

Allergen induced pulmonary inflammation enhances mammary tumor growth and metastasis: Role of CHI3L1

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ABSTRACT

Metastasis is the primary cause of mortality in women with breast cancer. Metastasis to the lungs is greater in patients with pulmonary inflammatory illnesses. It is unknown how pre-existing pulmonary inflammation affects mammary tumor progression. We developed a novel breast cancer model in which pulmonary inflammation is induced in mice prior to tumor cell implantation. In the present study, we determined how pre-existing allergen-induced inflammation changes the pulmonary microenvironment to exacerbate tumor metastasis. We showed that pre-existing pulmonary inflammation in mammary tumor bearers is associated with: 1) an increase in growth of the primary tumor and metastasis; 2) an increase in the expression of a glycoprotein known as CHI3L1; and 3) increase in the levels of myeloid populations in their lungs. We also showed that myeloid derived cells from the lungs of allergic tumor bearers produce higher amounts of CHI3L1 than the saline controls. We previously showed that CHI3L1 induces the expression of proinflammatory and protumorigenic molecules. In this study, we show that CHI3L1 knockout tumor bearers with pre-existing allergic pulmonary inflammation had decreased levels of myeloid-derived cells, decreased levels of proinflammatory mediators, and a significant reduction in tumor volume and metastasis compared with the wild-type controls. Pre-existing inflammation and CHI3L1 might be driving the establishment of a premetastatic milieu in the lungs and aiding in the support of metastatic foci. Understanding the role of allergen-induced CHI3L1 and inflammation in tumor bearers and its effects on the pulmonary microenvironment could result in targeted therapies for breast cancer. *J. Leukoc. Biol.* 97: 000-000; 2015.

Abbreviations: BALF = bronchoalveolar lavage fluid, CCL2 = c-chemokine ligand 2, CHI3L1 = chitinase-3-like-1 protein, CXCL2 = CXC chemokine ligand 2, MMP-9 = matrix metalloproteinase-9, MDCs = myeloid-derived cells

The online version of this paper, found at www.jleukbio.org, contains supplemental information.

Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer-related deaths. Metastasis is responsible for 90% of all cancer deaths despite significant improvements in diagnosis and treatments [1]. The incidence of metastasis to the lungs is greater in patients with breast cancer and chronic pulmonary inflammatory illnesses [2]. According to the American Lung Association, pulmonary inflammatory illnesses have been increasing throughout the world, with women being more susceptible than men. Therefore, it is important to determine how pre-existing inflammation affects breast cancer progression.

Patients with pulmonary inflammatory illnesses demonstrate higher levels of the glycoprotein CHI3L1 (also known as YKL-40, BRP-39) in their lungs and circulation [3]. Other conditions, such as chronic obstructive pulmonary disease, air pollution, allergies, and chronic respiratory infections, create the same inflammatory environment, with increased CHI3L1 expression [4, 5]. Furthermore, it has been reported that CHI3L1 levels are increased in the lungs of patients with allergic asthma and in mice with allergen-induced inflammation [6, 7]. Allergen-induced CHI3L1 knockout (KO) mice have decreased levels of inflammatory mediators in the circulation and in the pulmonary microenvironment [7]. We have previously shown that the levels of CHI3L1 are increased in the circulation of mammary tumor-bearing mice and that tumor cells, macrophages, and myeloid-derived cells (MDCs) express and secrete CHI3L1 [8]. CHI3L1 contributes to the pathological features associated with inflammation in the lungs of asthmatic patients by inducing the production of matrix degrading proteins and angiogenic molecules [9]. Our published results concur with these findings as we have also shown that CHI3L1 induces angiogenic and matrix degrading proteins [10]. The presence of these molecules at the inflammatory site might facilitate the growth of infiltrating tumor cells. A retrospective study of patients with breast cancer

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who had previously been diagnosed with asthma had an incidence of pulmonary metastases twice as great as those without asthma [2].

High levels of CHI3L1 have been correlated with disease severity and a poor prognosis, not only in patients with pulmonary inflammation but also in patients with different types of solid tumors, including breast cancer [11–14]. Recently, we have shown that CHI3L1 induces the expression of proinflammatory molecules in interstitial and alveolar macrophages and MDCs of 4T1 mammary tumor bearers [15]. CHI3L1 expression by these cells in the “premetastatic” lung plays a role in the increased occurrence of metastasis [15]. In the present study, we investigated whether pre-existing pulmonary inflammation can modulate breast cancer growth and metastasis. We report that mammary tumor-bearing mice with pre-existing allergic pulmonary inflammation have increased expression of proinflammatory molecules, including CHI3L1, in their lungs, expanded myeloid populations, accelerated primary tumor growth and metastases, and decreased survival. More importantly, we found a significant reduction in tumor growth and metastasis with increased survival in CHI3L1 KO mice, even in the presence of allergen-induced pulmonary inflammation. Because CHI3L1 is seen at higher levels in either patients with breast cancer or asthma [16–18] and the frequency of asthma is greater in patients with breast cancer [2], targeting lung inflammation and CHI3L1 could be a valuable therapeutic strategy for inhibiting breast cancer growth and metastasis.

MATERIALS AND METHODS

Mice and cell lines

Female BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) and CHI3L1 knockout mice (a gift from Dr. Alison Humbles, MedImmune, Gaithersburg, MD, USA), 8–10 weeks old were used in all studies and were housed and used according to the U.S. National Institutes of Health guidelines, under protocols approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Mammary tumor cells (4T1-luciferase [luc]-A4; Caliper Life Sciences, Hopkinton, MA, USA; 4T07 luc, a gift from Dr. William Schiemann, Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA; 67NR luciferase mammary tumor cells, a gift from Dr. Shoukat Dedhar, BC Cancer Research Agency, Vancouver, BC, Canada) were maintained in either RPMI 1640 medium (4T1 luc) or DMEM (67NR luc and 4T07 luc) with 10% FCS [19]. Next, 0.5×10^4 4T1 luc, 0.1×10^6 4T07 luc mammary tumor cells, or 2×10^6 67NR luc mammary tumor cells were implanted in the mammary fat pads of mice for the spontaneous metastasis studies. In other mice, mammary tumor cells were injected via the tail vein to assess lung-specific metastatic foci formation. 4T1 tumors metastasize to the lung at ~4–5 wk after tumor cell implantation. 67NR tumors do not metastasize to the lungs, but 4T07 cells form micrometastases and 4T1 cells form metastatic foci in the lungs [20]. The tumor bearers were assessed at 5 wk after tumor implantation for the expression of cytokines.

Induction of allergic pulmonary inflammation

Allergic pulmonary inflammation was induced in the mice using an established ragweed sensitization aerosol challenge model [21]. In brief, the mice were immunized with 2 intraperitoneal injections of endotoxin-free short ragweed allergen (<0.03 endotoxin unit/ml; Geer Laboratories, Lenoir, NC, USA), 100 μ g/dose (5000 protein nitrogen units/dose) suspended in 1:4

aluminum hydroxide adjuvant (Inject Alum; Thermo Fisher Scientific, Rockford, IL, USA) on days –14 and –10 (Fig. 1A). At 11 days (day –3) after the initial intraperitoneal sensitization, an intranasal challenge was performed with 100 μ g ragweed allergen. The control groups included immunized mice receiving an intranasal challenge of 0.1 ml saline (naïve I) or ragweed 100 μ g (naïve II) on day –3. On day –1, the mice were checked to ensure allergen sensitization. At 3 days after the intranasal challenge, these mice were injected with 4T1 luc, 4T07 luc, or 67NR luc tumor cells (day 0). The mice were again intranasally challenged on day 7. The tissue was harvested at approximately day 45 for the studies.

In vivo imaging and determination of metastasis

The mice were injected with 2.5 mg i.p. D-luciferin (Xenogen; Caliper Life Sciences, PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) dissolved in 100 μ l 0.9% saline, anesthetized with isoflurane, and imaged using the bioluminescence optical imager (IVIS Lumina LTE, Xenogen; Caliper Life Sciences). Maximal luciferase signals were quantified using Living Image 2.5 (Xenogen; Caliper Life Sciences) image analysis software. The luciferase signal is reported as the mean radiance photons/sec/cm²/steradian. Luciferase transfected 4T1 (either wild-type or CHI3L1 knockout mice), 4T07, and 67NR mice were also imaged at 4 different time points as indicated in the figures. Tissue-specific metastasis of 4T1 tumors was measured ex vivo by luminescence immediately after euthanasia on day 45.

