

Host-derived Interleukin-5 Promotes Adenocarcinoma-induced Malignant Pleural Effusion

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Rationale: IL-5 is a T helper 2 cytokine important in the trafficking and survival of eosinophils. Because eosinophils can be found in malignant pleural effusions (MPE) from mice and humans, we asked whether IL-5 is involved in the pathogenesis of MPE.

Objectives: To determine the role of IL-5 in MPE formation.

Methods: The effects of IL-5 on experimental MPE induced in C57BL/6 mice by intrapleural injection of syngeneic lung (Lewis lung cancer [LLC]) or colon (MC38) adenocarcinoma cells were determined using wild-type (*il5*^{+/+}) and IL-5-deficient (*il5*^{-/-}) mice, exogenous administration of recombinant mouse (rm) IL-5, and *in vivo* antibody-mediated neutralization of endogenous IL-5. The direct effects of rmlL-5 on LLC cell proliferation and gene expression *in vitro* were determined by substrate reduction and microarray.

Measurements and Main Results: Eosinophils and IL-5 were present in human and mouse MPE, but the cytokine was not detected in mouse (LLC) or human (A549) lung and mouse colon (MC38) adenocarcinoma-conditioned medium, suggesting production by host cells in MPE. Compared with *il5*^{+/+} mice, *il5*^{-/-} mice showed markedly diminished MPE formation in response to both LLC and MC38 cells. Exogenous IL-5 promoted MPE formation in *il5*^{+/+} and *il5*^{-/-} mice, whereas anti-IL-5 antibody treatment limited experimental MPE in *il5*^{+/+} mice. Exogenous IL-5 had no effects on LLC cell proliferation and gene expression; however, IL-5 was found to be responsible for recruitment of eosinophils and tumor-promoting myeloid suppressor cells to MPE *in vivo*.

Conclusions: Host-derived IL-5 promotes experimental MPE and may be involved in the pathogenesis of human MPE.

Keywords: pleural disease; lung cancer; allergy; eosinophils; myeloid suppressor cells

Malignant pleural effusions (MPE) occur frequently (~500 new cases per million population per yr) in patients with cancer, predominantly lung and breast adenocarcinoma (1–4). MPEs imply systemic spread of disease and reduce life expectancy and quality (5–8). Current treatment options, including needle aspiration, pleurodesis (chemical-induced pleural fibrosis), or indwelling pleural catheters, are aimed at draining or eliminating the pleural space. These treatments are nonspecific, often

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Malignant pleural effusion (MPE) is a common and severe clinical problem, for which no disease-specific therapy is available. Although tumor-specific biologic pathways that promote MPE formation have been previously identified, no host factors had been implicated in MPE formation to date.

What This Study Adds to the Field

Here we show how host-derived IL-5 enhances MPE progression by modulating the host inflammatory response to intrapleural adenocarcinoma. The effects of IL-5 bioactivity on MPE formation are amenable to exogenous manipulation. Hence, IL-5 can be a therapeutic target against MPE.

ineffective, associated with morbidity and mortality, and do not benefit a significant number of patients (9–11). Improved understanding of MPE formation and development of new treatments targeting this pathobiology may yield safer and more effective therapeutic options (12).

To aid in elucidating MPE pathobiology, we have developed an immunocompetent model of MPE using syngeneic mice (13–15). Immunocompetent models are vital, because recent evidence has linked inflammation with enhanced formation, growth, and metastasis of tumors (16–19). In this connection, mouse MPEs display a robust immune response, including varying degrees of mononuclear and eosinophil infiltration, similar to human MPEs (13–15, 20, 21). We and others have identified tumor-specific pathways that promote MPE formation, including vascular endothelial growth factor (VEGF), IL-6/signal transducer and activator of transcription-3, and nuclear factor (NF)- κ B/tumor necrosis factor (TNF)- α /monocyte chemoattractant protein (MCP)-1 (13–15, 22–24). However, host-derived factors that regulate MPE formation and progression have not been identified.

IL-5 is important for eosinophil differentiation, trafficking, and survival (25). Eosinophils are important effectors of allergy and of host defense against fungi, parasites, and protozoa (25). Eosinophils also have been associated with aggressive behavior of human malignancies (26, 27). IL-5 is involved in the eosinophilia occasionally observed with hematologic and solid tumors (28–30); however, a specific role for IL-5 in tumor progression has not been identified. In addition to eosinophil recruitment, IL-5 may have important effects on other components of the host inflammatory response, including lymphocytes and macrophages (31, 32).

In these studies, we asked whether IL-5 promotes the formation and progression of MPE. To address this question,

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we used two different models, Lewis lung cancer (LLC) and mouse colon adenocarcinoma (MC38)-induced MPE, in IL-5 competent and deficient mice (*il5*^{+/+} and *il5*^{-/-}, respectively). In addition, we delivered recombinant mouse (rm) IL-5 or neutralizing anti-IL-5 antibody (TRFK5) to mice with LLC-induced MPE. We found that IL-5 promotes MPE formation by adenocarcinoma through effects on the MPE-associated inflammatory response. Thus, IL-5 is the first host-derived mediator shown to play an important role in MPE formation.

METHODS

Detailed description of all methods is provided in the online supplement.

Reagents

Anti-IL-5 antibody was prepared from TRFK5 hybridoma (33).

Human Pleural Effusions

Pleural fluid and serum were obtained during initial diagnostic thoracenteses of 55 patients with MPE (tumors: 35 lung, 6 mesothelioma, 7 breast, 7 other) and 20 with congestive heart failure (CHF) at the General Hospital Evangelismos (September 2006 to May 2008). The study was approved by the hospital's ethics committee and all patients gave written informed consent. MPE was diagnosed by cytology and histology (2, 4) and CHF pleural effusion using clinical, radiologic, and laboratory findings (34).

Cell Culture

LLC mouse and A549 human lung adenocarcinoma, MC38 mouse colon adenocarcinoma, and mouse skin melanoma (B16F10) cells (ATCC, Manassas, VA; NCI, Frederick, MD) were cultured and *pNGL* MC38 cells stably expressing a NF- κ B reporter (NF- κ B.GFP.LUC; *pNGL*) were generated as described previously (13).

