tumorigenic microenvironment, potentially by suppressing

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IFN γ -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.

Keywords

IL-5; lung metastasis; eosinophils; CCL22; Treg

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). Interleukin-5 (IL-5) is a Th2 cytokine produced mainly by lymphocytes and to a lesser degree by mast cells, type 2 innate lymphoid (ILC2) cells, and eosinophils (4, 5). IL-5 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. IL-5 has been implicated in the pathogenesis of asthma, atopy, hypereosinophilic syndromes, and parasitic infections (7, 8). In addition, IL-5 appears to have a role in cancer biology, but existing evidence is contradictory. We previously reported that IL-5 does not alter lung tumor formation after treatment with the carcinogen urethane (9); however, we found that IL-5 enhances adenocarcinoma-induced malignant pleural effusion, a hallmark of progressive disease (10). In contrast, other studies have found that IL-5 facilitates immune surveillance against carcinogen-induced fibrosarcoma and melanoma (11, 12). In humans, elevated serum IL-5 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 IL-5 to tumor cell metastasis in the lungs. By employing a variety of preclinical models of metastasis, we found that IL-5 is a pivotal factor in determining metastatic colonization of the lungs.

Materials and Methods

Mouse models

All animal care and experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Sex-, weight-, and age-matched 8- to 10-wk-old wild type C57BL/6 mice, IL-5-deficient mice (C57BL/6-IL5^{tm1Kopf}/J, hereafter called IL-5KO) mice (9), c-kit mutant mice (B6.Cg-Kit^{W-sh}/HNihrJaeBsmGllJ, hereafter called c-kit^{W-sh}) (14) were used. For intravenous (IV) metastasis models, Lewis lung adenocarcinoma (LLC, 1.5×10⁵), B16-F10 mouse melanoma (2.5×10⁵) (American Type Culture Collection) or MC38 colon adenocarcinoma (0.5×10⁵) cells (from Dr. D. Lee Gorden, Vanderbilt University) 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 (SQ) and the primary tumor as well as lung metastases were assessed 28 days later. At the time of harvest, lungs were fixed in Bouin's fixative solution (Sigma-Aldrich) for 24 hours and surface tumors were counted as previously described (16). Tumor diameters were determined using digital calipers (Fisher Scientific).

Reconstitution or neutralization of IL-5 *in vivo*

To reconstitute IL-5 in IL-5KO mice, 50 ng rmIL-5 (R&D) in 100 µl phosphate-buffered saline (PBS) or PBS alone was delivered to mice by intraperitoneal (IP) injection every other day for 4 days prior to IV injection of LLC cells or every other day starting the day of tumor cell injection until harvest at day 14 (17). For IL-5 neutralization studies, mice received weekly IV injections of mouse IL-5 mAb (Clone TRFK5, R&D) (1 mg/kg) or isotype control (IgG) antibodies with the first dose given 3 hours prior to injection of tumor cells.

Measurement of IL-5 protein

Concentration of IL-5 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 rmIL-5 (10 ng/ml, R&D) or IL-5 antibodies (5 ng/ml, R&D).

Fluorescent microscopy and immunohistochemistry

For studies to detect LLC cells in the lungs, cells were stained with CellTracker™ Red CMTPX (Molecular Probes) prior to IV injection and lungs were harvested 24 hours later. Using Metamorph software, LLC cells were counted on 4 randomly selected fields of lung parenchyma (20x 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, Scottsdale, AZ) 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 20x 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 (Biolegend), CD3 - PE-Cy7, CD8 - Alexa Fluor 700, IFN γ – APC (BD Bioscience), 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 IFN γ , lung cells were stimulated with PMA (1ng/ml) and ionomycin (1uM) 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-IFN γ or anti-IL-4 antibodies. Flow cytometry was performed using BD LSR II flow cytometer (BD Bioscience) and data were analyzed with FlowJo software (TreeStar).