Metastasis in the lungs was assessed by India ink staining and bioluminescence signal detection. To assess metastasis using the India ink method, a solution of 15% India ink was injected intratracheally into the euthanized mice. The lungs were then excised, rinsed in PBS, immersed in Fekete's solution (~50% ethanol, 8% formaldehyde, and 4% acetic acid) for 24 h and photographed using a digital camera. For bioluminescence signal detection, the excised lungs were imaged using the IVIS Lumina imaging system, and the signals were quantified using the Living Image 2 analysis software, as described previously [8]. The bioluminescent signals were normalized by comparing the luciferase activity between the tumor-bearing and tumor-free mice. The metastatic tumor burden was determined by weighing the lungs (in grams).

Histologic examination of lung tissue

Formalin-fixed lung tissue from 4T1 saline wild-type, 4T1 ragweed wild-type, 4T1 saline CHI3L1 knockout, and 4T1 ragweed CHI3L1 knockout mice were embedded in paraffin and sectioned at 4- μ m thickness. The sections were deparaffinized and stained with H&E with automated Tissue Tek 2000 processor (Sakura Finetek, Torrance, CA, USA). The tissue was analyzed by a pathologist.

Isolation of pulmonary macrophages and MDCs

Bronchoalveolar macrophage isolation. The tracheas of the euthanized mice were cannulated and lavaged with 1 ml saline. The BALF was recovered to obtain alveolar macrophages, as described by Kogiso et al. [22]. Isolation of alveolar macrophages from the bronchoalveolar lavage fluid was done by purification based on CD11b expression and separation by Miltenyi magnetic beads (Miltenyi Biotec, Cambridge, MA, USA). The macrophages were then enriched by plastic adherence by incubating CD11b⁺ BALF cells (10^6 cells/ml) in media (RPMI 1640 with 20% FBS, 10 U/ml penicillin, and 10 μ g/ml streptomycin) for 1 h in a 5% CO₂ incubator at 37°C [23]. Nonadherent cells were removed, and the adherent macrophages were obtained by scraping with a rubber policeman. Viability was assessed by trypan blue exclusion and found to be >95% viable. The purity of CD11b⁺F4/80⁺ cells was >90% as assessed by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA, USA).

Isolation of cells from lungs. Immediately after collecting BALF specimens, interstitial macrophages were isolated as described previously [24, 25]. After BALF was collected, the lungs were removed and placed in a Petri dish with

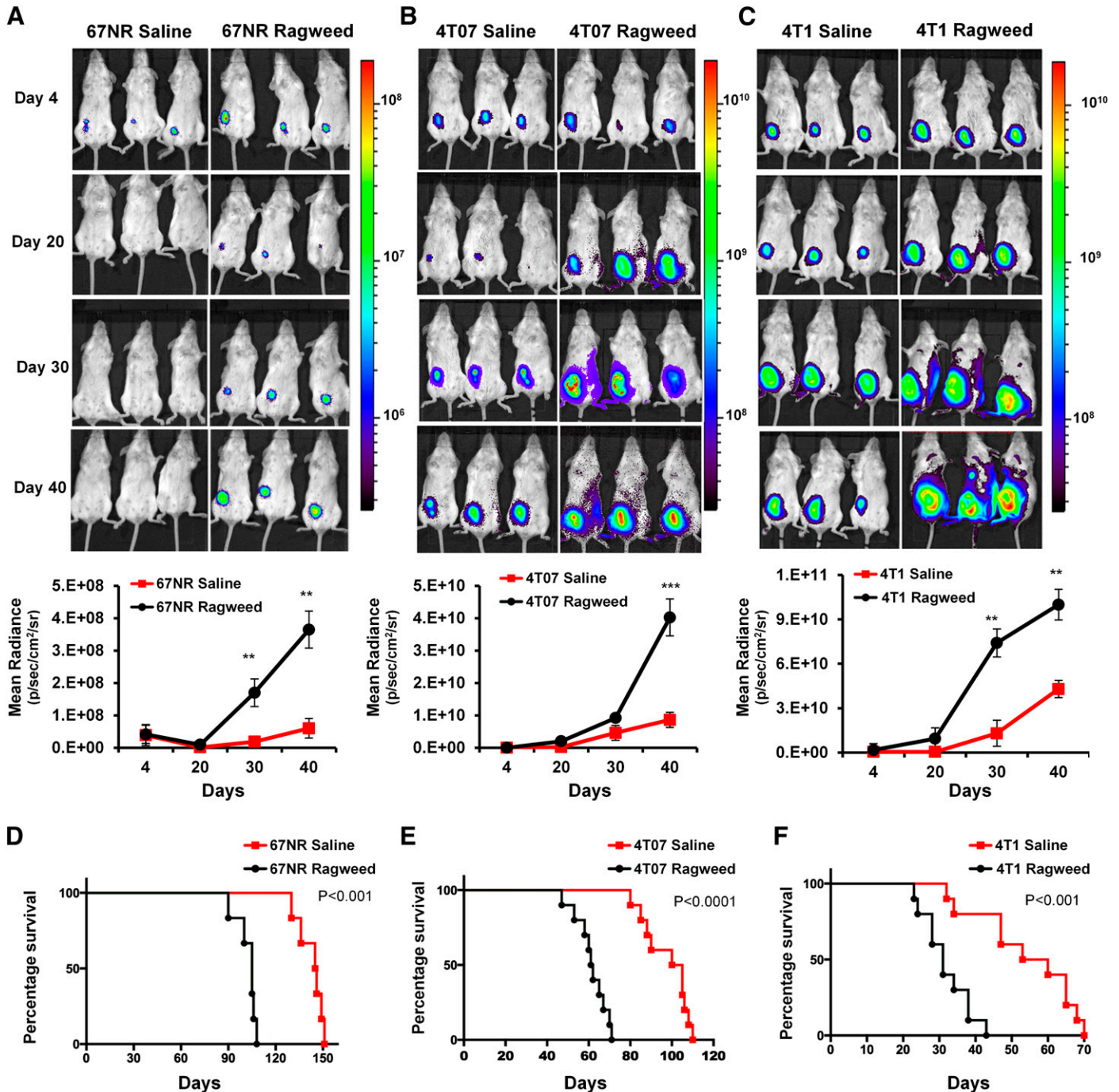


Figure 1. Tumor growth is increased as survival decreases in ragweed tumor bearers. Representative ventral image of 3 mice/group, with 3 independent experiments with 10 mice/group ($n = 30$). The mice were sensitized and challenged with ragweed before mammary tumor cell inoculation into the mammary fat pads. Bioluminescence images and quantification for tumor growth at days 4, 20, 30, and 40 in (A) 67NR saline and 67NR ragweed, (B) 4T07 saline and 4T07 ragweed, and (C) 4T1 saline and 4T1 ragweed. Kaplan-Meier survival plots of (D) 67NR, (E) 4T07, and (F) 4T1. $N = 10$ mice/group; representative data of three independent experiments are presented as mean $\pm$ SD of samples. $**P \leq 0.001$, $***P \leq 0.0001$.

1.4 mg/ml collagenase D (Sigma-Aldrich, St. Louis, MO, USA) and 300 Kunitz units/ml DNase I (Sigma-Aldrich) in HBSS with 5% FBS. The lungs were minced and agitated in a shaker at 37°C for 30 min. The cell aggregates were removed and redigested for an additional 20 min, as described above. The digested material was filtered through a 40 μ m cell strainer (BD Biosciences), and the lung cells were pelleted. The lung single cell suspensions were used

for additional purification of different cell populations, flow cytometric analysis, and ELISA.

Isolation of interstitial macrophages. To isolate interstitial macrophages, the lung cells were separated according to the density gradient using Lympholyte-M (Cedarlane, Burlington, NC, USA). The mononuclear layer containing lymphocytes and macrophages was further separated according to

the CD11b⁺ expression using Miltenyi magnetic beads. To purify the macrophages, the CD11b⁺ cells were plastic adhered, as described. The adherent populations were >95% viable according to Trypan blue exclusion and >90% CD11b⁺F4/80⁺, as determined using flow cytometric analysis.