Animal Models

il5^{+/+} and *il5*^{-/-} (35) C57BL/6 mice (Jackson, Bar Harbor, MN; BSRC Alexander Fleming, Vari, Greece) were inbred at Vanderbilt University and Evangelismos Hospital. Experiments were approved by both Institutional Animal Care and Use Committees. Intrapleural tumor cell injection (1.5×10^5), killing of mice, and specimen collection (Day 14 after LLC and Day 11 after MC38 cells) were described previously (13–15). For flank tumor formation, tumor cells (5×10^5) were subcutaneously injected, tumor dimensions ($\delta 1, \delta 2, \delta 3$) were measured weekly, and tumor volume (V) was determined ($V = \pi \times [\delta 1 \times \delta 2 \times \delta 3] / 6$).

Bioluminescence Imaging

Bioluminescence imaging of mice bearing *pNGL* cells was done using Xenogen IVIS (Alameda, CA).

Cytokine Determinations

Mouse and human IL-5 (detection limits, 7 and 3 pg/ml, respectively) were determined by ELISA (R&D, Minneapolis, MN).

Exogenous rmlL-5 Treatment

A total of 40 ng rmlL-5/100 μ l phosphate-buffered saline (PBS) or PBS alone were delivered to the retroorbital veins of mice every other day after LLC cells, a regimen that reconstitutes *il5*^{-/-} mice (36).

IL-5 Neutralization

Mice received 1 mg/kg intraperitoneal TRFK5, which provides prolonged bioactivity (37), in "prevention" (Days 0 and 8) and "regression" (Day 8) protocols, the latter aimed at therapy of established MPEs developing around Day 8 (13, 38).

Histology

Tissue sections were immunolabeled as described previously (13–15, 38), or stained with Biebrich scarlet for eosinophils (39).

Polymerase Chain Reaction and Gene Expression Profiling

Total RNA was isolated from LLC cells treated with PBS or rmlL-5 (22 pM) for 24 hours, reverse transcribed, and cDNA was labeled using amino-allyl cDNA. Primers and conditions for polymerase chain reaction have been described (40). Independent triplicate samples were mixed with isomolar reference sample and hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST Arrays (Santa Clara, CA) interrogating 28,853 genes. Differentially expressed genes were selected by analysis of variance without sample selection or filtering. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-2490.

Flow Cytometry

After red blood cell lysis, cells were suspended in PBS 1% bovine serum albumin, stained with phycoerythrin-Cy7-conjugated anti-CD11b and allophycocyanin-conjugated anti-Gr1 antibodies (0.1 μ g/10⁶ cells; 60 minutes), and analyzed on a BD FACS-SCAN (Palo Alto, CA). Data were analyzed using WinMDIv2.8 (J. Trotter, The Scripps Institute, La Jolla, CA; <http://facs.scripps.edu/software.html>).

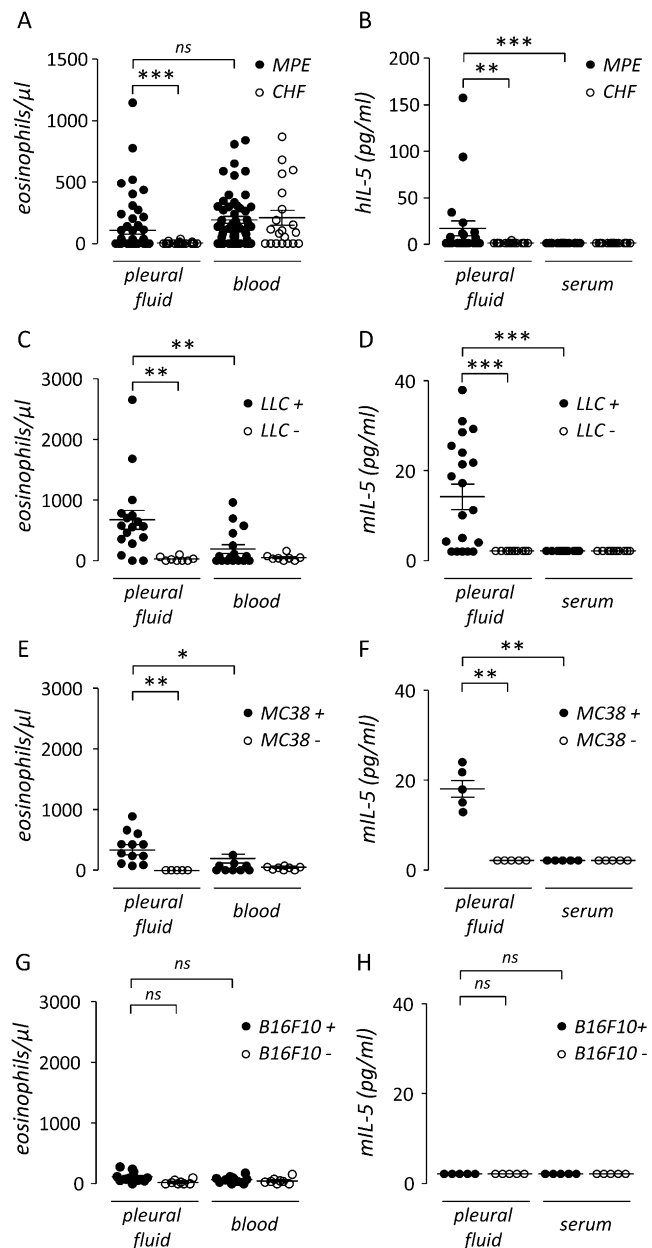
Statistics

All values represent mean \pm SEM. Intergroup differences in frequencies, means, and medians were examined by χ^2 test, *t* test, or one-way analysis of variance with least square difference *post hoc* tests, and Mann-Whitney or Kruskal-Wallis test with Dunn *post hoc* tests, respectively. Two-tailed *P* values less than 0.05 were considered significant. All analyses were performed using SPSSv.13.0.0 (Chicago, IL).