Adoptive transfer of eosinophils

Bone marrow derived eosinophils (BMDEos) were obtained as described (20) and 5×10^6 cells were injected via the tail vein 1 hour prior to injection of LLC cells.

Real-Time (RT) PCR

Total RNA was isolated from lung tissue using the RNeasy Mini kit (Qiagen) according to manufacturer's specifications. DNase-treated samples were subjected to quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems). PCR primers used were: IFN γ forward: 5'-GCGTCATTGAATCACACCTGA-3', reverse: 5'-CTCGGATGAGCTCATTGAATGC; IL-4 forward: 5'-TCCTCACAGCAACGAAGAAC-3', reverse: 5'-TGCAGCTCCATGAGAACAAC-3'; CCL17 forward: 5'-TGCTTCTGGGGACTTTTCTG-3', reverse: 5'-CATCCCTGGAACACTCCACT-3'; CCL20 forward: 5'-TTTTGGGATGGAATTGGACAC-3', reverse: 5'-TGCAGGTGAAGCCTTCAACC-3'; CCL22 forward: 5'-GGCACCTATCCAGTGCCACA-3', reverse: 5'-TGGTGGACCAGCCTGAAACTC-3'; IL-10 forward: 5'-ACCTGCTCCACTGCCTTGCT-3', reverse: 5'-GGTTGCCAAGCCTTATCGGA-3'; TGF- β forward 5'-CTTCAGCTCCACAGAGAAGA-3', reverse 5'-GACAGAAGTTGGCATGGTAG-3'; GAPDH forward: 5'-TGAGGACCAGGTTGTCTCCT-3', reverse: 5'-CCCTGTTGCTGTAGCCGTAT-3'. Relative mRNA expression in each sample was normalized to GAPDH and presented using the comparative Ct method (2^{-C_t}).

In vivo CCL22 and Treg cell depletion

For CCL22 depletion, mice received two consecutive daily IP injections with anti-CCL22 antibodies (100 μ g, R&D) or IgG2A isotype control antibodies (Biolegend) completed 24

hours prior to IV injection of LLC cells. For depletion of Tregs, mice received 500 µg of monoclonal anti-CD25 antibodies (clone PC61; American Type Culture Collection) or IgG1 isotype control antibodies (Biolegend) by IP injection two days prior to IV 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

IL-5 promotes metastasis to the lungs

To investigate whether IL-5 affects lung metastasis, we utilized a heterotopic model in which we injected Lewis lung carcinoma (LLC) cells (2.5×10^5 cells/mouse) subcutaneously (SQ) into the flank of WT and IL-5KO mice and measured primary tumor growth and metastatic lesions in the lungs 28 days later. While IL-5 deficiency did not alter the growth of primary tumors (Figure 1A), it almost completely abrogated pulmonary metastasis from these primary tumors (Figure 1B). To test whether protection from lung metastasis could be reproduced by neutralization of IL-5, we treated WT mice weekly with anti-IL-5 antibodies (IL-5 mAb, 1 mg/kg) or isotype control (IgG) antibodies at Days 0,7,14 after SQ injections of LLCs and analyzed lung metastasis. Consistent with results from IL-5KO mice, antibody-mediated depletion of IL-5 reduced lung metastasis of LLC cells in the flank tumor model (Figure 1C), but did not alter growth of the primary subcutaneous tumors (Figure 1D). We next investigated whether reconstitution of IL-5 in IL-5KO mice would increase LLC lung metastasis. For these studies, IL-5KO mice received SQ injection of LLC cells followed by IP injection of rmIL-5 (50 ng) or vehicle (PBS) every other day starting on the day of tumor cell injection. As shown in Figure 1E, reconstitution of IL-5 in IL-5KO mice significantly increased the number of pulmonary metastases in this model. Together, these findings show that IL-5 plays an important role in supporting pulmonary metastasis.