Isolation of MDCs. Myeloid-derived cells were purified from lungs using the Myeloid Derived Suppressor Cell Isolation kit (Miltenyi), in accordance with the manufacturer's suggested protocol. This separation resulted in isolation of monocytic (Gr1^{dim}Ly6G⁻) and granulocytic (Gr1^{hi}Ly6G⁺) populations.

Flow cytometry

Staining for flow cytometric analysis was performed on total lung single cells resuspended in FACS buffer (PBS with 1% BSA and 0.1% sodium azide). Fc-receptor-mediated nonspecific antibody binding was blocked by using anti-CD16/CD31 antibodies (BioLegend, San Diego, CA, USA). Total lung cells were stained for 30 min at 4°C. The following mAbs were used at the appropriate concentrations for staining: CD45 Pacific Blue (30-F11), CD11b FITC (M1/70; BD Biosciences), Ly6C APC-Cy7 (HK1.4), Ly6G APC (1-A8), F4/80 Brilliant Violet 605 (BM8), CD11c PerCP (N418), and CD68 PE-Cy7 (FA-11). All antibodies with the exception of CD11b were from BioLegend (San Diego, CA, USA). The cells were analyzed using Fortessa (BD Biosciences) flow cytometer, and the data were analyzed using Flow Jo X software (Tree Star, Inc., Ashland, OR, USA).

Cell cultures

The total lung homogenates, BALF cells, purified alveolar, interstitial macrophages, and MDCs were cultured at 2×10^6 cells/ml for 18 h in complete media as described previously [26], and the cell-free supernatants were collected and stored at -80°C.

Cytokine ELISA

BALF samples and culture supernatants from cells obtained from saline or ragweed mice (tumor-free) and saline or ragweed mammary tumor bearers were analyzed for CHI3L1, MMP-9, CXCL2/IL-8, CCL2/MCP-1, IL-13 (all from R&D Systems, Minneapolis, MN), IL-4, and IgE (BD Biosciences) by ELISA, according to the manufacturer's instructions. Absorbance at 450 nm with wavelength correction at 570 nm was measured using a Tecan SLT Rainbow Reader (Tecan US, Inc., Morrisville, NC, USA), and the OD values of the samples were converted to picograms against a standard curve plotted from the known quantities of recombinant murine cytokines.

Confocal microscopy

The lungs from 45-day mammary tumor-bearing mice were perfused with 70% optimal tissue cutting compound with 30% PBS. The lungs were then removed and snap frozen on dry ice. The 5 μ M cryostat sections were mounted on Plus slides (Thermo Fisher Scientific) and fixed in 4% paraformaldehyde. The cryostat sections were blocked in 10% normal horse serum before staining with primary antibodies, rabbit anti-mouse CHI3L1 (7 μ g/ml; provided by Dr. Alison Humbles, MedImmune) or rat anti-mouse Gr1 (clone RB6-8C5, 15 μ g/ml; BioXCell, West Lebanon, NH, USA) by incubating overnight at 4°C. The cryostat sections were washed and labeled with the following secondary antibodies: PE donkey anti-rat, and FITC donkey anti-rabbit (1:2000; both from Invitrogen, Life Technologies, Carlsbad, CA, USA). To visualize the nuclei, the sections and cells were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined by confocal microscopy (LSM 700, Carl Zeiss Microimaging, Thornwood, NY, USA).

Statistical analysis

The results are expressed as the group mean $\pm$ SD. Statistical analyses were performed using Prism 6 software (GraphPad Software, Incorporated, La Jolla, CA, USA). Statistical comparisons were performed using an unpaired 2-tailed Student *t* test, with significance at $P < 0.05$. Nonparametric analysis was used to determine tumor growth. For the survival analysis, the Kaplan-Meier method was used.

RESULTS

Establishment of allergic pulmonary inflammation

It is well established that inflammation within the tumor microenvironment fosters tumor growth and metastasis. CHI3L1 is a proinflammatory glycoprotein that is overexpressed in various types of solid tumors and during inflammatory illnesses [3, 17, 27, 28]. To determine whether pre-existing pulmonary inflammation and the associated CHI3L1 expression have an effect on tumor growth and metastasis, we developed a novel mouse model by combining an established model of allergic pulmonary inflammation with a model of breast cancer (Supplemental Fig. 1A). Pulmonary inflammation was established by initial sensitization by intraperitoneal injections with short ragweed allergen, which were then followed by an intranasal challenge with the same antigen (Supplemental Fig. 1A). Before inoculation with mammary tumor cells, the inflammatory status in the lungs of the mice was assessed. We found higher levels of IgE in the serum and higher levels of IL-4 and IL-13 in the BALF of ragweed allergen-induced mice (Supplemental Fig. 1B–D). CHI3L1 levels were also higher in the BALF of ragweed mice compared with the saline tumor-free mice (Supplemental Fig. 1E). The serum level of the ragweed mice and the saline, tumor-free mice was $159 \pm 34 \times 10^3$ ng/ml ($\pm$ SEM) and $12 \pm 19 \times 10^3$ ng/ml ($P \leq 0.001$), respectively. Furthermore, H&E analysis of the pulmonary tissue indicated increased inflammatory cellular infiltrates in the ragweed mice compared with the saline controls (Supplemental Fig. 1F). Taken together, these data confirm the presence of pulmonary inflammation in the ragweed mice.

Tumor growth is increased in mice with allergic pulmonary inflammation

Allergen-induced pulmonary inflammation is characterized by recruitment of proinflammatory cells and increased levels of growth and angiogenic factors, including CHI3L1. We hypothesized that this environment might have an effect on the growth of the tumor, facilitate tumor cell recruitment, and enable the establishment of metastasis. To determine whether pre-existing pulmonary inflammation has an effect on primary breast cancer growth, mice with previously induced allergic pulmonary inflammation were orthotopically implanted with mammary tumor cells with varying degrees of growth and metastatic potential. Toward this end, a series of isogenic mammary tumor cells lines, 67NR, 4T07, and 4T1, which are derived from the 4T tumor, were used.

Implantation of 67NR cells, a cell line that grows slowly and fails to disseminate from the primary tumor into mammary fat pads, resulted in increased tumor growth in the allergen-sensitized mice compared with the saline controls (Fig. 1A). Furthermore, survival was decreased in the 67NR ragweed mice compared with the 67NR saline controls ($P < 0.001$; Fig. 1D). The 4T07 cells are known to metastasize to the lungs and form small nodules, but these nodules fail to proliferate [20]. Similar to what was observed with 67NR ragweed, increased tumor growth was seen in the 4T07 ragweed mice compared with the 4T07 saline mice (Fig. 1B). Survival in the 4T07 ragweed tumor mice was decreased compared with that of the 4T07 saline mice

($P < 0.0001$; Fig. 1E). The most metastatic of the 4T1 tumor cell lines is 4T1, which, when transplanted into a mammary fat pad, metastasizes to the bone, brain, liver, and lungs. The tumor growth in the 4T1 ragweed mice was also greater than that in the 4T1 saline control mice (Fig. 1C). Similar to the other 4T1 ragweed mice, survival of the 4T1 ragweed mammary tumor mice was decreased compared with that of the 4T1 saline controls ($P < 0.001$; Fig. 1F). Although survival was decreased in all types of 4T1 ragweed tumor bearers, the survival for the 4T1 ragweed mice was the lowest. Taken together, these data suggest that inflammation at a distant site does have an effect on primary tumor growth and survival.

A high expression of CHI3L1 is correlated with survival [14, 16]. We previously reported increased expression of CHI3L1 in mammary tumor-bearing mice [8]. We sought to determine the CHI3L1 expression in the three 4T1 mammary tumor cell lines. Western blot and ELISA of tumor cell culture supernatants revealed the highest expression in the 4T1-bearing mice, with the least expression in 67NR; 4T07 had average levels of CHI3L1 (Supplemental Fig. 2).