RESULTS

Eosinophils and IL-5 are Present in Human and Mouse MPE

Initially, we determined the abundance of eosinophils in pleural fluid and blood from patients with MPE using patients with pleural effusions caused by CHF as a control group. We found equivalent eosinophil numbers in peripheral blood from patients in these two groups; however, significantly more eosinophils were present in pleural fluid from patients with MPE compared with pleural effusions from patients with CHF (Figure 1A). Although eosinophils were detected in 40 of 55 patients with MPE, only 7 (13%) met the clinical definition of eosinophilic pleural effusion, usually defined as greater than 10% eosinophils (3, 22). Because IL-5 is important in eosinophil homeostasis, we went on to assess the levels of this cytokine in the previously mentioned biologic samples. IL-5 was detected in 9 of 22 human MPEs tested, but in only 1 of 16 effusions caused by CHF (detection limit, 3 pg/ml; $\chi^2 = 5.74$; *P* = 0.017). IL-5 levels were significantly elevated in MPE compared with pleural effusions caused by CHF and with serum from either patient group. The increased IL-5 concentration in MPE compared with serum suggests local production in the malignancy-affected pleural space (Figure 1B). We subsequently sought to assess eosinophils and IL-5 in a mouse model of lung adenocarcinoma-induced MPE (13–15, 38). Pleural accumulation of eosinophils was observed 14 days after intrapleural injection of LLC cells into C57BL/6 mice (Figure 1C). In this model, $6.8 \pm 0.8\%$ of inflammatory cells in pleural fluid were eosinophils, and 3 of 17 effusions would be classified as eosinophilic using the clinical definition. Consistent with data from human MPE, IL-5 was also found to be present in mouse MPE; however, IL-5 was not detected in matched serum or in pleural fluid or serum from PBS-treated controls without MPE (Figure 1D). To determine whether this was unique to the LLC model of MPE, we developed an additional mouse model of MPE using intrapleural delivery of MC38 colon adenocarcinoma cells (1.5×10^5 cells, 11-day latency). Similar to our observations with LLC cells, eosinophils and IL-5 locally accumulated in MPEs induced by MC38 cells. In the MC38 model, $5.3 \pm 1.2\%$ of inflammatory



cells in MPE were eosinophils, and 2 of 12 effusions would be classified as eosinophilic (Figure 1E). IL-5 was also present in MC38-induced MPE, but not in matched serum or in pleural fluid or serum from PBS-treated controls without MPE (Figure 1F). In contrast to our findings with LLC and MC38 cell injections, intrapleural injection of live mouse skin melanoma (B16F10) cells, which are also syngeneic to the C57BL/6 mouse strain, did not result in substantial MPE formation (15), eosinophil recruitment, or IL-5 production (Figures 1G and 1H). In separate experiments, IL-5 was not detected in media conditioned by mouse (LLC) and human (A549) lung and mouse colon (MC38) adenocarcinoma cells by ELISA (data not shown). Collectively, these results implicated intrapleural IL-5 production by the host with associated eosinophil recruitment in response to intrapleural dissemination of adenocarcinoma during MPE formation.

Host-Derived IL-5 Promotes MPE Formation

Because IL-5 was present in MPE, we investigated whether host-derived IL-5 contributes to MPE formation. For these

Figure 1. Eosinophils and IL-5 in mouse and human malignant pleural effusion (MPE). (A) Pleural fluid and blood eosinophils in humans with pleural effusion caused by malignancy (MPE, n = 55) and heart failure (congestive heart failure [CHF], n = 20). Pleural eosinophils are increased in patients with MPE, compared with CHF. (B) Pleural fluid and serum IL-5 levels in humans with MPE (n = 22) and CHF (n = 16). IL-5 is locally increased in the pleural fluid of patients with MPE. (C) Pleural fluid and blood eosinophil numbers in C57BL/6 mice 14 days after intrapleural delivery of LLC cells (LLC⁺, n = 17) or phosphate-buffered saline (PBS) control (LLC⁻, n = 8). Pleural eosinophil accumulation is observed after tumor cell injection only. (D) Pleural fluid and serum IL-5 levels in C57BL/6 mice 14 days after intrapleural delivery of LLC cells (LLC⁺, n = 20) or PBS control (LLC⁻, n = 10). (E) Pleural fluid and blood eosinophil numbers in C57BL/6 mice 11 days after intrapleural delivery of MC38 cells (MC38⁺, n = 12) or PBS control (MC38⁻, n = 5). Pleural eosinophil accumulation is observed after tumor cell injection only. (F) Pleural fluid and serum IL-5 levels in C57BL/6 mice 11 days after intrapleural delivery of MC38 cells (MC38⁺, n = 5) or PBS control (MC38⁻, n = 5). (G) Pleural fluid and blood eosinophil numbers in C57BL/6 mice 14 days after intrapleural delivery of B16F10 cells (B16F10⁺, n = 12) or PBS control (B16F10⁻, n = 8). (H) Pleural fluid and serum IL-5 levels in C57BL/6 mice 14 days after intrapleural delivery of B16F10 cells (B16F10⁺, n = 5) or PBS control (B16F10⁻, n = 5). Dots = raw data points; lines = mean; bars = SE. *P < 0.05, **P < 0.01, ***P < 0.001. B16F10 = mouse skin melanoma; CHF = congestive heart failure; h = human; LLC = Lewis lung cancer; m = mouse; MC38 = mouse colon adenocarcinoma; MPE = malignant pleural effusion; ns = not significant.

studies, C57BL/6 *i5*^{+/+} and *i5*^{-/-} mice (35) received intrapleural LLC cells (or MC38 cells) and MPEs were evaluated at Day 14 (or Day 11 after MC38 cell injection). Compared with *i5*^{+/+} mice, *i5*^{-/-} mice exhibited a marked reduction in MPE formation (6 of 13 *i5*^{-/-} and 17 of 17 *i5*^{+/+} mice developed MPE in response to LLC cells, $\chi^2 = 11.94$, $P = 0.001$; 3 of 7 *i5*^{-/-} and 7 of 7 *i5*^{+/+} mice developed MPE in response to MC38 cells, $\chi^2 = 5.6$, $P = 0.018$). Of mice that developed MPEs, the volume of fluid was reduced in *i5*^{-/-} mice (mean MPE volume in *i5*^{-/-} and *i5*^{+/+} mice after LLC cells, 217 \pm 81 and 662 \pm 70 μ l, respectively, $P < 0.001$; mean MPE volume in *i5*^{-/-} and *i5*^{+/+} mice after MC38 cells, 173 \pm 47 and 593 \pm 65 μ l, respectively, $P < 0.001$) (Figure 2A). In addition, the number of individual pleural tumors was decreased in *i5*^{-/-} mice (mean number of LLC pleural tumors in *i5*^{-/-} and *i5*^{+/+} mice, 3.2 \pm 0.4 and 6.1 \pm 0.8, respectively, $P = 0.001$; mean number of MC38 pleural tumors in *i5*^{-/-} and *i5*^{+/+} mice, 11 \pm 1.1 and 16.4 \pm 1.9, respectively, $P = 0.04$) (Figure 2B). We next used intrapleurally injected LLC and MC38 cells stable expressing a constitutive NF- κ B reporter (*pNGL*), to verify noninvasively the reduced MPE formation and integrate pleural tumor load in *i5*^{-/-} mice (13–15). Indeed, in these models designed to monitor tumor-specific NF- κ B and hence tumor mass (13), *i5*^{-/-} mice bearing intrapleural *pNGL* LLC or *pNGL* MC38 cells displayed markedly reduced chest photon emission before sacrifice compared with *i5*^{+/+} mice, consistent with decreased intrapleural tumor load (Figure 2C). The concentration of several tumor cell-derived mediators important in MPE formation, including VEGF, TNF- α , MCP-1, and IL-6, was similar in pleural fluid from both genotypes (data not shown). These results indicate that IL-5 deficiency in the host reduces intrapleural fluid accumulation and tumor dissemination of experimental MPE.