In order to confirm our findings in the heterotopic model and begin to investigate the mechanisms by which IL-5 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 (1.5×10^5 cells/mouse) into WT and IL-5KO mice and measured lung metastases 14 days later. As with the heterotopic model, IL-5 deficiency almost completely abrogated metastatic lesions in the lungs (Figure 2A-B); however, no effect on the size of metastatic lesions was identified (Figure 2C). Similarly, antibody-mediated IL-5 depletion in WT mice significantly reduced lung metastases after IV delivery of LLC cells (Figure 2D). In contrast, reconstitution of IL-5 in IL-5KO mice significantly increased the number of metastatic lesions in the IV lung metastasis model (Figure 2E). Together with studies in the heterotopic lung metastasis model, these studies suggest that IL-5 functions to regulate metastasis in the distal organ (lung) after hematogenous dissemination of tumor cells.

To determine whether pro-metastatic effects of IL-5 were broadly applicable to other tumor types, we injected B16-F10 melanoma (2.5×10^5) or MC38 colon adenocarcinoma (0.5×10^5) cells IV via the lateral tail vein into WT and IL-5KO mice and measured lung metastases 14 days later. As with LLC cells, IL-5KO mice developed significantly fewer lung metastases than WT mice after injection of these tumor cell types (Figures 2F-G). As with LLC cells, the mean size of lung metastatic foci was not different between WT and IL-5KO mice (Figures 2H-I). In combination, these studies indicate that IL-5 plays an important role in lung metastasis of a variety of different tumor cell types and models without affecting growth of the primary or metastatic lesions.

To determine whether IL-5 production is increased in the setting of lung metastasis, we analyzed IL-5 protein expression in the lungs, blood, spleen, and bone marrow of WT mice after IV injection of LLC cells. We found a transient increase in IL-5 in lung and bone marrow at 24 hours after IV LLC cell injection, but no change in IL-5 levels was observed in blood and spleen (Figure 3A). Since IL-5 levels were low and increased only at a very early time point, we postulated that IL-5 might affect the early stages of metastasis. Therefore, we tested whether reconstitution of IL-5 before LLC cell injection would augment pulmonary metastasis. To this end, IL-5KO mice received rmIL-5 every other day for 4 days prior to IV injection of LLC cells. At day 14 after LLC injection, IL-5KO mice pre-treated with rmIL-5 displayed significantly increased number of pulmonary metastases compared to IL-5KO mice injected with LLC cells and similar to the number of metastases in IL-5KO mice injected with IL-5 every other day for the entire 14 days (Figure 3B). These findings suggest that IL-5 is most critical during early stages of lung metastasis. In the IV LLC model, the majority of tumor cells are cleared from the lung vasculature by 24 hours while a small number of surviving cells extravasate and begin the process of establishing a metastatic niche (15). We next investigated whether IL-5 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 IV injection to WT and IL-5KO 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 IL-5KO mice (Figures 3C-D). These findings indicate that IL-5 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 IL-5 directly modulates survival or proliferation of tumor cells. LLC cells in culture expressed neither IL-5 nor IL-5 receptors (data not shown). We then conducted *in vitro* experiments in which LLC cells were incubated in the presence of PBS (control), neutralizing anti-IL-5 mAb, or rmIL-5. Serial assessment revealed no differences in cell number between treatment groups at any time point (Figure 3E), suggesting that IL-5 facilitates pulmonary metastasis indirectly, by influencing cells in the local lung microenvironment, rather than through directly affecting tumor cells.