Metastasis to the lungs is increased in mice with allergic pulmonary inflammation

It is well established that metastasis accounts for most of the deaths of patients with cancer. Of the cell lines used in our studies, the 4T1 cells were the only cells known to complete all the steps of metastasis. Because our studies involved the effect of

pulmonary inflammation on metastasis of breast cancer cells to the lungs, we focused the rest of our studies on using the 4T1 mammary tumor cells. Furthermore, the 4T1 tumor model shares many of the characteristics present in human patients with breast cancer. Ragweed-induced 4T1 tumor-bearing mice showed increased metastasis using the India ink technique (Fig. 2A). A 3-fold increase in metastatic foci was found in 4T1 ragweed mice compared with the 4T1 saline controls (Fig. 2A). The lungs were then weighed to determine the effect of the tumor load. Although the lungs of the saline 4T1 tumor bearers had an average weight of 0.19 ± 0.09 g (SEM), the weight of the lungs in the ragweed tumor bearers was almost doubled, with an average weight of 0.37 ± 0.10 g (SEM) (Fig. 2B). These findings suggest that pulmonary inflammation can be correlated with an increased incidence of metastasis to the lung ($P \leq 0.001$). Detection using bioluminescence signals revealed a 6-fold increase in metastatic foci in the 4T1 ragweed mice (Fig. 2C). Histologic assessment on H&E-stained lung tissue revealed tumor lesions in the 4T1 ragweed mice compared with the 4T1 saline controls (Fig. 2D).

In the previous section, we evaluated the levels of spontaneous metastasis in ragweed mice implanted with tumor cells in the mammary fat pads. To determine whether colonization of tumor cells in a target organ such as the lung is affected by pre-existing allergen-induced pulmonary inflammation, we injected 4T1 luc mammary tumor cells via the tail vein. We found a 10-fold increase in tumor cell colonization in the 4T1 ragweed mice

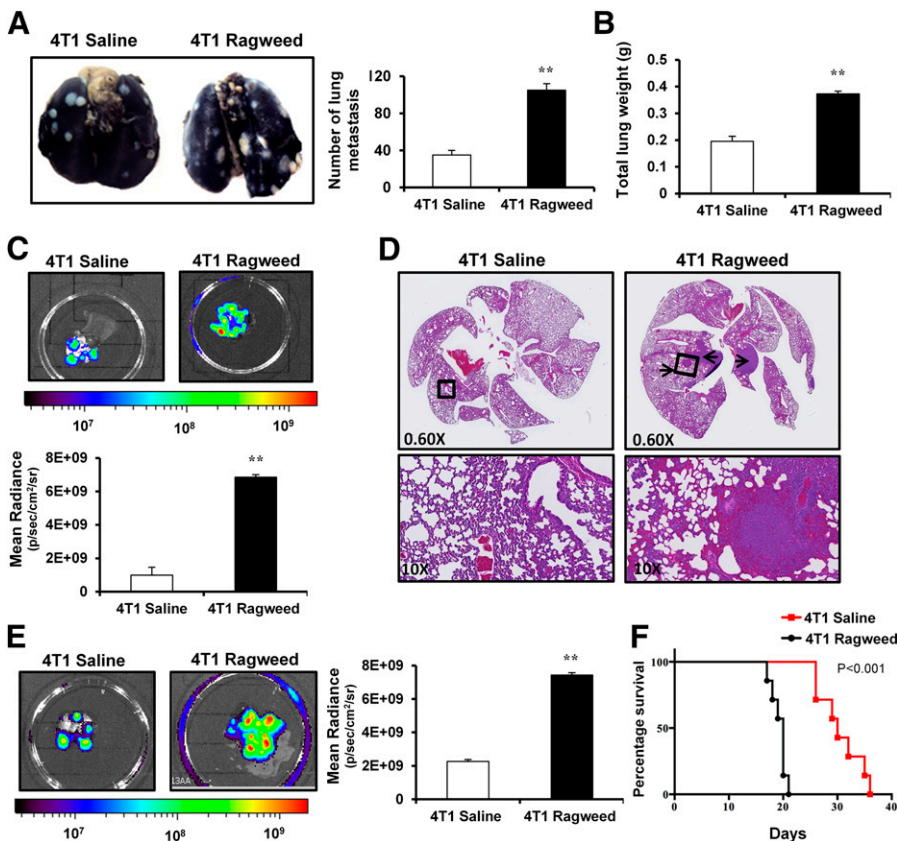


Figure 2. Increased spontaneous and experimental metastasis in lungs of 4T1 ragweed mice. Mice were euthanized on day 45, and the lungs were assessed for metastasis. (A–D) Spontaneous metastasis in lungs of mice implanted with tumor cells in mammary fat pads. Photograph of India ink-perfused lungs and number of tumor nodules in the lungs (A), weight of excised lungs (B), bioluminescence images of excised lungs (C), and H&E stain of 5- μ m lung sections at $\times 6$ and $\times 100$, showing extensive tumor metastasis with infiltration of mononuclear cells and neutrophils (D). (E and F) Experimental metastasis in mice injected with mammary tumor cells via tail vein injection. Lungs were excised and imaged for bioluminescence (E). Kaplan-Meier survival plot (F). $N = 10$ mice/group; representative data of three independent experiments are presented as mean $\pm$ SD of samples. $**P \leq 0.001$.

compared with the 4T1 saline controls (Fig. 2E). Similar to the findings reported in the previous section, 4T1 ragweed mice had significantly reduced survival rates compared with the 4T1 saline controls ($P < 0.001$; Fig. 2F). These results suggest that pulmonary inflammation accelerates the metastasis occurring naturally from the mammary fat pads and colonization when tumor cells are introduced via the tail vein.

CHI3L1 expression is higher in ragweed tumor-bearing mice

We previously reported that the proinflammatory glycoprotein CHI3L1 is increased in the circulation and in the lung microenvironment of 4T1 mammary tumor bearers [15]. Because CHI3L1 is increased during inflammatory conditions, we investigated whether pre-existing pulmonary inflammation had additional effects on CHI3L1 expression in tumor bearers. CHI3L1 expression, as analyzed by ELISA, was increased in the serum of the 4T1 ragweed mammary tumor-bearing mice compared with that in the 4T1 saline controls (Fig. 3A). The higher CHI3L1 in circulation could have partly resulted from contributions from the BALF and lung homogenates, in which CHI3L1 was increased in the 4T1 ragweed tumor bearers with respect to the 4T1 saline controls (Fig. 3B and C). To obtain additional insight into CHI3L1 localization in the lungs, confocal microscopy was performed. Confocal microscopy revealed localization of CHI3L1 in the cytoplasm of GR1⁺ myeloid cells

and in the metastatic foci (Fig. 3D). The GR1 marker identifies both monocytic and granulocytic immature myeloid populations. These monocytic and granulocytic populations were separated using Miltenyi magnetic bead separation. Quantification of CHI3L1 expression by ELISA in monocytic and granulocytic myeloid populations revealed higher expression in the 4T1 ragweed tumor bearers than in the 4T1 saline tumor bearers (Fig. 3E). We have previously shown that CHI3L1 plays a role in establishing a premetastatic niche in the lungs of 4T1 mammary tumor bearers and that myeloid cells and macrophages express CHI3L1 [8, 15]. Because macrophages are associated with pathologic responses in both allergic inflammatory conditions and tumors, we assessed these cells in the lungs of the 4T1 ragweed mammary tumor bearers. Toward this, macrophages were purified from BALF (alveolar) and total lung tissue homogenates (interstitial) by CD11b magnetic bead separation followed by plastic adherence. Analysis of alveolar and interstitial macrophages revealed a similar upregulation in the production of CHI3L1 in the ragweed tumor-bearing mice compared with the saline tumor-bearing mice (Fig. 3F and G).

