Regulation of Carcinogenesis by IL-5 and CCL11: A Potential Role for Eosinophils in Tumor Immune Surveillance[1]

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The role of the immune system in the surveillance of transformed cells has seen a resurgence of interest in the last 10 years, with a substantial body of data in mice and humans supporting a role for the immune system in host protection from tumor development and in shaping tumor immunogenicity. A number of earlier studies have demonstrated that eosinophils, when recruited into tumors, can very effectively eradicate transplantable tumors. In this study, we investigated whether eosinophils also play a role in tumor immune surveillance by determining the incidence of methylcholanthrene (MCA)-induced fibrosarcomas in IL-5 transgenic mice that have greatly enhanced levels of circulating eosinophils, *CCL11* (eotaxin-1)-deficient mice that lack a key chemokine that recruits eosinophils into tissues, and the eosinophil-deficient mouse strains, *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA*. It was found that MCA-induced tumor incidence and growth were significantly attenuated in IL-5 transgenic mice of both the BALB/c and C57BL/6 backgrounds. Histological examination revealed that the protective effect of IL-5 was associated with massively enhanced numbers of eosinophils within and surrounding tumors. Conversely, there was a higher tumor incidence in *CCL11*$^{-/-}$ BALB/c mice, which was associated with a reduced eosinophil influx into tumors. This correlation was confirmed in the eosinophil-deficient *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* mouse strains, where tumor incidence was greatly increased in the total absence of eosinophils. In addition, subsequent in vitro studies found that eosinophils could directly kill MCA-induced fibrosarcoma cells. Collectively, our data support a potential role for the eosinophil as an effector cell in tumor immune surveillance. ***The Journal of Immunology,*** **2007, 178: 4222–4229.**

The requirement for a cooperative interaction between the innate and adaptive immune systems for effective tumor immunotherapy and tumor immune surveillance is widely recognized. Most importantly, the host immune status has been identified as critical in controlling the spread and growth of metastatic tumors (1). Until now, many tumor immune surveillance studies have focused on the role of the adaptive dendritic cell-driven CD8$^+$ CTLs response in tumor elimination following recognition of tumor Ags presented by MHC class I molecules on transformed cells. It should be noted, however, that the ability of the host to generate an effective innate immune response is fundamental to the generation of an adaptive CTL-response, and previous studies have confirmed the importance of macrophages and NK cells in this process (2). In addition, studies have highlighted the role of CD4$^+$ T cells in both the activation of innate effector cells in the primary response and subsequent cooperation with innate cells in tumor cell killing (3).

The role of Th2-mediated events in tumor immune surveillance and, in particular, the eosinophilic granulocyte, has not been thoroughly investigated. Eosinophils have been implicated in resistance to tissue-invasive parasitic helminths and in the pathology of atopic conditions including asthma and atopic dermatitis; however, the true immunological role(s) of this cell have yet to be elucidated. Eosinophils are commonly found in infiltrates of many different cancers (4), and controversy exists as to whether they are passive bystanders or active cellular agents in host immune responses. Several in vivo studies have found a strong link between tumor eradication and eosinophil recruitment. Studies by Tepper et al. (5) using IL-4-transfected tumor cell lines found that IL-4 displayed potent antitumor activity, primarily mediated by eosinophils and cooperative killing by macrophages. The antitumor effect of transplanted IL-4-secreting tumor cells was associated with the IL-4-induced local production of the eosinophil-specific chemokine, CCL11 (eotaxin-1) (6). Previous studies in our laboratory have demonstrated that tumor-specific CD4$^+$ T cells, exhibiting a cytokine profile characteristic of Th2 cells, were capable of clearing established lung and visceral metastases of a CTL-resistant melanoma, with degranulating eosinophils within the tumors inducing regression in a CCL11-dependent manner (7).

Expressed constitutively in many tissues (8), CCL11 is regarded as one of the most potent and specific effector molecules targeting eosinophils, its pleiotropic effects including the bone marrow maturation and release of eosinophils (9) and the directed trafficking of eosinophils into tissues (10). During Th2-mediated inflammatory conditions, epithelial cells and fibroblasts provide the main source of CCL11. In contrast, IL-5 is

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produced by activated T lymphocytes, mast cells, and NK cells, and has several functions in eosinophil biology. Although initially identified as a B cell growth and differentiation factor (11), studies in vitro (12) and in IL-5 transgenic (Tg)[3] (13) and *IL-5*$^{-/-}$ mice (14) have established that the major role for IL-5 is in eosinophilopoiesis (12), in eosinophil trafficking and survival (15), and in eosinophil activation, and the subsequent release of cytotoxic granule proteins (16). In the present study, we were interested in investigating the role of Th2-mediated signals and eosinophils in tumor immune surveillance, using the methylcholanthrene (MCA)-induced fibrosarcoma model. To address this question, we used genetically modified mice known to express low and high endogenous levels of eosinophils, namely *CCL11*$^{-/-}$ (17) and IL-5 Tg mice (13), and the eosinophil-deficient mouse strains, *IL-5*/*CCL11*$^{-/-}$ and Δ*dblGATA*.