IL-5 Does Not Directly Enhance Tumor Growth

Based on the finding of decreased tumor burden in the pleural space of *i5*^{-/-} mice compared with *i5*^{+/+} mice, we evaluated

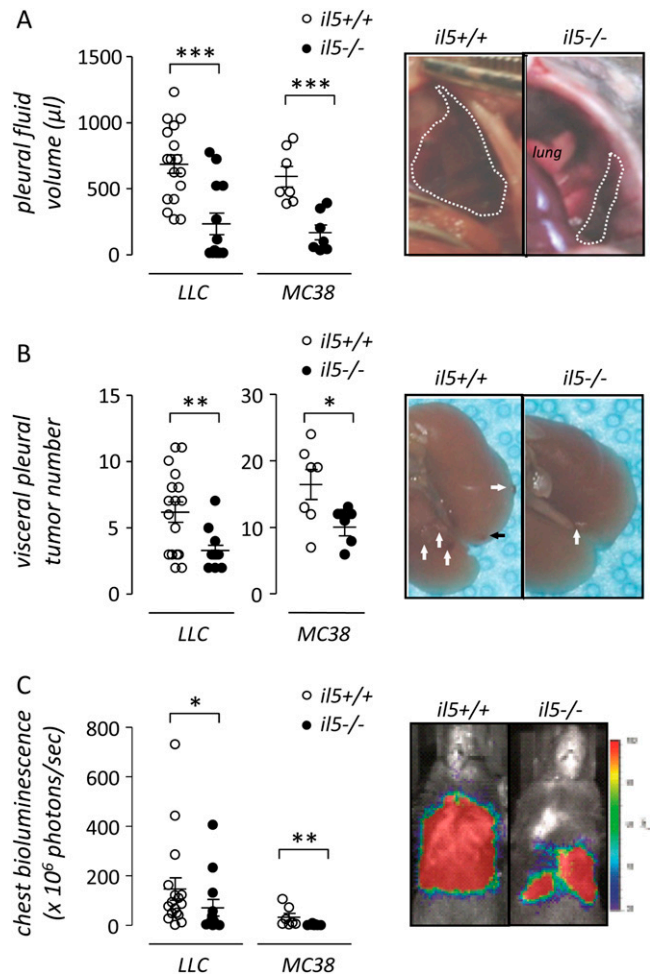


Figure 2. IL-5 promotes experimental lung and colon adenocarcinoma-induced malignant pleural effusion (MPE). (A) Pleural fluid volume, (B) pleural tumor number, and (C) bioluminescence of MPEs generated in wild-type (*il5*^{+/+}, n = 27) and IL-5 knock-out (*il5*^{-/-}, n = 23) mice on the C57BL/6 background 14 and 11 days after intrapleural delivery of LLC (n = 30) and MC38 (n = 14) cells, respectively. Genetic IL-5 deficiency limits pleural fluid accumulation and intrapleural adenocarcinoma dissemination. Photographs show representative images of MPEs (A, transdiaphragmatic views), of pleural tumors (B, post-lung explantation from thorax; arrows point toward visceral pleural tumors), and of bioluminescence emission (C, day of sacrifice) from *il5*^{+/+} and *il5*^{-/-} mice bearing intrapleural LLC cells. Dots = raw data points; lines = mean; bars = SE. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. LLC = Lewis lung cancer; MC38 = mouse colon adenocarcinoma.

whether host-derived IL-5 directly impacts tumor growth *in vivo* using the flank tumor growth model. For these studies, *il5*^{+/+} and *il5*^{-/-} mice received subcutaneous LLC or MC38 cells and the volume of the resulting tumors was measured weekly. We found no difference in the growth of subcutaneous LLC and MC38 tumors between *il5*^{+/+} and *il5*^{-/-} mice (Figure 3A). We also made observations regarding the proliferation rate of LLC and MC38 cells exposed to rmIL-5 *in vitro*. Exogenous IL-5 did not alter LLC or MC38 cell proliferation even after prolonged exposure times (4 days) (Figure 3B). These findings support the conclusion that the impact of IL-5 on tumor dissemination and effusion formation in the pleural space are dependent on local microenvironmental factors rather than a general effect of IL-5 on tumor cell proliferation or survival.

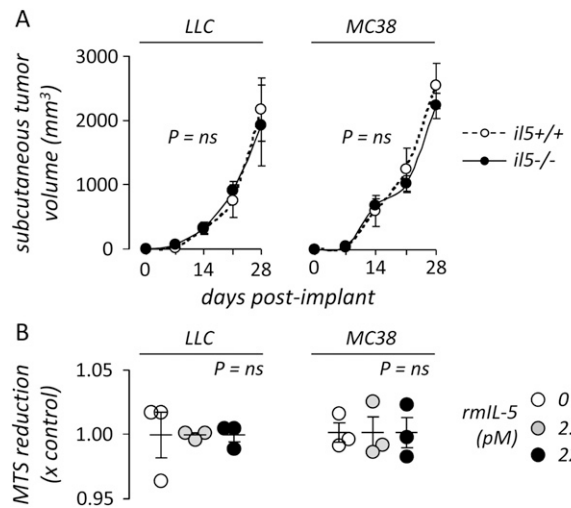


Figure 3. IL-5 does not enhance tumor growth *in vivo* and *in vitro*. (A) Volume of subcutaneous flank tumors induced in wild-type (*il5*^{+/+}, n = 10) and IL-5 knock-out (*il5*^{-/-}, n = 10) mice after subcutaneous injection of 5×10^5 Lewis lung carcinoma (LLC; n = 10) or colon adenocarcinoma (MC38; n = 10) cells. Dots = mean; bars = SE. (B) *In vitro* cell proliferation rate of LLC and MC38 cells 4 days after exposure to varying concentrations of recombinant mouse IL-5, as determined by a substrate (MTS) reduction assay. Note that 22 pM is the binding constant (kilodalton) of IL-5 for its receptor. Dots = raw data points; lines = mean; bars = SE. LLC = Lewis lung cancer; MC38 = mouse colon adenocarcinoma; ns = not significant.