Proinflammatory/protumorigenic molecules are increased in ragweed tumor-bearing mice

Infiltration of metastatic lungs by MDCs and macrophages producing inflammatory cytokines is characteristic of tumor-promoting inflammation [29, 30]. Likewise, allergic pulmonary

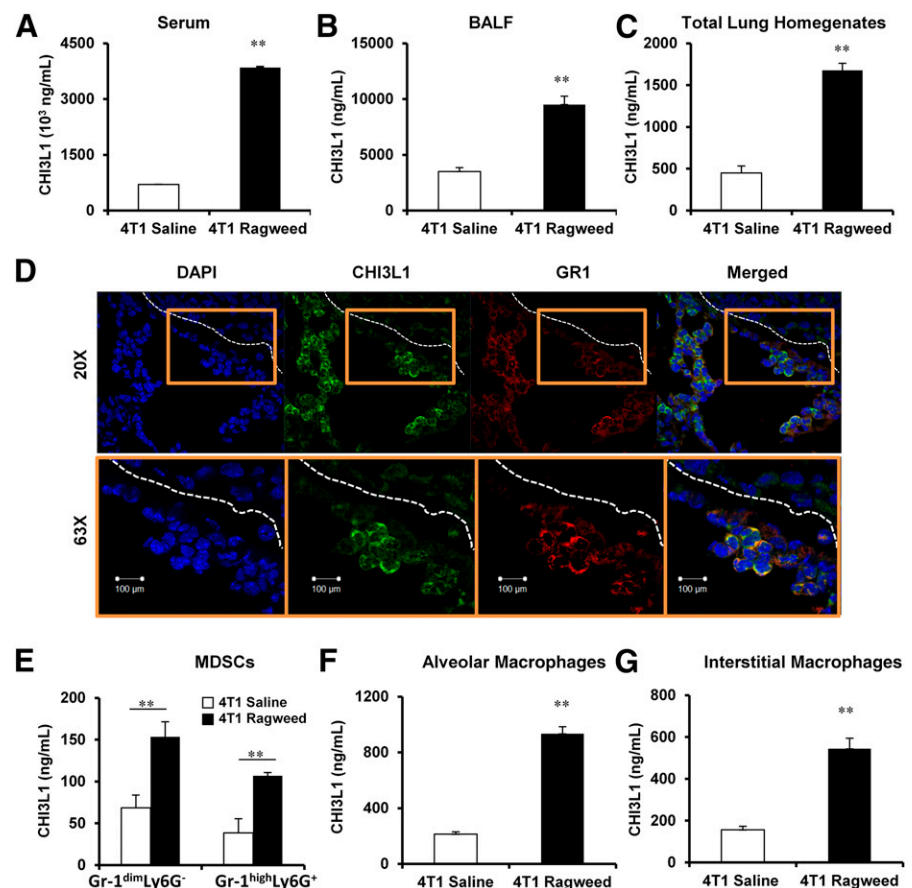


Figure 3. CHI3L1 expression is increased in ragweed mammary tumor-bearing mice. Mice sensitized and challenged with ragweed allergen were implanted with 4T1 mammary tumor cells into mammary fat pads. CHI3L1 expression was assessed at day 45 after tumor cell implantation. ELISA findings of CHI3L1 expression in serum (A), BALF (B), and total lung homogenates (C). (D) Immunofluorescence image showing CHI3L1 expression in 7-μm lung section of ragweed tumor bearer. CHI3L1 expression by ELISA of MDCs (E), alveolar macrophages (F), and interstitial macrophages (G). $N = 10$ mice/group; representative data of three independent experiments are presented as mean $\pm$ SD of samples. ** $P \leq 0.001$.

inflammation is characterized by MDC and macrophage infiltration of the lungs [31, 32]. We have previously shown that proinflammatory/protumorigenic molecules are upregulated in 4T1 mammary tumor bearers [8]. MDC populations of monocytic lineage ($\text{Gr1}^{\text{dim}}\text{Ly6G}^-$) produced higher levels of CXCL2/IL-8, CCL2/MCP-1, and MMP-9 compared with the 4T1 saline tumor bearers. The granulocytic lineage ($\text{Gr1}^{\text{hi}}\text{Ly6G}^+$) of the 4T1 ragweed tumor bearers produced higher levels of CCL2/MCP-1 and MMP-9 (Fig. 4A–C). However, no significant difference was found in the production of CXCL2/IL-8 in the granulocytic population (Fig. 4A). Furthermore, both alveolar and interstitial macrophages produced greater amounts of CXCL2/IL-8, CCL2/MCP-1, and MMP-9 in the 4T1 ragweed mice than in the 4T1 saline tumor bearers (Fig. 4D–I).

Allergic pulmonary inflammation alters the distribution of myeloid cells in tumor-bearing mice

Because the 4T1 ragweed tumor-bearing mice presented with increased metastasis to the lungs and had higher levels of proinflammatory/protumorigenic molecules, we characterized the whole-lung tissue using flow cytometry. Single cell suspensions of whole lungs were initially gated on CD45^+ cells, and populations of immune cells were then verified according to their forward and side scatter profiles (Fig. 5A). From these, CD11b^+ cells were selected and analyzed further according to Ly6G expression. The Ly6G^+ granulocytic lineage ($\text{F4/80}^- \text{Ly6C}^{\text{mid}}$) was not altered in either the total numbers or percentages in the 4T1 ragweed vs. 4T1 saline tumor bearers (Fig. 5B). The Ly6G^- lineage was also characterized further. Three distinct populations were identified according to Ly6C and F4/80 expression. Population I ($\text{Ly6C}^{\text{hi}}\text{F4/80}^-$) showed decreased percentages in the 4T1 ragweed tumor-bearing mice. However, the total numbers of cells were unaltered (Fig. 5C). The percentages and numbers of population II ($\text{Ly6C}^{\text{mid}}\text{F4/80}^+$) and population III ($\text{Ly6C}^- \text{F4/80}^+$) were significantly increased in the 4T1 ragweed tumor-bearing mice (Fig. 5C). Additional

characterization of these 3 populations using CD68 and CD11c showed a homogeneous distribution in each of the populations, with population I as $\text{CD68}^{\text{low}}\text{CD11c}^-$, population II as $\text{CD68}^{\text{hi}}\text{CD11c}^{\text{hi}}$, and population III as $\text{CD68}^- \text{CD11c}^{\text{lo}}$.

Tumor growth and metastases are decreased in CHI3L1 KO mice

To confirm the role of proinflammatory CHI3L1 in tumor growth and metastasis, pulmonary inflammation-induced CHI3L1 KO and wild-type (WT) mice were implanted with 4T1-luc mammary tumor cells. A significantly reduced tumor volume was observed in the 4T1 ragweed CHI3L1 KO mice compared with that in the 4T1 ragweed WT tumor bearers (Fig. 6A, panels 3 and 4). Likewise, the 4T1 saline CHI3L1 KO mice had a significantly reduced tumor volume compared with the 4T1 WT saline controls (Fig. 6A, panels 1 and 2). Quantification of the bioluminescence signals showed a 10-fold decrease in the signals in the 4T1 ragweed CHI3L1 KO mice compared with the 4T1 ragweed WT mice. In sharp contrast, no significant difference was seen in the signals between the 4T1 saline CHI3L1 KO and 4T1 ragweed CHI3L1 KO mice (Fig. 6A). As already shown, the 4T1 ragweed WT mice had decreased survival compared with the 4T1 saline WT mice. However, the 4T1 ragweed CHI3L1 KO mice had increased survival compared with the 4T1 ragweed WT mice ($P < 0.001$). No significant difference in survival was seen between the 4T1 ragweed CHI3L1 KO and 4T1 saline CHI3L1 KO mice (Fig. 6B). A comparison of median survival in these mice showed that survival was 31 days in the 4T1 ragweed WT mice, 56 days in the 4T1 saline WT mice, 87 days in the 4T1 ragweed CHI3L1 KO mice, and 99 days in the 4T1 saline CHI3L1 KO mice, indicating increased survival for the CHI3L1 KO mice. It is well known that metastasis correlates with decreased survival [14, 16]. Significantly, no tumor metastases were present in either the 4T1 saline CHI3L1 KO or 4T1 ragweed CHI3L1 KO mice ($P \leq 0.0001$). However, large metastatic foci