Notably, IL-5 mRNA expression, regulated by the dominant controlling region of the human *CD2* gene in IL-5 Tg mice, has been determined to be restricted to s.c. lymph nodes, the thymus, bone marrow, and Peyer's patches, with no mRNA expression detected in the skeletal muscle, heart, or brain (13). The resulting phenotype of the IL-5 Tg mouse is characterized by 25–50% eosinophils in the circulating leukocyte pool (compared with <3% in wild type (WT)), and elevated levels of eosinophils in the spleen, bone marrow, and peritoneal cavity (13), thus providing an ideal model for studying the role of these cells in tumor immune surveillance. In stark contrast, the targeted disruption of the *CCL11* gene results in the impaired recruitment of eosinophils, highlighted by a 2- to 3-fold decrease in circulating eosinophils in naive and allergic mice when compared with WT cohorts (17). In addition to the impaired eosinophil recruitment model, we used the eosinophil-deficient *IL-5*/*CCL11*$^{-/-}$ and Δ*dblGATA* mouse strains. Deletion of the high-affinity GATA-binding site in the *GATA-1* promoter has been found to lead to the selective loss of the eosinophil-lineage (18) and a subsequent reduction in airways remodeling associated with chronic asthma (19). Mice deficient in both IL-5 and CCL11 provide another useful model for eosinophil deletion, being eosinopoiesis-deficient and eosinophil recruitment impaired, resulting in no blood or tissue eosinophilia in response to allergic modeling (20).

In this study, a significant reduction in tumor establishment and growth was identified in IL-5 Tg mice that directly correlated with a high level of eosinophil recruitment to both the tumor and surrounding connective tissue. This result was substantiated by an elevated tumor incidence and reduced influx of eosinophils into tumors observed in *CCL11*$^{-/-}$ mice of the BALB/c background. Furthermore, studies in eosinophil-deficient *IL-5*/*CCL11*$^{-/-}$ and Δ*dblGATA* mice confirmed the correlation of high tumor incidence with an absence of eosinophils within tumors and the surrounding connective tissue. These novel observations suggest that the role of Th2-derived cytokines and, in particular, of eosinophils warrants further investigation to clarify their role in tumor immune surveillance.

Materials and Methods

Mice

Six- to 9-wk-old BALB/c and C57BL/6 (B6) WT male mice were obtained from the specific pathogen-free facilities of either the John Curtin School of Medical Research (Australian National University, Canberra, Australia) or the Walter and Eliza Hall Institute of Medical Research. The following genetically modified mice, all 6- to 9-wk-old males, were bred and maintained by the Gene Targeting Facility at the John Curtin School of Medical Research and the Peter MacCallum Cancer Centre: *CCL11*$^{-/-}$ mice (17) backcrossed to a BALB/c or C57BL/6 background for 10 and 11 generations, respectively; BALB/c and C57BL/6 IL-5 Tg mice (13, 21) backcrossed to their respective backgrounds for 20 generations; BALB/c Δ*dblGATA* mice backcrossed for 10 generations, followed by four intercrosses; and BALB/c *IL-5*/*CCL11*$^{-/-}$ mice backcrossed for 20 generations as IL-5$^{-/-}$ followed by backcrossing with *CCL11*$^{-/-}$ mice for an additional 10 generations. Mice were treated according to the Australian National University and Peter MacCallum Cancer Centre Animal Welfare guidelines and were housed in an approved containment facility.

Fibrosarcoma induction by MCA

BALB/c and B6 WT, CCL11$^{-/-}$ and IL-5 Tg, and BALB/c *IL-5*/*CCL11*$^{-/-}$ and Δ*dblGATA* mice were inoculated s.c. in the left hind leg with 0.1 ml of corn oil containing 1–400 μg of MCA (Sigma-Aldrich) and monitored weekly over a 26-wk period for the establishment and growth of fibrosarcomas. Mean tumor size was calculated as the product of the two perpendicular axes of the tumor (cm^2), measured using Vernier calipers. Small protrusions (<2 mm in diameter), histologically determined to be encapsulated MCA, were evident in the majority of mice in all low-dose (e.g., 5 μg) MCA groups. Therefore, protrusions >4 mm in diameter and demonstrating progressive growth over three or more weeks were recorded as fibrosarcoma positive. Mice were sacrificed before fibrosarcomas reaching 1.5 cm^2 or, alternatively, at the conclusion of the 180-day experiment. Fibrosarcomas were aseptically removed and fixed in 10% phosphate-buffered formalin for 72 h before histological staining.

Histological analysis

Fibrosarcomas were sectioned and stained with Carbol's chromotrope-hematoxylin for identification of eosinophils. Eosinophils were quantified (10 fields/section, one section/animal at ×400 magnification) and represented as the mean ± SEM of five mice from each group unless otherwise specified. Photomicrographs of histological preparations were taken at ×100 to ×400 magnification (as specified for each figure) using a BX50 microscope with CCD capability (Olympus).

Lectin perfusion and vascular staining

Fluorescein (FITC)-labeled lectin (*Lycopersicon esculentum*; Sigma-Aldrich) perfusion was performed as described previously (22). Briefly, mice were anesthetized, injected i.v. with 50 μg of FITC-labeled lectin, which was allowed to circulate for 10 min, and heart perfusion was performed with 10% zinc-buffered formalin. Tumors were removed, fixed for 4 h in 4% paraformaldehyde, processed through 30% sucrose, and then embedded in Tissue-Tek OCT freezing medium (Sakura Finetek). Sections (35 μm) were cut and lectin staining was visualized using the Olympus fluorescence microscope.

Measurement of in vitro eosinophil-mediated tumor eradication

Preparation of eosinophils. OVA-activated eosinophils were generated in BALB/c IL-5 Tg mice by i.p. sensitization with 50 μg of OVA/10% aluminum hydroxide in normal saline on day 0, followed by i.p. challenge with 50 μg of OVA in normal saline on days 12, 13, and 14 before harvesting eosinophils on day 15. Eosinophils were harvested from the peritoneal cavity by washing the cavity with HBSS and purified by sorting on a BD FACSVantage (BD Biosciences) based on forward and side scatter parameters and polarized light. The purity of the eosinophil-enriched population was >95% as determined by differential staining with May-Grüwald Giemsa.