IL-5 facilitates pulmonary metastasis by regulating eosinophils in the lungs

We postulated that immune/inflammatory cells regulated by IL-5 might facilitate pulmonary metastasis. Since IL-5 promotes recruitment and expansion of tissue eosinophils (7, 8), we analyzed lungs from WT and IL-5KO mice for infiltration with eosinophils. We immunostained lung sections from WT and IL-5KO mice harvested at Day 14 after IV injection of LLC cells using eosinophil-specific anti-MBP-1 antibodies (18). As shown in Figures 4A-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 IL-5KO mice. To determine whether IL-5-deficiency results in decreased infiltration of lungs with eosinophils at early time points after injection of LLC cells, we harvested lungs from WT and IL-5KO mice Days 0, 1 and 3 after IV injection of LLC cells and performed immunohistochemistry using anti-MBP-1 antibodies. Similar to the results at Day 14, MBP-1-positive eosinophils were markedly reduced in the lungs from IL-5KO mice compared to WT mice (Figures 4C-D). To confirm these findings, we performed flow cytometry analysis of lung eosinophils from WT and IL-5KO mice at days 0, 1, and 3 after IV 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 Figure S1) as previously reported (24). As shown in Figure 4E, lungs of IL-5KO mice contained very few eosinophils before or after LLC cell injection and WT lungs contained more than 10-fold greater eosinophils than IL-5KO mice at each time point (Figure 4F). Together, these studies demonstrate that IL-5 is important for the recruitment and maintenance of pulmonary eosinophils during metastatic colonization of the lungs.

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 IL-5KO mice. The purity of cultured bone marrow-derived eosinophils (BMDEos) was confirmed to be >98% by analysis of cell morphology (Figure 4G). WT and IL-5KO mice received BMDEos (5×10^6) by IV injection one hour prior to IV 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 IL-5KO mice while WT mice showed a trend towards increased numbers of pulmonary metastases ($p=0.07$) (Figure 4H). Based on these results, we concluded that eosinophils are important for IL-5-mediated promotion of pulmonary metastasis.

Since mast cells have been previously reported to produce IL-5 (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 IV injection of LLCs and found that eosinophils were markedly reduced in the lungs of c-kit^{W-sh} mice (Figure 4I). In addition, we detected significantly fewer lung metastases in c-kit^{W-sh} mice compared to WT mice after IV injection of LLC cells (Figure 4J). These studies suggest that c-kit⁺ cells regulate the number of eosinophils in the lungs, possibly through local production of IL-5.

Eosinophil-secreted CCL22 facilitates metastasis through local recruitment of regulatory T cells (Tregs)

In addition to exerting effects on eosinophils, IL-5 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 IL-5KO mice before and after IV injection of LLC cells. Compared to untreated mice, we detected increased total CD4⁺ lymphocytes and NK cells in the lungs of both WT and IL-5KO mice at 24 hours after LLC cell injection, but no differences were found between WT and IL-5KO mice (Supplementary Figure S2). In contrast, CD8⁺ lymphocytes and B lymphocytes were not increased in the lungs after LLC cell injection in either WT or IL-5KO mice (Supplementary Figure S2).

Compared to IL-5KO mice, we detected increased numbers of CD4⁺/CD25⁺/FoxP3⁺ regulatory T cells (Tregs) in the lungs of WT mice at baseline and 24 hours after LLC cell injection (Figures 5A-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 two days prior to IV injection of LLC cells and assessed lung metastases after 14 days. As indicated in Figures 5C-D, Treg depletion significantly decreased pulmonary metastases.

To explain the reduction of Tregs found in IL-5KO mice, we wondered whether IL-5 could affect local generation of Tregs from naïve T cells through TGFβ- and/or IL-10-dependent mechanisms (27). We therefore measured TGFβ and IL-10 mRNA expression in lung tissue by quantitative RT-PCR at 24 hours after IV injection of LLC cells and found no differences between WT and IL-5KO mice (Figures 6A-B). Next, we evaluated whether IL-5 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 IL-5KO mice compared with WT mice (Figures C-E). Since both Tregs and eosinophils were reduced in lungs of IL-5KO 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 (Figure 6F). Consistent with these findings, eosinophils isolated from lungs of WT mice at 24 hours after IV LLC injection expressed higher mRNA levels of CCL22 in comparison to lung eosinophils obtained from untreated WT mice (Figure 6G). In contrast, no differences in CCL22 mRNA expression were detected in lung macrophages from these mice following IV 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 antibodies (100µg/mouse) or isotype-control IgG completed 24 hours prior to IV delivery of LLC cells. We found that neutralization of CCL22 significantly reduced lung Tregs at 24 hours after LLC cell injection (Figure 6H). To determine whether CCL22 regulates lung metastasis, we injected WT mice with CCL22 or IgG-control antibodies prior to IV injection of LLC cells (as described above) and analyzed lung metastases 14 days