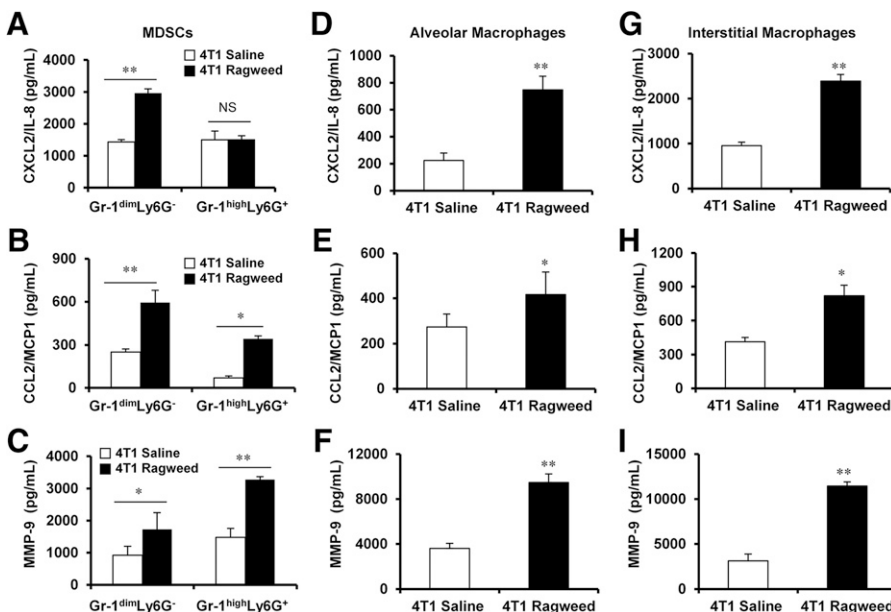


Figure 4. ELISA findings of supernatants from MDCs, interstitial and alveolar macrophages at day 45 after tumor cell implantation. Mice sensitized and challenged with ragweed allergen were implanted with 4T1 mammary tumor cells into mammary fat pads. MDCs and macrophages were isolated and cultured overnight. Cell-free supernatants were then assessed for proinflammatory mediators. CXCL2/IL-8 levels in MDCs (A), alveolar macrophages (D), and interstitial macrophages (G). CCL2/MCP-1 levels in MDCs (B), alveolar macrophages (E), and interstitial macrophages (H). MMP-9 levels in MDCs (C), alveolar macrophages (F), and interstitial macrophages (I). $N = 10$ mice/group; representative data of three independent experiments are presented as mean $\pm$ SD of samples. * $P \leq 0.05$, ** $P \leq 0.001$.

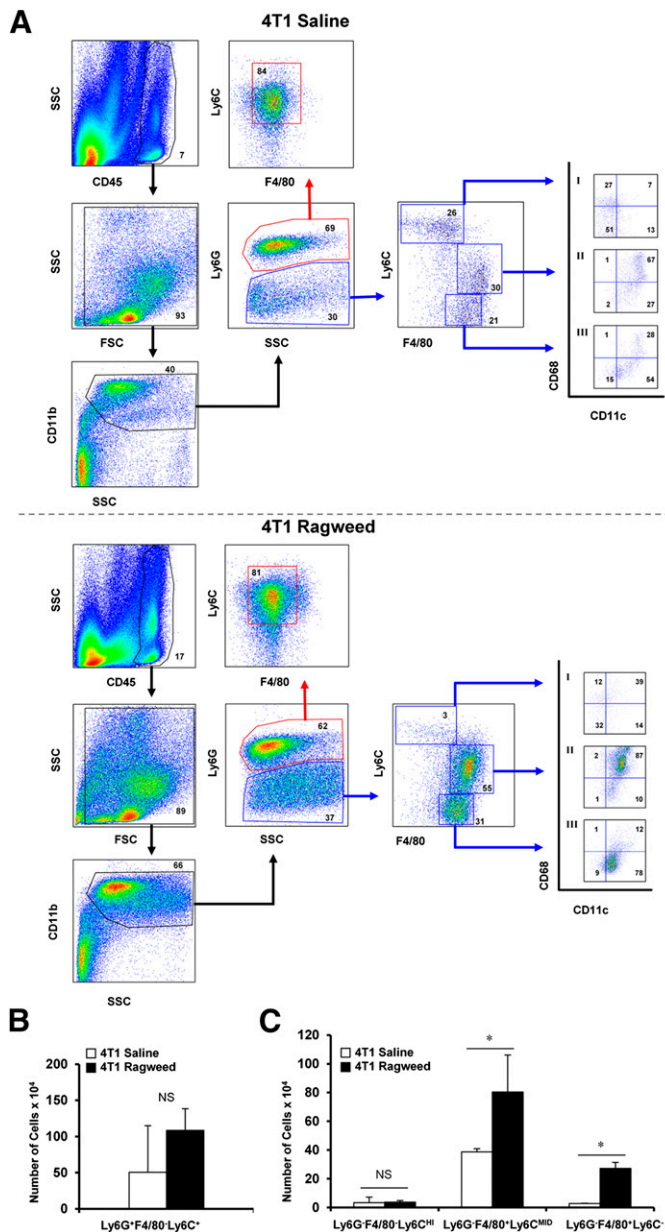


Figure 5. Flow cytometric analysis of single cell suspensions of total lung homogenates reveals an altered cellular distribution in ragweed 4T1 tumor bearers. (A) Gating strategy. The number of cells in 4T1 saline vs. 4T1 ragweed mice in Ly6G⁺ (B) and Ly6G⁺ (C) subpopulations. *N* = 10 mice/group; representative data of three independent experiments are presented as mean ± SD of samples. **P* ≤ 0.05.

were seen in the 4T1 ragweed WT and 4T1 saline WT mice, determined by the bioluminescence signals (Fig. 6C). It is important to note that the observed numbers of tumor lesions were greater in the 4T1 ragweed WT mice than in the 4T1 ragweed CHI3L1 KO mice. Histologic sections of H&E-stained lung tissue further confirmed these findings. Large tumor clusters were seen in the lungs of both 4T1 saline WT and 4T1 ragweed WT mice at day 45. In contrast, at the same time point, no tumor cells were seen in either the 4T1 saline CHI3L1

KO or 4T1 ragweed CHI3L1 KO lungs (Fig. 6D). These findings suggest an important role for inflammation in that decreased inflammation in the CHI3L1 knockout mice resulted in smaller tumors and a decreased incidence of pulmonary metastasis.

Proinflammatory/protumorigenic mediators are decreased in CHI3L1 KO mice

Because proinflammatory mediators are induced by CHI3L1 [8, 11, 15, 33] and secreted by MDCs and macrophages, we determined the CXCL2/IL-8, CCL2/MCP-1, and MMP-9 levels in CHI3L1 KO mice. The CXCL2/IL-8, CCL2/MCP-1, and MMP-9 levels were decreased in 4T1 saline WT, 4T1 ragweed CHI3L1 KO, and 4T1 saline CHI3L1 KO mice compared with the 4T1 ragweed WT mice (Fig. 7A–C). No significant differences were found between the 4T1 saline CHI3L1 KO and 4T1 ragweed CHI3L1 KO mice. As shown in Supplemental Fig. 2, 4T1 tumor cells produce CHI3L1. To determine the contribution of tumor-derived CHI3L1 in the CHI3L1 KO mice, CHI3L1 ELISA was performed using serum and lung homogenates. The serum level of the 4T1 ragweed CHI3L1 KO mice was 104 ± 45 ng/ml (SEM) compared with $684 \pm 495 \times 10^3$ ng/ml (SEM) in the 4T1 ragweed WT mice. Furthermore, the CHI3L1 levels in the total lung homogenates were significantly decreased in the KO mice (Fig. 7D).

Ablation of CHI3L1 reverts distribution of myeloid cells observed in ragweed WT tumor bearers

Because no significant differences were seen in tumor progression or proinflammatory mediator expression in the 4T1 saline CHI3L1 KO and 4T1 ragweed CHI3L1 KO mice, we focused our attention on the comparison of the 4T1 ragweed WT and 4T1 ragweed CHI3L1 KO mice. Given that the distribution of cells in the ragweed vs. saline mice was altered, we analyzed the cellular composition of the lungs in the 4T1 ragweed WT and 4T1 ragweed CHI3L1 KO mice using flow cytometry. A similar gating strategy was used for the whole-lung homogenates of the 4T1 ragweed WT and 4T1 ragweed CHI3L1 KO tumor bearers, as described in the section, “Allergic pulmonary inflammation alters the distribution of myeloid cells in tumor-bearing mice”. (Figs. 5A and 8A). The CD11b⁺Ly6G⁺ granulocytic lineage was similar in percentages and absolute numbers in both groups (Fig. 8B). Among the CD11b⁺Ly6G⁺ cells, all 3 populations (I, II, III) showed a decrease in the percentages and absolute number of cells in the 4T1 ragweed CHI3L1 KO mice (Fig. 8C). In this experiment, population I was defined as CD11b⁺Ly6G⁺F4/80⁺Ly6C^{hi}, population II as CD11b⁺Ly6G⁺F4/80⁺Ly6C^{mid}, and population III as CD11b⁺Ly6G⁺F4/80⁺Ly6C^{lo} (Fig. 8A).