Coculture of eosinophils with MCA-induced fibrosarcoma cells. To measure eosinophil cytotoxic activity against MCA-induced fibrosarcoma cells in vitro, fibrosarcoma cells were seeded into a 96-well plate at 4.15 × 10^3 cells/well in F15/10% FCS and grown for 20–24 h before coculturing with sort-purified eosinophils in RPMI 1640/10% FCS alone or supplemented with PMA (10^{-7} M) at various E:T ratios. Thirty hours after coculture, 0.5 μCi of tritiated ([^{3}H])-thymidine was added to each culture well in 20 μl of RPMI 1640/10% FCS and [^{3}H]thymidine incorporation measured for 18 h. At the end of the culture period, cell culture plates were centrifuged and cell supernatants discarded. Cells were washed once with PBS, then deadhered using 100 μl/well trypsin/EDTA at 37°C for 7 min. Cell cultures were then stored at −20°C until harvesting.

Measuring [^{3}H]thymidine incorporation. Using a Filtermate 196 harvester, cell cultures were harvested onto glass fiber filters (Packard Bioscience). Filters were dried and placed in Omnifilter plates, 20 μl of Microscint-O scintillation fluid was added to each well, and the plate was sealed with TopSeal-A adhesive film (PerkinElmer). [^{3}H]Thymidine incorporation was measured using a TopCount NXT (PerkinElmer).

[3] Abbreviations used in this paper: Tg, transgenic; MCA, methylcholanthrene; WT, wild type; HPF, high-powered field.

Statistical analysis

Statistically significant differences were analyzed by using the Mann-Whitney *U* test for tumor growth rate data, with $p < 0.05$ considered as significant. A paired Student's *t* test was used to calculate the statistical significance of the tumor incidence data. Significant differences between the eosinophil contents of tumors were calculated using unpaired Student's *t* tests.

Results

IL-5 overexpression protects mice from MCA-induced fibrosarcoma establishment and growth

We were interested in the potential role for Th2-mediated events, in particular eosinophils, in tumor immune surveillance. The IL-5 Tg mouse was used in these studies due to its high constitutive level of circulating eosinophils, whereas *CCL11*$^{-/-}$ mice were used to determine the impact of impaired eosinophils recruitment. Initially, we examined the effect of MCA dose on tumor incidence in WT, IL-5 Tg, and *CCL11*$^{-/-}$ BALB/c mice (Fig. 1*A*). It was noted that, across the MCA dose range examined, IL-5 Tg mice were uniformly more resistant to MCA-fibrosarcoma induction compared with WT mice, with a 50% reduction in tumor incidence in the IL-5 Tg group at the 100 μg MCA dose. In contrast, *CCL11*$^{-/-}$ mice were consistently more sensitive to chemical carcinogenesis than their WT controls. Indeed, at the 100 μg dose 90% of *CCL11*$^{-/-}$ mice, compared with only 60% of WT mice, had developed fibrosarcomas during the course of the 24 wk of the experiment. Furthermore, at the lowest MCA dose tested (1 μg), only *CCL11*$^{-/-}$ mice developed tumors. We next examined larger cohorts of mice at a low dose of MCA (5 μg) to confirm statistical significance and to elucidate any genetic background-specific effects by using mice with either the Th2-oriented BALB/c or Th1-oriented C57BL/6 backgrounds. It was confirmed that only 6% (1 of 16) of BALB/c IL-5 Tg mice and 20% (4 of 19) of C57BL/6 IL-5 Tg mice developed progressively growing fibrosarcomas compared with 40% (8 of 20) of BALB/c WT and 65% (13 of 20) of C57BL/6 WT controls during the 24-wk experiment (Fig. 1*B*). The difference in tumor incidence between IL-5 Tg and WT groups of both genetic backgrounds was found to be highly significant ($p < 0.001$) from wk 16 in the BALB/c background and wk 18 in C57BL/6 mice (Fig. 1*B*). Furthermore, the onset of fibrosarcomas was considerably delayed in BALB/c IL-5 Tg mice, being wk 18 in BALB/c IL-5 Tg mice compared with wk 12 in the syngeneic WT group. In stark contrast, the BALB/c *CCL11*$^{-/-}$ cohort displayed a significant ($p = 0.017$) increase in tumor incidence (72%, 13 of 18 mice) and an earlier onset of fibrosarcoma development (ie, wk 10). Interestingly, no significant change in tumor incidence or onset was observed in C57BL/6 *CCL11*$^{-/-}$ mice (Fig. 1*B*), implying that the genetic background of mice influences the effect of CCL11 deficiency.