Exogenous IL-5 Treatment Enhances MPE Formation

During subsequent studies aimed at identifying a mechanism of MPE promotion by IL-5, we used the better established and characterized LLC-induced MPE model. To show that reduced MPE formation in IL-5-deficient mice was directly related to loss of IL-5 and not a developmental or compensatory effect, we reconstituted *il5*^{-/-} mice with IL-5 during MPE formation. For this study, *il5*^{-/-} mice on the C57BL/6 background received intrapleural LLC cells followed by intravenous PBS or rmIL-5 treatment every other day until harvest. Fourteen days later, rmIL-5-treated *il5*^{-/-} mice exhibited increased MPE formation (mean MPE volume in PBS- and rmIL-5-treated mice, respectively: 94 ± 94 and 368 ± 109 µl; *P* < 0.05) and intrapleural tumor dissemination (mean number of pleural tumors in PBS- and rmIL-5-treated mice, respectively: 4.3 ± 1 and 8.8 ± 1.1 ; *P* < 0.05) compared with PBS-treated *il5*^{-/-} mice (Figure 4A).

Next, we asked whether exogenous IL-5 administration *in vivo* would impact MPE formation in wild-type (C57BL/6 *il5*^{+/+}) mice. In these studies, we treated mice as described previously with intravenous PBS or rmIL-5 every other day after intrapleural LLC cells. Fourteen days later, rmIL-5-treated mice exhibited increased MPE formation (mean MPE volume in PBS- and rmIL-5-treated mice, respectively: 662 ± 72 and 911 ± 73 µl; *P* < 0.001) and intrapleural tumor dissemination (mean number of pleural tumors in PBS- and rmIL-5-treated mice, respectively, 6.1 ± 0.6 and 10.1 ± 1.1 ; *P* < 0.001) (Figure 4B). The levels of VEGF, TNF-α, MCP-1, and IL-6 were similar in MPEs from both experimental groups (data not shown). Collectively, these results supported the conclusion that IL-5 directly promotes MPE formation.

IL-5 Neutralization Limits Experimental MPE

We next sought to assess whether neutralization of endogenous IL-5 bioactivity *in vivo* favorably impacts experimental MPE.

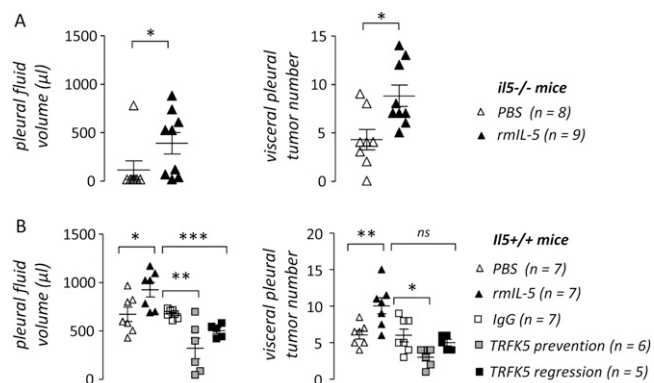


Figure 4. Exogenous IL-5 directly promotes and IL-5 neutralization limits experimental lung adenocarcinoma-induced malignant pleural effusion (MPE). (A) Pleural fluid volume (left) and pleural tumor number (right) of MPEs generated in IL-5-deficient C57BL/6 (*iL5*^{-/-}) mice 14 days after intrapleural delivery of Lewis lung cancer (LLC) cells, when mice were treated with phosphate-buffered saline (PBS) control or recombinant mouse (rm) IL-5 (*n* = 8 and 9, respectively). Exogenous IL-5 enhances pleural fluid accumulation and intrapleural adenocarcinoma dissemination in *iL5*^{-/-} mice. (B) Pleural fluid volume (left) and pleural tumor number (right) of MPEs generated in wild-type C57BL/6 (*iL5*^{+/+}) mice 14 days after intrapleural delivery of LLC cells, when mice were treated with PBS control, recombinant mouse (rm) IL-5, IgG control, or anti-IL-5 antibody (TRFK5) in both a prevention (Days 0 and 8) and a regression (Day 8) trial (*n* = 7, 7, 7, 6, and 5, respectively). Exogenous IL-5 enhances, whereas antibody-mediated IL-5 neutralization decreases pleural fluid accumulation and intrapleural adenocarcinoma dissemination. Points = raw data; lines = mean; bars = SE. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for comparison with appropriate control. Ig = immunoglobulin; MPE = malignant pleural effusion; PBS = phosphate-buffered saline; rm = recombinant mouse; TRFK5 = anti-IL-5 neutralizing antibody.

For these studies, wild-type (C57BL/6 *iL5*^{+/+}) mice received intrapleural LLC cells followed by 1 mg/kg intraperitoneal IgG2a (control) or anti-IL-5 antibody (TRFK5) in two protocols designated as prevention (Days 0 and 8 after LLC cells) or regression studies (Day 8 after LLC cells). This latter study design was aimed at unveiling potential therapeutic effects of TRFK5 against already established mouse MPE, which develop around Day 8 (13, 38). Fourteen days after LLC cell injection, all mice developed MPE; however, TRFK5-treated mice in both prevention and regression studies displayed decreased MPE formation and intrapleural tumor growth compared with IgG-treated controls (Figure 4B). Again, pleural fluid VEGF, TNF- α , MCP-1, and IL-6 levels were similar between groups (data not shown). In addition to further supporting a MPE-promoting role for IL-5, these studies indicated that targeting IL-5 could have therapeutic benefit in reducing MPE formation.

IL-5 Enhances *In Vivo* Tumor Cell Survival and MPE Formation through Modulation of the Host Immune Response

To determine whether IL-5 directly affects tumor cells, we studied LLC cells in culture. Cultured LLC cells did not express the IL-5 receptor (IL-5R α) as determined by reverse transcriptase-polymerase chain reaction (data not shown). As mentioned previously, treatment of LLC cells *in vitro* with IL-5 at concentrations equaling its binding constant with IL-5R α (1 ng/ml = 22 pM) had no effect on LLC cell proliferation as determined by MTS assay, NF- κ B activation as determined by a relevant reporter, *Ras/RhoA* expression as determined by Western blot, or apoptosis as determined by terminal deoxynucleotidyl nick-end labeling (TUNEL) (data not shown). Microarray-assisted inter-

rogation of 28,853 genes showed that IL-5 treatment for 24 hours altered the expression of only 139 putative genes with no known function (see Table E1 in the online supplement). The greatest changes in gene expression observed were modest (less than two-fold). Moreover, these modest differences in 139 of 28,853 genes were fewer than those expected to appear by chance: when the threshold of significance (*P* < 0.05) was divided by the number of comparisons made (28,853), these changes in gene expression were not significant. Most importantly, microarray data and validation at the protein level showed that exogenous IL-5 induced no significant change in the expression of genes previously implicated in MPE pathogenesis (Table 1). These results indicated that host-derived IL-5 does not directly impact MPE formation via effects on tumor cells, but rather seems to facilitate MPE formation through alteration of the microenvironment of the pleural space.