DISCUSSION

In the present study, we showed that pre-existing pulmonary inflammation is associated with higher CHI3L1 expression and modifies the microenvironment to accelerate tumor growth, increase metastasis, and decrease survival. Immune cellular

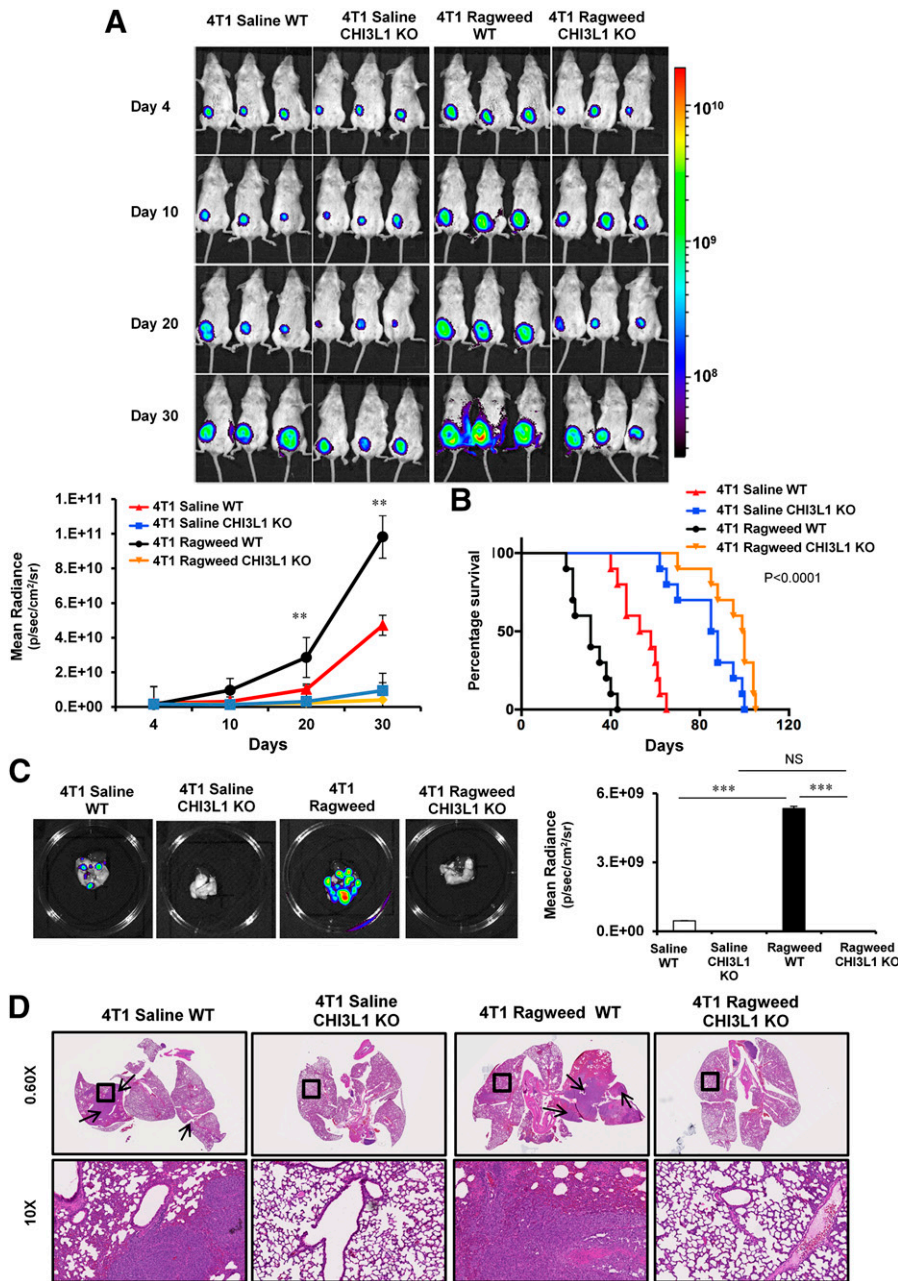


Figure 6. Tumor growth and metastasis is decreased in CHI3L1 KO mice. WT and CHI3L1 KO mice sensitized and challenged with ragweed allergen were implanted with 4T1 mammary tumor cells into mammary fat pads and tissue was harvested at day 45 after tumor cell inoculation. Representative ventral image of saline tumor and ragweed tumor bearers showing 10 mice/group with 3 independent experiments with total of 30 mice/group (A). Saline tumor bearers: WT (panel 1) and CHI3L1 KO (panel 2); ragweed tumor bearers: WT (panel 3) and CHI3L1 KO (panel 4). Quantification of bioluminescence signals is shown below. Kaplan-Meier survival curve showing increased survival in 4T1 KO mice (B). Bioluminescence image of excised lungs from 4T1 WT and 4T1 CHI3L1 KO mice indicating decreased metastasis in knockout mice (C). H&E staining of lung sections from 4T1 WT and 4T1 CHI3L1 knockout mice showing decreased metastasis in KO mice (original magnification, $\times 6$ and $\times 100$) (D). $N = 10$ mice/group; representative data of three independent experiments are presented as mean $\pm$ SD of samples. $**P \leq 0.001$, $***P \leq 0.0001$.

composition altered by pre-existing pulmonary inflammation in mammary tumor bearers might establish a premetastatic niche. Given that CHI3L1 is involved in both allergic inflammation and breast cancer, we explored the possibility that CHI3L1-associated inflammation might accelerate tumor progression.

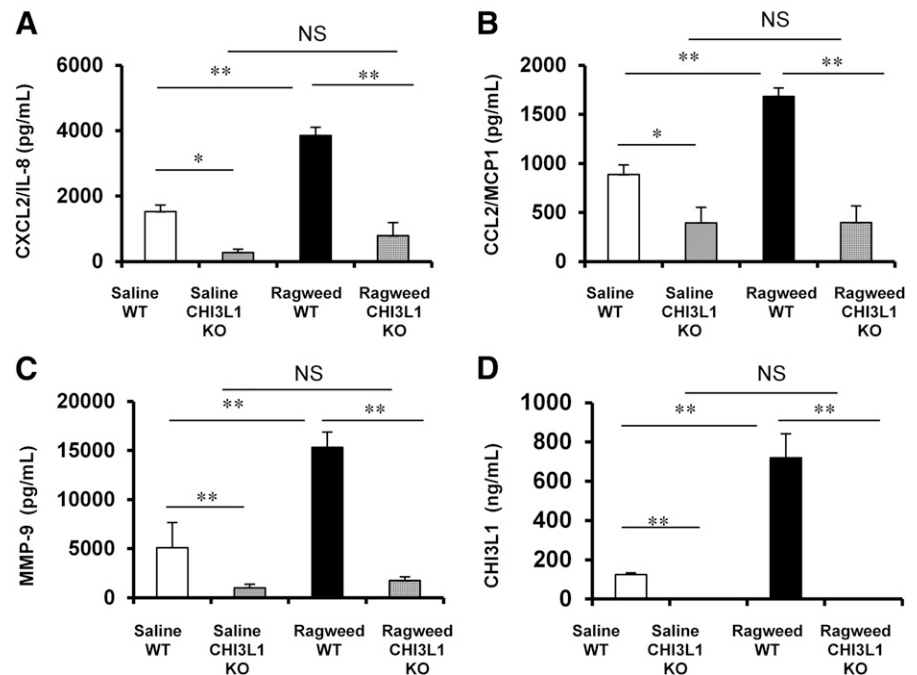
Lee et al. [7] established that CHI3L1 expression is increased in the lungs of mice exposed to an allergen. Elevated CHI3L1 expression has also been correlated with solid tumors such as glioblastoma, colorectal cancer, and breast cancer [16, 34, 35]. Our previous study examined the expression of CHI3L1 in the “premetastatic” lungs of 4T1 mammary tumor-bearing mice and found that it plays a role in establishing a niche for infiltrating mammary tumor cells [15]. In the present study, we linked

CHI3L1 expression in the pulmonary microenvironment to accelerated primary breast cancer tumor growth and metastasis to the lung. We found that pre-existing allergic pulmonary inflammation synergizes with tumor-induced inflammation to enhance CHI3L1 expression and increase primary tumor growth and metastasis. We found that CHI3L1 expression is progressively greater in 4T1-related tumor cells (least in 67NR and highest in 4T1) (Supplemental Fig. 2). These studies indicated that, irrespective of the tumor type, distant pre-existing inflammation can still have growth potentiating effects on tumor progression.