Further evaluation of tumor progression highlighted a strong reduction in the growth rate of MCA-induced fibrosarcomas (5 μg dose) with IL-5 Tg mice of both genetic backgrounds (Fig. 2*A*). Mean growth rates were determined by comparing the primary fibrosarcoma growth phase of individual tumors in each group and representative trend lines were produced. Using the Mann-Whitney *U* test, it was found that, irrespective of the genetic background (ie, BALB/c or C57BL/6), the growth rate of MCA-induced tumors in the IL-5 Tg mice was significantly slower than in WT or *CCL11*$^{-/-}$ mice (Fig. 2*B*). In contrast, when comparing the fibrosarcoma growth rates over the entire time course of the experiment, no significant differences were observed between the growth rates of the tumors induced in the *CCL11*$^{-/-}$ and WT groups. However, once tumors reached 0.5 cm^2 in size, their growth rate was substantially (28%) faster in BALB/c *CCL11*$^{-/-}$ mice than in WT BALB/c mice ($p < 0.05$). The tumor growth rate curves (Fig. 2*A*) also highlight the slow progress of tumor development within the IL-5 Tg mice of both backgrounds. In particular, the BALB/c IL-5 Tg group displayed several tumors (3 of 16) exhibiting the arrested growth characteristic of tumor dormancy and in one mouse (1 of 16), tumor regression. To further highlight the growth retardation of these tumors, ex vivo cultures of fibrosarcoma

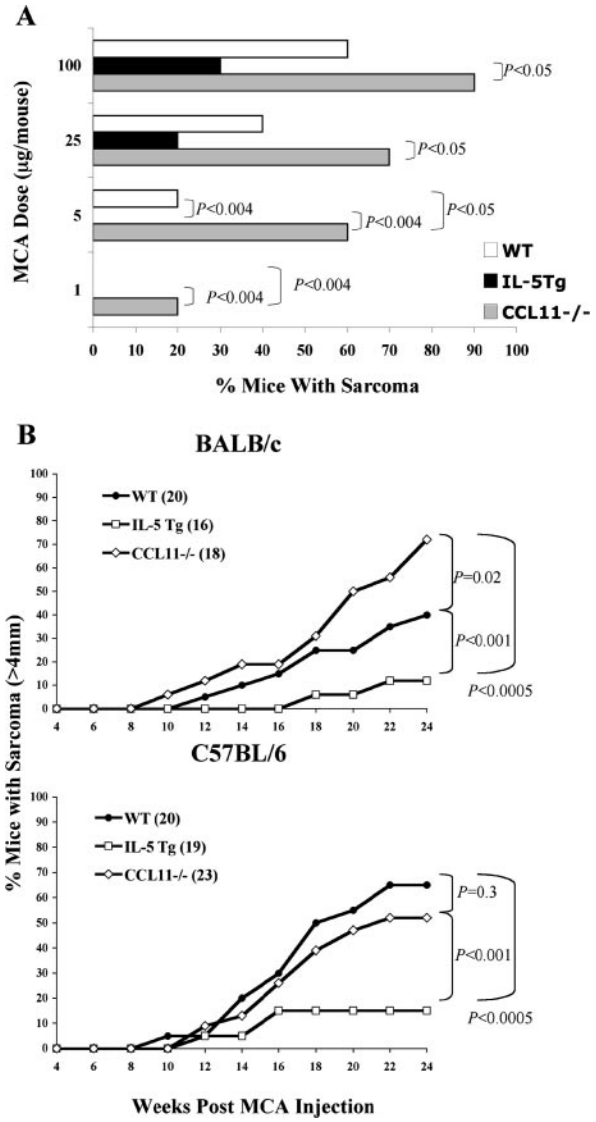
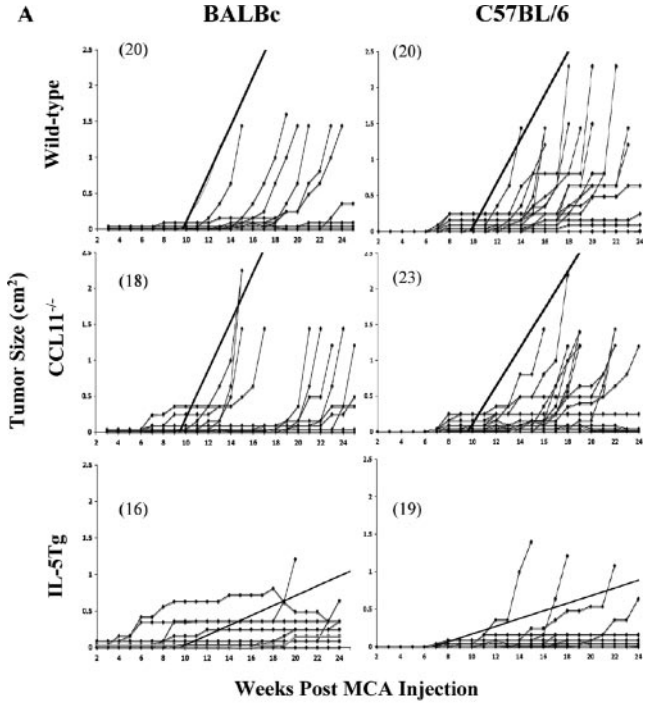


FIGURE 1. Overexpression of IL-5 protects mice from MCA-induced fibrosarcomas, whereas lack of CCL11 renders animals more susceptible to MCA-induced carcinogenesis. *A*, Dose-dependent increase in incidence of MCA-induced fibrosarcomas is limited in IL-5 Tg BALB/c mice and increased in *CCL11*$^{-/-}$ BALB/c mice relative to syngeneic WT animals. Groups of WT, *CCL11*$^{-/-}$, and IL-5 Tg BALB/c mice were injected s.c. in the hind flank with 1–100 μg of MCA diluted in 0.1 ml of corn oil and observed weekly for 24 wk for the development of tumors. Tumor incidence at 24 wk was recorded as a percentage of tumor-positive mice in each group. Significant differences between genetically modified groups and WT were determined at 24 wk by a Fisher's exact test as shown. *B*, Incidence of fibrosarcoma development over time in IL-5 Tg and *CCL11*$^{-/-}$ mice with BALB/c and C57BL/6 backgrounds. Mice were injected s.c. in the hind flank with 5 μg of MCA diluted in 0.1 ml of corn oil and observed weekly for tumor development over the course of 24 wk. Significant differences between genetically modified groups and WT were determined for the entire time course by a paired Student's *t* test. Number of mice in each treatment group is shown in parentheses.