To investigate the microenvironmental impact of host-derived IL-5 in the pleural space, we assessed tumor cell proliferation, apoptosis, and angiogenesis *in vivo*. We performed immune-labeling of pleural tumor tissue sections obtained from LLC-induced MPE-bearing C57BL/6 *iL5*^{+/+} and *iL5*^{-/-} mice, and C57BL/6 *iL5*^{+/+} mice treated with rmIL-5, TRFK5 antibodies, and controls for proliferating cell nuclear antigen, TUNEL, and factor VIII-related antigen, indicators of cell proliferation, apoptosis, and angiogenesis, respectively (Figure 5). We found no significant intergroup differences in tumor cell proliferation and angiogenesis (Figures 5A and 5C). However, pleural tumor cell apoptosis was significantly increased in *iL5*^{-/-} mice compared with *iL5*^{+/+} mice and in TRFK5-treated compared with IgG-treated mice (Figure 5B).

We also investigated inflammatory cells in MPEs from *iL5*^{+/+} and *iL5*^{-/-}, and PBS-, rmIL-5-, IgG-, and TRFK5-treated mice. We found no differences in the absolute or relative abundance of macrophages, lymphocytes, and neutrophils (Figures 6A and 6B). However, we found significantly decreased numbers of eosinophils in MPE fluid from *iL5*^{-/-} mice and significantly lower numbers of these cells in MPEs from mice treated with TRFK5 (Figure 6C). These findings were recapitulated when pleural tumor tissue was examined for eosinophils using the Luna technique (39): significantly decreased numbers of these cells were observed in pleural tumor tissue from *iL5*^{-/-} mice compared with *iL5*^{+/+} mice (Figure 6D). Mice treated with rmIL-5 had significantly higher numbers of eosinophils in pleural tumors and mice treated with TRFK5 had significantly

TABLE 1. CHANGES IN THE EXPRESSION OF GENES (Δ GE) OF PROVEN OR POTENTIAL IMPORTANCE IN MALIGNANT PLEURAL EFFUSION PATHOGENESIS INDUCED IN LEWIS LUNG ADENOCARCINOMA CELLS BY 22 pM RECOMBINANT MOUSE IL-5*

Gene Symbol	Gene Name	Microarray		ELISA/CBA	
		Δ GE	<i>P</i>	Δ GE	<i>P</i>
<i>Ccl2</i>	Chemokine (C-C motif) ligand 2	1.080	0.310	1.029	0.885
<i>Ccl12</i>	Chemokine (C-C motif) ligand 12	1.080	0.330	1.101	0.741
<i>Cxcl2</i>	Chemokine (C-X-C motif) ligand 2	1.020	0.800	-1.090	0.720
<i>IIfng</i>	Interferon- γ	1.180	0.230	1.100	0.691
<i>Il6</i>	IL-6	1.020	0.660	1.011	0.978
<i>Il10</i>	IL-10	-1.020	0.770	1.038	0.776
<i>Il12b</i>	IL-12b	1.090	0.260	1.243	0.489
<i>Tnf</i>	Tumor necrosis factor	1.180	0.230	1.171	0.515
<i>Vegfa</i>	Vascular endothelial growth factor A	-1.020	0.650	1.046	0.852

Definition of Abbreviations: CBA = cytometric bead array; *P* = probability.

* Determined at the RNA level by microarray and at the protein level by ELISA or CBA.

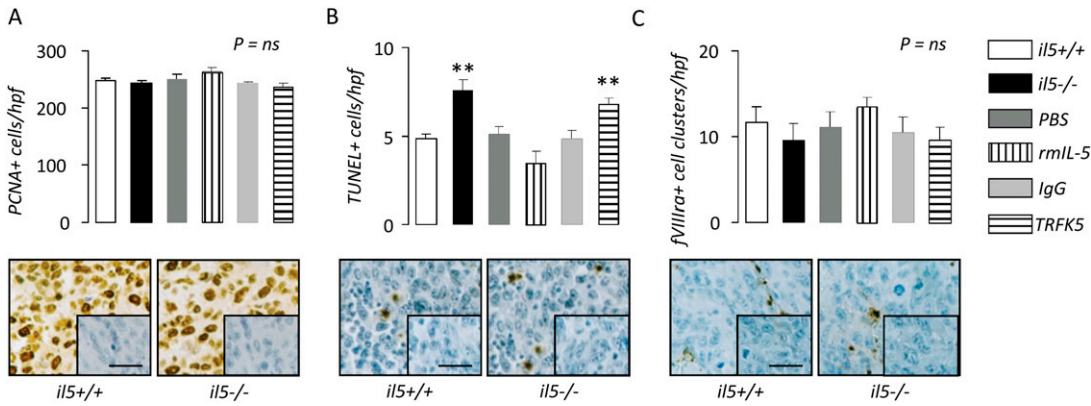


Figure 5. IL-5 promotes pleural tumor cell survival. Immunodetection of (A) proliferating cell nuclear antigen, (B) TUNEL, and (C) fVIIIra in pleural tumor tissue obtained from wild-type (*il5*^{+/+}), IL-5 knock-out (*il5*^{-/-}), and phosphate-buffered saline-, rmlL-5-, IgG-, and TRFK5-treated *il5*^{+/+} C57BL/6 mice (n = 7/group) 14 days after intrapleural delivery of LLC cells. Endogenous IL-5 deficiency enhances, exogenous IL-5

delivery decreases, and IL-5 blockade increases pleural tumor cell apoptosis. Microphotographs show representative images of staining from *il5*^{+/+} and *il5*^{-/-} mice (inlays = isotype controls; bars = 50 μm; original magnification ×400; brown = immunoreactivity; blue = nuclear hematoxylin counterstaining). Columns = mean; bars = SE. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, respectively, for comparison with appropriate control. fVIIIra = factor VIII-related antigen; Ig = immunoglobulin; LLC = Lewis lung cancer; PBS = phosphate-buffered saline; PCNA = proliferating cell nuclear antigen; rm = recombinant mouse; TRFK5 = anti-IL-5 neutralizing antibody; TUNEL = terminal deoxynucleotidyl nick-end labeling.

lower numbers of eosinophils in pleural tumors, compared with the respective controls. Based on previous reports of experimental tumor promotion by CD11b+GR1+ myeloid suppressor cells (41), we examined the numbers of these cells in blood and MPE from our experimental mice (Figure 7). We found significantly decreased numbers of myeloid suppressor cells in MPE and blood from *il5*^{-/-} compared with *il5*^{+/+} mice. In addition, mice treated with rmlL-5 showed increased circulating suppressor cells in the blood, and mice treated with TRFK5 had significantly lower numbers of these cells in MPE fluid, compared with respective controls. Collectively, these results suggested that enhanced eosinophil and myeloid suppressor cell recruitment may contribute to the MPE-promoting effects of host-derived IL-5.