later. As shown in Figures 6I-J, mice pre-treated with CCL22-neutralizing antibodies had significantly fewer pulmonary metastases compared with isotype-treated controls. Collectively, these 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 IFN γ 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 IFN γ and IL-4 in the lungs of LLC cell-injected WT and IL-5KO mice and found increased expression of the Th1 cytokine IFN γ in lungs of IL-5KO mice, but not in WT mice. In contrast, no differences in IL-4 expression were evident between groups (Figures 7A-B). To determine the cell type(s) responsible for increased IFN γ expression in IL-5KO mice, we performed flow cytometry to identify IFN γ expressing CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, and NK cells. As shown in Figures 7C-D, the proportion of CD4⁺ and CD8⁺ T lymphocytes expressing IFN γ after LLC cell injection was similar in WT and IL-5KO mice; however, IFN γ -expressing NK cells were significantly increased in IL-5KO mice (Figure 7E). Consistent with mRNA expression results, we found no differences in the percentage of lung lymphocytes producing IL-4 in WT and IL-5KO mice at 24 hours after IV 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 μ g/mouse) or isotype-control IgG followed by IV injection of LLC cells. At 24 hours after LLC cell injection, we found significantly increased numbers of NK cells expressing IFN γ in mice treated with anti-CCL22 antibodies (Figure 7F). These results suggest that Tregs may support tumor cell survival through suppression of IFN γ production by NK cells.

Since IFN γ is a well-known stimulus for anti-tumor 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 IV LLC cell injection. Using flow cytometry, we found that more lung macrophages from IL-5KO mice expressed MHC II and fewer expressed CD206 compared to WT mice, indicating a shift towards M1 macrophage polarization in IL-5KO mice (Figures 7G-I). Together, these findings suggest that Tregs alter the lung microenvironment during lung metastasis by suppressing IFN γ 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 pro-survival or anti-tumor 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 IL-5 is important for survival of metastatic cancer

cells in the lungs. Using genetic and antibody-mediated IL-5 depletion models, we found reduced metastasis of LLC cells to the lungs, while introduction of recombinant IL-5 to IL-5KO mice significantly increased pulmonary metastasis. Equivalent pro-metastatic effects of IL-5 were detected in the context of metastasis of colon or melanoma tumor cells, establishing the broad relevance of this finding. The pro-metastatic properties of IL-5 are linked with the presence of pulmonary eosinophils, which aid in generating a favorable pulmonary microenvironment for tumor cells during the early stages of metastatic colonization. We found that eosinophils express CCL22, which supports local recruitment of Tregs that assist tumor cells in establishing a metastatic niche. Tregs appear to function by suppressing protective host immune responses by NK cells and macrophages. Together, these studies provide a novel framework by which allergic inflammatory circuitry predisposes to pulmonary metastasis (Figure 7J) and indicate that interventions targeting this pathway may represent new therapeutic approaches for prevention of metastasis to the lung.