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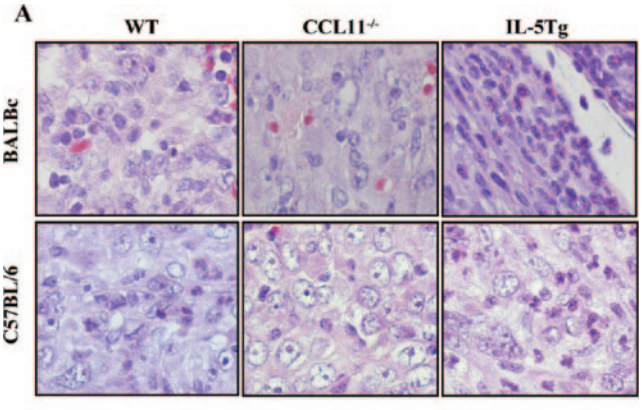
Background	Strains	P value
BALB/c	CCL11$^{-/-}$ vs WT	0.65
	CCL11$^{-/-}$ vs IL-5Tg	***0.03***
	IL-5Tg vs WT	***0.05***
C57BL/6	CCL11$^{-/-}$ vs WT	0.73
	CCL11$^{-/-}$ vs IL-5Tg	***0.0001***
	IL-5Tg vs WT	***0.0001***

FIGURE 2. Reduced growth rates of MCA-induced tumors in IL-5 Tg mice. Groups of WT, *CCL11*$^{-/-}$, and IL-5 Tg mice (number of mice in parentheses) of BALB/c and C57BL/6 background were injected s.c. in the hind flank with 5 μg of MCA diluted in 0.1 ml of corn oil and observed weekly over 24 wk for tumor growth. *A*, Tumors >2 mm in diameter were measured weekly and the size was calculated as the product of two perpendicular diameters (cm^2). Each line with ♦ represents data for an individual animal, whereas the lines of best fit for each group (demonstrating mean growth rate) are represented by a solid trend-line. *B*, Statistical analysis of differences between trend lines was tested with the Mann-Whitney *U* test.



B

Background	Eosinophil No./10 HPF		
	Wild-type	CCL11$^{-/-}$	IL-5Tg
BALB/c	9±3	6±1	*186*
C57BL/6	7±1	9±2	*67±2* *

FIGURE 3. Eosinophil content of MCA-induced tumors from WT, CCL11$^{-/-}$, and IL-5 Tg mice. Fibrosarcomas induced by 5 μg of MCA (Fig. 1*B*) in WT, CCL11$^{-/-}$, and IL-5 Tg mice of BALB/c and C57BL/6 background were excised at 24 wk and examined histologically for eosinophil content. *A*, Representative fields from the fibrosarcomas were stained using Carbol's chromotrope-hematoxylin for the identification of eosinophils (×400 magnification). *B*, Eosinophil content of tumors from different experimental groups were quantified, and data are presented as mean eosinophil number per 10 high-powered fields (HPFs) ± SEM of tumors from five mice of each WT and CCL11$^{-/-}$ group being examined. In the IL-5 Tg groups, only progressive tumors were examined, hence one mouse of BALB/c and four mice of C57BL/6 background were assessed. Significant differences between groups were calculated using an unpaired Student's *t* test (*, $p < 0.001$).

lines from IL-5 Tg mice were extremely difficult to propagate compared with the rapid growth of fibrosarcoma lines derived from the tumors of WT and *CCL11*$^{-/-}$ mice (data not shown).

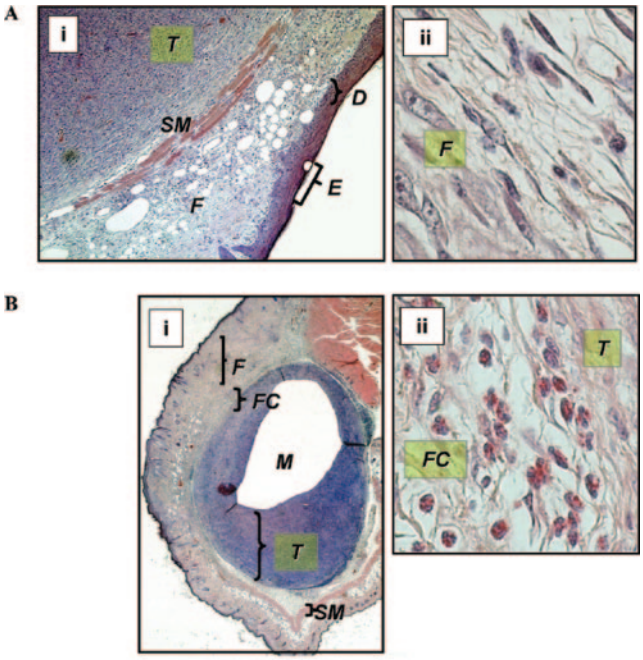
Tumor-associated eosinophilia correlates with IL-5-mediated tumor suppression