We have previously shown that CHI3L1 induces the production of CXCL2/IL-8, CCL2/MCP-1, and MMP-9 in the macrophages of 4T1 mammary tumor bearers [8]. CXCL2/IL-8

Total Lung homogenates

Figure 7. Proinflammatory/protumorigenic mediators are down-regulated in 4T1 CHI3L1 KO mammary tumor bearers. Whole lung homogenates were cultured for 24 h, and the cell-free supernatants were analyzed by ELISA for CXCL2/IL-8 (A), CCL2/MCP-1 (B), MMP-9 (C), and CHI3L1 (D) production. ELISA of $n = 10$ mice/group; representative data of three independent experiments are presented as mean $\pm$ sd of samples. * $P \leq 0.05$, ** $P \leq 0.001$.



and CCL2/MCP-1, in addition to their angiogenic activity, act as chemoattractant molecules that recruit, not only tumor cells, but also MDCs and macrophages that provide growth factors supporting the immigrant population of tumor cells [36–39]. In the present study, we have shown that MDCs and macrophages from 4T1 ragweed tumor bearers also produce high levels of proangiogenic chemokines, CCL2/MCP-1, CXCL2/IL-8, and CHI3L1, lending support to the accelerated tumor growth and metastasis. MDCs and macrophages are known to promote metastasis through secretion of MMP-9, which enhances the invasion and metastasis of tumor cells to the lungs [40–42]. It is possible that the increased levels of chemokines might attract leukocytes to the site of inflammation. These cells would now produce proinflammatory mediators that would further enhance the adverse effects on the tumor-bearing host.

In further characterizing the populations in the lungs, we found that pulmonary inflammation alters the distribution of myeloid populations in tumor bearers. The $CD11b^+Ly6G^+Ly6C^{mid}F4/80^-$ granulocytic cells were not altered by the presence of pulmonary inflammation. Previous studies have shown that this population does not contribute to immunosuppression in tumor-bearing mice [43, 44]. However, $CD11b^+Ly6G^-Ly6C^{mid}F4/80^+CD68^{hi}CD11c^+$ and $CD11b^+Ly6G^-Ly6C^-F4/80^+CD68^-CD11c^+$ are increased in ragweed tumor bearers, indicating that inflammation induces a trend toward a more mature macrophage phenotype. It is established that $F4/80^+CD68^{hi}CD11c^+$ are bona fide macrophages [45, 46]. Our results are consistent with other reports that inflammation plays a role in the accumulation of myeloid cells with immunosuppressive properties [44, 47].

In the present study, we have shown that CHI3L1 is up-regulated during ragweed allergen-induced pulmonary

inflammation before tumor cell inoculation, similar to what was observed by Lee et al. [7] using ova allergen sensitization. Lee et al. [7] showed that CHI3L1 KO mice had decreased inflammatory mediators in the circulation and pulmonary microenvironment. Recently, we have shown that CHI3L1 induces the expression of proinflammatory molecules in both interstitial and alveolar macrophages in the “premetastatic” lung that might contribute to an increased incidence of pulmonary metastasis [15]. That CHI3L1 KO mice exhibit decreased levels of proinflammatory/protumorigenic mediators with reduced tumor growth and metastasis further supports the role of CHI3L1 in promoting inflammation and tumor progression. Similar to our studies, Lee et al. [7] reported that ova-sensitized tumor-free CHI3L1 KO mice had decreased infiltration of myeloid populations. The effects of allergic pulmonary inflammation on the myeloid cell phenotype distribution were abrogated in 4T1 CHI3L1 KO mice. Additional studies are planned to characterize the functional role of these cells in our system.

We are the first to show that pre-existing allergic inflammation and CHI3L1 accelerates primary tumor growth and metastasis to the lungs. This was validated using CHI3L1 KO mice, which exhibit decreased primary tumor growth and metastasis even in the presence of pre-existing pulmonary inflammation. Given that breast cancer is a multifactorial disease, it is critical to understand how comorbidities such as allergic inflammation can influence the outcomes of patients with the disease. By use of a preclinical model that combines pre-existing pulmonary inflammation with mammary tumors, we have shed light on further understanding the links between these conditions to better design targeted therapies for cancer.

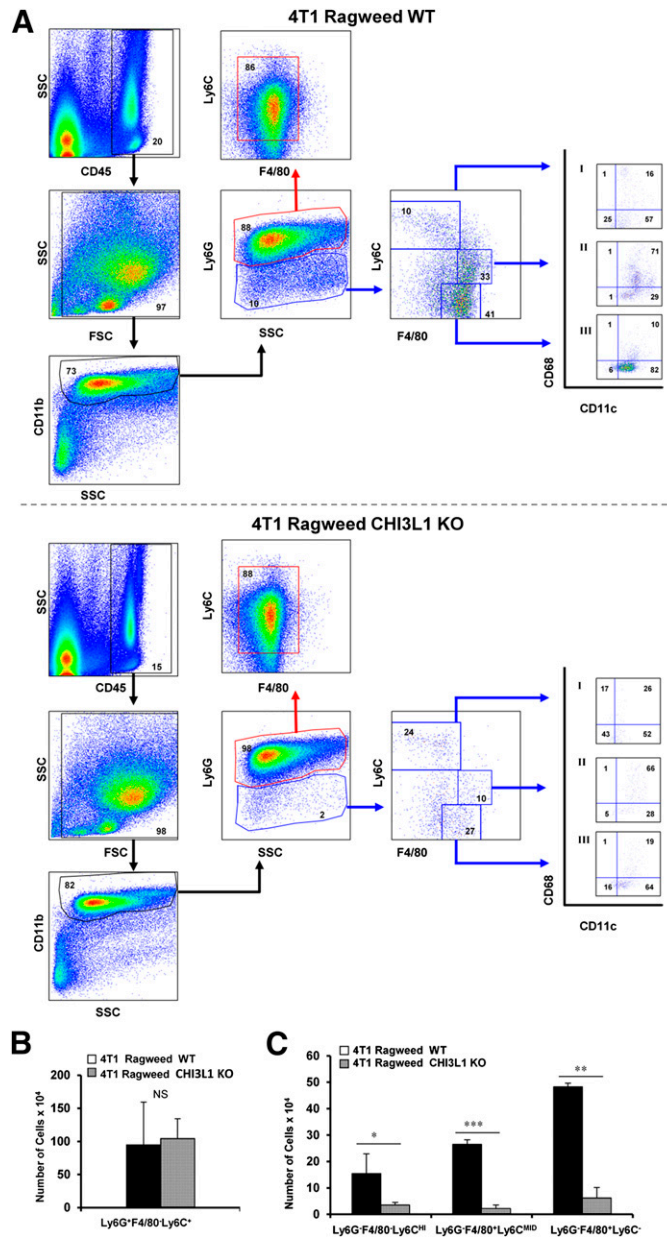


Figure 8. Flow cytometric analysis of single cell suspensions of total lung homogenates reveals a reversal of phenotype in ragweed 4T1 CHI3L1 KO mice. (A) Gating strategy. The numbers of cells in the ragweed 4T1 WT vs. ragweed 4T1 CHI3L1 KO mice were assessed. (B) Ly6G⁺ and (C) Ly6G⁻ subpopulations. *N* = 10 mice/group; representative data of three independent experiments are presented as mean ± SD of samples. ***P* ≤ 0.001, ****P* ≤ 0.0001.

AUTHORSHIP

S.L. as primary author, designed, conducted, and performed the experiments and prepared the manuscript; R.G.-A. conducted and performed some of the experiments; P.K. performed the flow cytometry studies; N.G. induced ragweed allergen pulmonary inflammation in mice and contributed to execution of the experiments; P.R. interpreted the histology results; A.H.

performed CHI3L1 knockout mouse studies; V.I.-C. performed experimental design and manuscript preparation.

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DISCLOSURES

The authors declare no competing financial interests.

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KEY WORDS:

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