IL-5 is a Th2 cytokine implicated in the pathogenesis of allergic inflammatory responses, but its role in tumor biology has been unclear (5). Increased expression of IL-5 in breast carcinomas has been linked to higher rates of development of distant metastasis and poor prognosis (29). Similarly, we reported that host-derived IL-5 is critical for formation of malignant pleural effusions induced by adenocarcinoma cells (10). In the current study, we employed a variety of cancer cell lines and models to elucidate an important role for IL-5 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 *et al.* that utilized another IL-5 deficient mouse model and found that IL-5 suppressed metastasis of B16F10 melanoma cells to the lungs (12). While explanation of these discrepant findings is not clear, the preponderance of evidence now indicates that IL-5 is a pro-metastatic factor.

The effects of IL-5 on primary tumors remain to be fully elucidated. In these studies, we found that IL-5 did not affect growth of primary tumors or metastatic lesions. These findings are consistent with our previous study showing that IL-5 does not alter lung tumor formation after treatment with the carcinogen urethane (9). In contrast, IL-5 was shown to increase immune surveillance in a carcinogen-induced fibrosarcoma model (11). Also, IL-5 expression has been detected in some non-small cell lung cancer cell lines (30) and primary non-small cell lung tumors (13) and Lee *et al.* showed that IL-5 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 IL-5 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 to IL-5KO mice under basal conditions and during lung metastasis. Like IL-5, 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 anti-tumor effects of eosinophils in primary colorectal, gastrointestinal, head and neck cancers and melanoma (12, 32-36). While 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 IL-5KO mice, indicating a pro-metastatic role for pulmonary eosinophils. In addition, we found that c-kit⁺ cells support lung localization of eosinophils during metastasis. ckit 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 IL-5 in the lungs (4). Therefore, further studies are required to determine specific c-kit⁺ cell subsets contributing to promotion of IL-5-mediated lung metastasis. Cumulatively, available data suggest that eosinophils support metastasis of tumor cells to the lungs but the effects of eosinophils, like IL-5, 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 anti-tumor 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 IL-5KO mice after LLC cell injection. In conjunction with these findings, we identified increased expression of the anti-tumor cytokine IFN γ by NK cells in IL-5KO 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 account 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 IL-5 is involved in facilitating colonization of the lungs with tumor cells and suggests that IL-5 neutralizing therapy could be beneficial for the prevention of pulmonary metastases. Humanized monoclonal antibodies against IL-5 (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.

Supplementary Material

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Figure 1.

Deficiency of IL-5 reduces lung metastasis in a heterotopic flank tumor model. A) Flank tumor volume and B) number of metastases to the lungs of wild type (WT) or IL-5KO mice at 28 days after subcutaneous (SQ) injection of Lewis Lung Carcinoma (LLC) cells (2.5×10^5 /mouse, $n=8$ mice per group). C) Number of lung metastases and D) size of primary flank tumors in mice at day 25 after SQ injection of LLC cells. Mice were treated with intraperitoneal (IP) injection of anti-IL-5 (1 mg/kg) or control (IgG) antibodies on day 0 (day of LLC cell injection) and days 7 and 14 ($n=6-7$ mice per group, $*p < 0.05$). E) Number of lung metastases at day 25 after SQ injection of LLC cells into WT and IL-5KO mice that were treated every other day by IP injection of PBS-vehicle (veh) or rmIL-5 (50 ng/mouse) starting on day 0 ($n=7-12$ mice per group, $*p < 0.05$ compared with the IL-5KO+veh group). Data are presented as mean $\pm$ SEM.

Figure 2.

Deficiency of IL-5 reduces lung metastases after intravenous (IV) delivery of a variety of different tumor cells. A) Representative images of lungs with metastatic foci (arrows), B) number of lung metastases and C) size of lung metastases at day 14 after IV injection of LLC cells into WT and IL-5KO mice (1.5×10^5 cells/mouse, $n=17-19$ mice per group, $*p < 0.05$). D) Number of lung metastases in WT mice at day 14 after IV injection of LLC cells followed by intraperitoneal (IP) injection with IL-5 antibodies (IL-5 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 IV injection of LLC cells into WT and IL-5KO mice treated every other day with IP injection of PBS-vehicle (veh) or rmIL-5 (50 ng/mouse, n=7-12 mice per group starting the day of LLC injection, * $p < 0.05$ compared with the IL-5KO+veh group). F-G) The number of lung metastases and H-I) size of lung metastases in WT and IL-5KO mice at day 14 after IV injection of B16-F10 melanoma cells (2.5×10^5 /mouse, n=8 mice per group) or MC38 colon cancer cells (0.5×10^5 /mouse, n= 5-7 mice per group), * $p < 0.05$.