Consistent with observations made in many human tumors (4) and murine tumor studies, histological examination revealed the presence of a small population of eosinophils in the tumors of WT mice of both the BALB/c and C57BL/6 backgrounds. When compared with syngeneic WT mice, tumor-infiltrating eosinophil numbers were increased 20-fold in the BALB/c IL-5 Tg progressive tumor and up to 9-fold in the C57BL/6 IL-5 Tg tumors (Fig. 3*B*; *, $p < 0.001$ compared with WT), revealing a strong correlation with reduced tumor incidence (Fig. 1), delayed tumor establishment (Fig. 2), and slower growth rate of tumors in these mice (Fig. 2). Conversely, although not quite statistically significant, eosinophil numbers were reduced by approximately one-third in BALB/c *CCL11*$^{-/-}$ tumors (Fig. 3*B*), whereas, consistent with no significant change in tumor incidence (Fig. 1*B*), minimal change was detected in eosinophil numbers in the tumors of C57BL/6 *CCL11*$^{-/-}$ mice when compared with WT syngeneic mice (Fig. 3*B*).

The presence of residual eosinophils in the tumors of *CCL11*$^{-/-}$ cohorts of both genetic backgrounds (Fig. 3, *A* and *B*) suggests that other chemokines/cytokines are also involved in the recruitment of eosinophils in the tumor environment. However, the relative reduction in eosinophil numbers observed in the tumors of the BALB/c *CCL11*$^{-/-}$ group (Fig. 3*B*), when compared directly to the tumors of WT BALB/c mice, correlates with the increase in tumor incidence and more rapid growth rate of established tumors observed in this group (Fig. 1*B*). In contrast, the maintenance of eosinophil numbers equivalent to WT levels in the C57BL/6 *CCL11*$^{-/-}$ tumors directly correlates with the lack of a significant change in tumor incidence and growth rate in these mice (Fig. 1*B*). Collectively, these data suggest that differences in the tumor incidence in *CCL11*$^{-/-}$ BALB/c and C57BL/6 mice is due to the relative importance of CCL11 in recruiting eosinophils into tissues in these two mouse strains rather than the Th2-oriented nature of the BALB/c background vs the Th1-oriented nature of the C57BL/6 background.

Persistent tumor encapsulation and ongoing immune surveillance in IL-5 Tg mice

Low-dose (5 μg) MCA BALB/c mice of the WT, *CCL11*$^{-/-}$, and IL-5 Tg backgrounds displayed varying levels of MCA encapsulation in the first 4 wk after MCA injection (data not shown). The majority of mice in the WT and *CCL11*$^{-/-}$ groups that had initially displayed partial MCA encapsulation proceeded to develop rapidly outgrowing fibrosarcomas, with tumor cells penetrating the surrounding connective tissue layers to the overlying epidermis



C

BALB/c	Eosinophil No./10 HPF	
Strain/Tumor Types	Tumor Tissue	Connective Tissue
WT Progressive	9±3	16±5
IL-5Tg Progressive	186	465
IL-5Tg Non-Progressive	*222±13* *	*1605±138* *

FIGURE 4. Tumor encapsulation and poor tumor outgrowth is evident in IL-5 Tg BALB/c mice following low-dose (5 μg) MCA inoculation. Mice were injected s.c. in the hind flank with 5 μg of MCA diluted in 0.1 ml of corn oil, and MCA-induced fibrosarcomas were monitored over the 24 wk of the experiment. Upon completion of the experiment (wk 24), poorly growing fibrosarcomas from the IL-5 Tg BALB/c group were histologically examined and compared with WT and "progressive" IL-5 Tg tumors following staining with Carbol's chromotrope-hematoxylin. *A*, Representative tumor in BALB/c WT mice viewed at ×100 (*i*) and surrounding connective tissue at ×400 (*ii*) magnification (F, fibroblasts and connective tissue; SM, smooth muscle; D, dermal tissue; E, epidermal tissue; T, tumor tissue). *B*, Encapsulated "nonprogressive" fibrosarcoma representative of 4 of 16 mice in the BALB/c IL-5 Tg group (5 ± 1 mm diameter) viewed at ×40 (*i*) and surrounding fibrotic capsule at ×400 (*ii*) magnification (FC, fibrotic capsule; F, fibroblast and connective tissue; M, MCA injection site; SM, smooth muscle; T, tumor). Carbol's chromotrope-stained eosinophils appear with distinctive red cytoplasmic granules in (*ii*). *C*, Eosinophil influx was compared between BALB/c WT, progressive IL-5 Tg, and nonprogressive IL-5 Tg tumors from the 5 μg of MCA groups. Eosinophils were quantified (10 fields/tumor/four mice per group at ×400 magnification) within the tumor and surrounding connective tissue and represented as mean per 10 HPFs ± SEM of four mice. Significant differences between groups were calculated using an unpaired Student's *t* test (*, $p < 0.001$).