DISCUSSION

In these studies, we identified both IL-5 and eosinophils in MPEs from humans and immunocompetent mice, and showed that IL-5 plays an important role in formation and progression of experimental MPE. Importantly, host-derived rather than tumor-derived IL-5 seems to regulate MPE through alterations on the pleural microenvironment. Moreover, our results indicate that host-derived IL-5 does not exert direct effects on cancer cells, but rather on other host cells. In this connection, we observed enhanced eosinophil and myeloid suppressor cell recruitment to experimental MPE in an IL-5-dependent manner. Although IL-5 is well known as a chemotactic agent for eosinophil recruitment, it is uncertain whether IL-5 also exerts direct effects to recruit myeloid suppressor cells. The presence

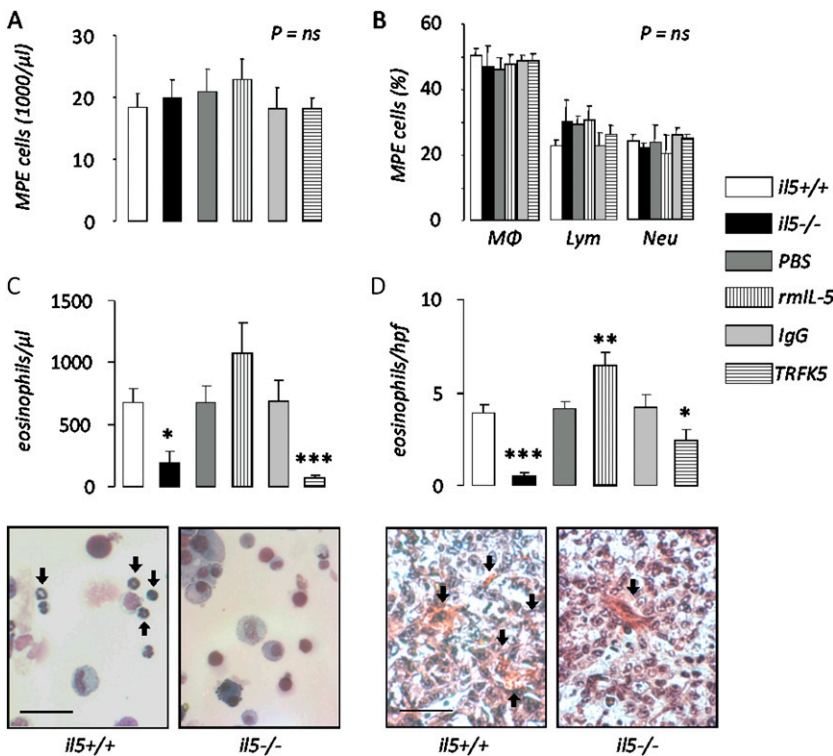


Figure 6. IL-5 promotes eosinophil accumulation in malignant pleural effusion (MPE) and pleural tumors. Total nucleated cells (A), differential cell counts (B), and eosinophil number (C) in MPEs and eosinophil counts in pleural tumor tissue (D) obtained from wild-type (*il5*^{+/+}), IL-5 knock-out (*il5*^{-/-}), and phosphate-buffered saline-, rmlL-5-, IgG-, and TRFK5-treated *il5*^{+/+} C57BL/6 mice (n = 17, 13, 7, 7, 7, and 11, respectively) 14 days after intrapleural delivery of LLC cells. IL-5 deficiency ablates, exogenous IL-5 enhances, and IL-5 neutralization inhibits eosinophil recruitment to MPE. Microphotographs show representative images of MPE (left, May-Gruenwald-Giemsa stain; bar = 50 μm; original magnification ×400; arrows, eosinophils) and tissue (right, Biebrich scarlet stain; bar = 50 μm; original magnification ×400; arrows, eosinophils) eosinophil staining from *il5*^{+/+} and *il5*^{-/-} mice. Columns = mean; bars = SE. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, respectively, for comparison with appropriate control. Ig = immunoglobulin; LLC = Lewis lung cancer; Lym = lymphocytes; MΦ = macrophages; MPE = malignant pleural effusion; Neu = neutrophils; PBS = phosphate-buffered saline; rm = recombinant mouse; TRFK5 = anti-IL-5 neutralizing antibody.

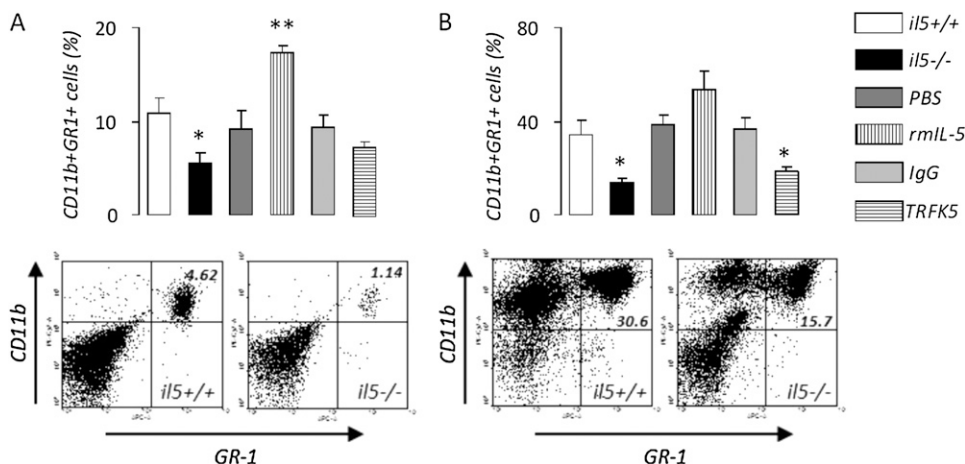


Figure 7. IL-5 promotes myeloid suppressor cell recruitment to malignant pleural effusion (MPE). CD11b+GR1+ myeloid suppressor cells in (A) blood and (B) MPEs obtained from wild-type (*il5*^{+/+}), IL-5 knock-out (*il5*^{-/-}), and phosphate-buffered saline-, rmlL-5-, IgG-, and TRFK5-treated *il5*^{+/+} C57BL/6 mice (A: n = 8, 8, 5, 3, 5, and 4, respectively; B: n = 8, 3, 5, 3, 5, and 4, respectively) 14 days after intrapleural delivery of LLC cells. IL-5 deficiency decreases, exogenous IL-5 enhances, and IL-5 neutralization inhibits recruitment of these cells in response to MPE. Images show representative flow cytometry results of blood (left; numbers, percentage of cells in quadrant) and MPE (right;