Figure 3.

IL-5 generates a favorable pulmonary microenvironment for tumor cells during the early stages of metastatic colonization. A) Time course for IL-5 expression in bone marrow, lung, blood and spleen after IV injection of LLC cells (1.5×10^5 cells/mouse, $n=3$ mice per group, $*p < 0.05$ compared with the day 0). B) The number of pulmonary metastases in IL-5KO mice treated with rmIL-5 (50 ng/mouse) every other day for 4 days prior to IV injection of LLC cells or every other day starting the day of tumor cell injection until harvest at day 14 [$n=5-7$ mice per group, $*p < 0.05$ compared with the IL-5KO mice injected with LLCs only (no treatment with rmIL-5)]. C) Representative microphotograph of lung section from WT and IL-5KO mice and D) number of LLC cells (red) labeled with the CellTracker™ Red CMTPIX dye per unit area of lung parenchyma at 24 hours after the IV injection (1.5×10^5 cells/mouse). E) Relative light units (RLU) as measure of the number of LLC cells during different intervals of culture in presence of rmIL-5 (10 ng/ml) or IL-5 antibodies (5 ng/ml).

Figure 4.

Eosinophils are important for IL-5-mediated promotion of pulmonary metastasis. A-B) Representative photograph of lung section from WT and IL-5KO mice immunostained with antibodies to major basic protein-1 (MBP-1) for detection of lung eosinophils and average number of MBP-1⁺ lung eosinophils per field (20X magnification) at day 14 after IV injection of LLC cells (n=4-5 mice per group for each time interval, *p < 0.05). Arrows indicate MBP-1⁺ eosinophils. C) Representative photograph of lung sections from WT and IL-5KO mice immunostained for MBP-1 prior to injection of LLC cells and D) average

number of MBP-1⁺ lung eosinophils at days 0-3 after IV injection of LLC cells (n=4-8 mice per group for each time interval, *p < 0.05). E) Percentages and F) absolute numbers of CD45⁺/CD11c⁻/F4/80⁺/siglec-F⁺ eosinophils in lungs of WT and IL-5KO mice during different intervals after IV injection of LLC cells (1.5×10⁵/mouse, n=3 per group for each time interval, *p < 0.05). G) Representative photomicrograph of bone marrow-derived eosinophils (BMDEos) injected to WT and IL-5KO mice. H) The number of metastases in lungs of WT and IL-5KO mice at day 14 after IV injection of LLC cells (1.5×10⁵/mouse). One hour prior to the injection of LLC cells, mice received IV injection of 5 × 10⁶ BMDEos (n=6 - 8 mice per group, *p < 0.05). I) Average number of MBP-1⁺ lung eosinophils per field taken at 20X magnification in WT and c-kit-deficient mice (c-kit^{W-sh}) at 24 hours after IV injection of LLC cells (n=5 mice per group, *p < 0.05). J) The number of metastases in lungs of WT and c-kit^{W-sh} mice at day 14 after IV injection of LLC cells (1.5×10⁵/mouse, n=5-7 mice per group, *p < 0.05).

Figure 5.