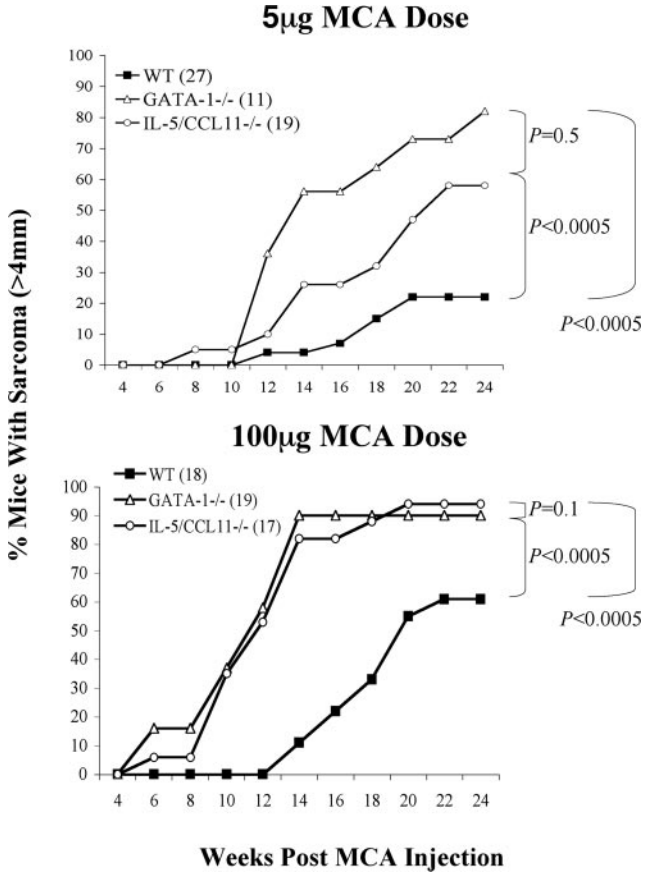


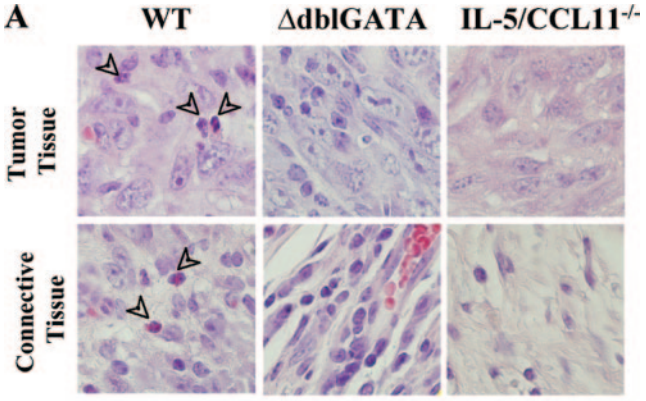
FIGURE 5. Eosinophil depletion in *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* mice is associated with increased susceptibility to MCA-induced carcinogenesis. A higher incidence of MCA-induced fibrosarcomas is observed in *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* BALB/c mice relative to syngeneic WT animals at both low and high doses of MCA. Groups of WT, *IL-5/CCL11*$^{-/-}$, and Δ*dblGATA* BALB/c mice were injected s.c. in the hind flank with 5 or 100 μg of MCA diluted in 0.1 ml of corn oil and observed weekly for 24 wk for the development of tumors. Tumor incidence over the 24 wk was recorded as the percentage of tumor-positive mice in each group. Significant differences between groups were determined for the entire time course using a paired Student's *t* test as shown.

(Fig. 4*Ai*) and underlying skeletal muscle (data not shown). Histological analysis revealed a low level of infiltrating eosinophils and other leukocytes (Fig. 4*Aii*). This result suggests that any protective effect mediated by the surrounding connective and fibrous tissue and associated recruited innate inflammatory cells was limited by the host's inability to eradicate cells transformed progressively by the carcinogen.

In contrast, in the IL-5 Tg groups encapsulation persisted throughout the 24 wk of the experiment. Upon histological analysis (Fig. 4*Bi*) it was revealed that in 4 of 16 BALB/c IL-5 Tg mice, the persistent fibrotic capsule (size 5 ± 1 mm) contained a high influx of eosinophils and other leukocytes (Fig. 4*Bii*) occupying ~70% of the encapsulated area, surrounding a small cluster of tumor cells. Tumor growth had been arrested, and in one case regression induced, from the time of capsule formation at 6–12 wk throughout the course of the experiment (Fig. 3). As previously mentioned, we were unable to successfully culture cell lines from these dormant IL-5 Tg mouse "tumors," suggesting poor cellular growth in vivo and ex vivo. The most striking feature of these lesions was a persistently high level of eosinophils within both the tumor stroma and surrounding connective tissue (Fig. 4*B*). Eosinophil numbers in the connective tissue (fibrotic capsule) surrounding the nonprogressive tumor were found to be 100-fold higher than observed in WT syngeneic mice and 3.5-fold higher than the progressive IL-5 Tg BALB/c tumor. Visualization of tumor vasculature using FITC-lectin perfusion showed no significant differences between WT and nonprogressive tumors in the IL-5 Tg strain (data not shown).

Eosinophil deficiency correlates with an increased tumor incidence

To further investigate the potential role of the eosinophil in tumor immune surveillance, the eosinophil-deficient *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* BALB/c mutant strains were treated with MCA to



B

Background	Eosinophil No./10 HPF		
	Wild-type	IL-5/CCL11$^{-/-}$	dblGATA
Tumor Tissue	4±1	*0*	*0*
Connective Tissue	15±8	*0*	*0*

FIGURE 6. Eosinophil content of MCA-induced tumors from WT, *IL-5/CCL11*$^{-/-}$, and Δ*dblGATA* mice. Fibrosarcomas induced by 5 μg of MCA (Fig. 5) in WT, *IL-5/CCL11*$^{-/-}$, and Δ*dblGATA* mice of BALB/c background were excised at 24 wk and examined for eosinophil content. *A*, Representative fields from the fibrosarcomas were stained using Carbol's chromotrope-hematoxylin for the identification of eosinophils (×400 magnification). Representative eosinophils are indicated by arrows. *B*, Eosinophil content of tumors from different experimental groups were quantified, and data are presented as mean eosinophil number per 10 HPFs ± SEM of tumors from five mice of each group examined.