numbers, percentage of cells in quadrant) from *il5*^{+/+} and *il5*^{-/-} mice. Columns = mean; bars = SE. **P* < 0.05 and ***P* < 0.01, respectively, for comparison with appropriate control. Ig = immunoglobulin; LLC = Lewis lung cancer; MPE = malignant pleural effusion; PBS = phosphate-buffered saline; rm = recombinant mouse; TRFK5 = anti-IL-5 neutralizing antibody.

of these leukocytes in pleural tumors and MPEs was associated with reduced tumor cell apoptosis *in vivo*. Collectively, our data support a model in which pleural invasion by adenocarcinoma cells induces IL-5 expression by the host, leading to recruitment of eosinophils and myeloid suppressor cells (and possibly other immune and inflammatory cells) that facilitate tumor cell survival in the pleural space and enhance vascular permeability, leading to MPE.

Although these studies support the involvement of IL-5 in the pathogenesis of experimental adenocarcinoma-induced MPE, the relevance of our findings to human MPE remains to be fully clarified. We identified significant numbers (>10%) of eosinophils in 13% and levels of IL-5 in 41% of human MPEs, suggesting that IL-5 could impact tumor progression in a substantial percentage of human MPEs. Indeed, the low concentration of IL-5 and relatively small number of eosinophils in our experimental MPE models support the idea that IL-5 protumorigenic pathways may be broadly relevant to human MPEs and not limited to the fraction of MPEs clinically defined as eosinophilic.

Our results are consistent with recent evidence that supports a protumorigenic role for inflammatory cells and pathways in epithelial carcinogenesis, tumor progression, and metastasis (16–19). However, the precise role of the various inflammatory cells and mediators in each step of tumor progression is largely unknown. This is especially true for MPE, because of the lack, until recently, of immunocompetent animal models (12, 13, 38, 42). Experimental MPE progression is intimately associated with an inflammatory response, and several tumor-specific MPE-promoting pathways are proinflammatory, including VEGF/VEGFR1, IL-6/signal transducer and activator of transcription-3, and NF- κ B/TNF- α /MCP-1 (13–15, 22–24). Available data suggest that: (1) the inflammatory response that accompanies MPE may actually promote it (13–15, 22–24); (2) this effect of inflammation may be greater with MPE compared with solid tumors, a difference possibly explained by the pleural microenvironment, which is a privileged niche for inflammation and vascular hyperpermeability (42); and (3) tumor and host components contribute to the inflammatory cascade in MPE, with each playing distinct roles (42, 43). Although prior studies have identified several tumor cell-specific biologic pathways that promote experimental MPE by modifying the host immune and inflammatory response, to our knowledge this is the first example of a host factor (IL-5) that enhances experimental MPE progression by modulating the inflammatory response.

IL-5 is important in allergic inflammatory responses, eosinophil recruitment, and regulation of eosinophil lifespan (25). Prior information suggesting a possible role for the IL-5/eosinophil axis in progression of malignancies includes (1) the presence of eosinophils in MPE and other tumors (30, 44); (2) the role of IL-5 in tumor-associated eosinophilia (26–29); (3) the oncogenic effects of IL-5R β (or β c) signaling in hematopoietic cells (45); and (4) the observation that IL-5-dependent eosinophil recruitment in response to eukaryotic microorganisms is not always protective (46, 47). Indeed, our results using genetic and cytokine/antibody-based approaches all converged toward a marked MPE-promoting effect of IL-5. Although most studies have focused on the impact of IL-5 on eosinophil differentiation, trafficking, and tissue survival (25), IL-5 has also been shown to be involved in mononuclear cell recruitment to inflammatory sites (46). It is important to determine in future studies whether the important role for IL-5 in experimental MPE formation is mediated primarily through eosinophil recruitment and activation; recruitment of other immune inflammatory cells (like the myeloid suppressor cells); or other effects of IL-5 signaling.

In experimental MPE, recruitment of eosinophils and CD11b+GR1+ myeloid suppressor cells is associated with IL-5 production. Eosinophils are seminal players in allergic inflammation and host immune responses against eukaryotic microorganisms (25). Recent experimental work and older clinical observation have linked these cells with enhanced progression of solid and hematologic malignancies (26–30, 48), although the nature of this relationship is not well defined. Similarly, CD11b+GR1+ myeloid suppressor cells are tumor-promoting bone marrow-derived inflammatory cells produced in response to tumors (41). These cells are important mediators of tumor-induced immune dysfunction via suppressing effector T cells (49). Based on the absence of direct effects of IL-5 on tumor cells, we examined the abundance of these cells in our experimental animals. We found consistent increases in the relative abundance of myeloid suppressor cells in MPE and blood from mice with increased IL-5 bioactivity. On the contrary, genetic and antibody-mediated interventions to reduce IL-5 bioactivity resulted in decreased numbers of these cells. We speculate that IL-5 may be involved in the differentiation of myeloid suppressor cells, because its receptor shares a common β -subunit with the granulocyte-macrophage colony-stimulating factor, the primary stimulus for their development in the bone marrow of patients with cancer (49). Hence, recruitment of

specific inflammatory cell populations by IL-5 is likely to be critical for mediating the intrapleural fluid accumulation and tumor dissemination in experimental MPE.

Conclusions

Herein we describe an unprecedented biologic effect of host-derived IL-5 in malignant progression to form MPE. Because this cytokine is present in a significant proportion of human MPEs, it is possible that IL-5 may function to promote the human condition. Because anti-IL-5 directed therapies are currently being tested for benign human diseases, the present work may serve, pending further validation, to set a rational framework for future trials of IL-5 neutralization against malignant pleural disease.

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