IL-5 deficiency reduces infiltration of lungs with pro-metastatic regulatory T cells (Tregs). A) Representative flow cytometry plots and B) the percentages of CD4⁺/CD25⁺/FoxP3⁺ Tregs in lungs of WT and IL-5KO mice prior to (pre-LLCs) or 24 hours after IV injection of LLC cells (post-LLCs) (1.5×10^5 cells/mouse, $n=3-6$ mice per group, $*p < 0.05$). C) Representative photographs of lungs with metastases and D) number of lung metastases in WT mice pre-treated with isotype control antibodies (IgG) or anti-CD25 antibodies (500 μ g/mouse) two days prior to IV injection of LLC cells (1.5×10^5 /mouse) and harvested at day 14 after LLC injection. $*p < 0.05$.

Figure 6.

IL-5 supports metastasis of LLC cells to the lungs through eosinophil-mediated CCL22 production and recruitment of Tregs. A-E) Expression of TGFβ, IL-10, CCL17, CCL20 and CCL22 in lungs from WT or IL-5KO mice at 24 hours after IV injection of LLC cells (1.5×10^5 cells/mouse, * $p < 0.05$). F) Expression of CCL22 by bone marrow-derived eosinophils from WT mice alone or after 4 hours of incubation in media supplemented with 40% media from LLC cells (* $p < 0.05$), or G) Expression of CCL22 by lung CD45⁺/CD11c⁻/F4/80⁺/Siglec-F⁺ eosinophils that were FACS sorted from WT mice prior (pre-

LLCs) or at 24 hours after IV injection of LLC cells (post-LLCs). 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 pre-treated with isotype control antibodies (IgG) or anti-CCL22 antibodies (100 µg/mouse) prior to injection of LLC cells (1.5×10^5 /mouse) and analyzed by flow cytometry at 24 hours after LLC cell injection (n=3-4 mice per group, *p < 0.05). I-J) Representative photographs of lungs and number of lung metastases in WT mice pre-treated with isotype control antibodies (IgG) or anti-CCL22 antibodies (100 µg/mouse) at 48 and 24 hours prior to IV injection of LLC cells (1.5×10^5 /mouse). Lungs were harvested at day 14 after LLC injection (n=9 mice per group, *p < 0.05).

Figure 7.

IL-5 deficiency results in increased IFN γ -producing NK cells and M1-polarized macrophages in the lungs after LLC cell injection. A-B) mRNA expression of IFN γ and IL-4 in lungs from WT (black bars) and IL-5KO mice (open bars) prior to (pre-LLCs) or 24 hours after (post-LLCs) IV injection of LLC cells. C-E) Percentage of IFN γ -expressing CD8⁺ lymphocytes (C), CD4⁺ lymphocytes (D), and CD3⁺/CD49b⁺ NK cells (E) isolated from lungs of WT and IL-5KO mice at 24 hours after IV injection of LLC cells. Cells were re-stimulated with PMA/ionomycin for 6 hours *in vitro* and analyzed by flow cytometry. F) Percentage of IFN γ -expressing CD3⁺/CD49b⁺ NK cells isolated from lungs of WT mice at 24 hours after IV injection of LLC cells. Mice were treated with two consecutive daily injections of anti-CCL22 antibodies (100 μ g/mouse) or isotype-control IgG completed 24 hours prior to IV delivery of LLC cells (n=3 mice per group, *p < 0.05). G-I) Percentages and total numbers of M1-polarized CD45⁺/F4/80⁺/MHC-II⁺ macrophages or M2-polarized CD45⁺/F4/80⁺/CD206⁺ macrophages in lungs of WT and IL-5KO mice at 24 hours after IV injection of LLC cells (1.5 $\times$ 10⁵/mouse, n=3 mice per group, *p < 0.05). J) Schematic for facilitation of lung metastasis through allergic inflammatory circuitry. c-kit⁺ cells regulate numbers of sentinel eosinophils in the lungs, likely through local IL-5 production. Tumor cells induce eosinophils to express CCL22, which recruits Tregs, leading to impaired immune surveillance through inhibition of host protective functions by NK cells and cytotoxic macrophages.