induce fibrosarcomas. At the 5 and 100 μg dose of MCA, both strains showed a significant increase ($p < 0.005$) in tumor incidence when compared with syngeneic WT controls (Fig. 5). Indeed, at the 5 μg dose, 58% of the *IL-5/CCL11*$^{-/-}$ and 82% of the Δ*dblGATA* mice had developed fibrosarcomas during the 24-wk experiment, compared with an incidence of only 22% in WT mice. In addition, 90 and 94% of the *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* mice, respectively, had developed tumors at the 100 μg MCA dose compared with only 61% of the WT cohort. Although there was no significant difference in tumor incidence between the *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* cohorts at either 5 or 100 μg MCA, the Δ*dblGATA* mice were particularly sensitive to MCA treatment, with tumor incidence being extremely high at both the low and high MCA doses, at 82 to 90%, respectively.

A histological examination was performed on the connective tissue surrounding the tumors, the tumor tissue, and necrotic cores (data not shown), confirming a low infiltration of eosinophils throughout these regions in all WT mice (Fig. 6). However, no eosinophils were observed in these regions at either the 5 or 100 μg MCA dose in the *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* cohorts (data not shown), revealing a strong correlation with reduced tumor incidence in these groups (Fig. 5). As seen in the first series of experiments (Fig. 1), WT mice exhibited MCA encapsulation in the first 4 wk after MCA injection (data not shown), whereas several animals in the *IL-5/CCL11*$^{-/-}$ and Δ*dblGATA* groups displayed MCA-encapsulation, characterized by a less severe protrusion and thinner fibrotic capsule (data not shown), which progressed to develop into rapidly outgrowing fibrosarcomas.

Eosinophils can directly mediate fibrosarcoma killing in vitro

In conjunction with in vivo immune surveillance experiments, the ability of eosinophils to mediate direct killing of fibrosarcoma cells in vitro was investigated. A fibrosarcoma cell line derived from a MCA-induced tumor, following the administration of 5 μg of MCA in a WT BALB/c mouse, was used as the target in these experiments, whereas OVA-stimulated eosinophils mimicking a Th2-primed tumor environment were used as effector cells. PMA-stimulated OVA-activated eosinophils were able to instigate an almost complete eradication of MCA-fibrosarcoma cells at target ratios of 25:1 and above (Fig. 7, *Aiii* and *Aiv* and *B*). In addition, unstimulated OVA-activated eosinophils also illustrated effective fibrosarcoma killing (Fig. 7*Aii*), with only 60-40% of fibrosarcoma cells remaining viable following coincubation with eosinophils at E:T ratios of 50:1 and 100:1, respectively (Fig. 7*B*). These results confirm that eosinophils can mediate the direct killing of MCA-induced fibrosarcoma cells.

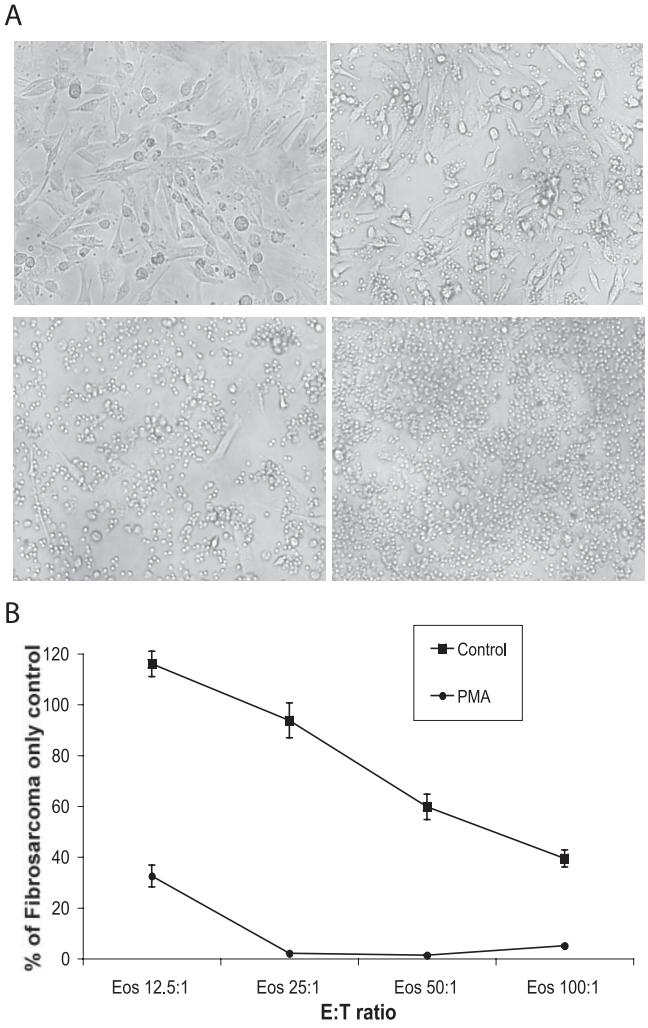


FIGURE 7. In vitro eosinophil-mediated killing of MCA-induced fibrosarcoma cells. A MCA-fibrosarcoma cell line, derived from a tumor established in a WT BALB/c mouse following 5 μg of MCA administration, was used to investigate the ability of eosinophils to mediate fibrosarcoma cell death. *A*, Bright field images (×400 magnification) illustrate fibrosarcoma viability following various treatments: *i*, fibrosarcoma alone; *ii*, fibrosarcoma with unstimulated OVA-activated eosinophils at an E:T ratio of 25:1; *iii*, fibrosarcoma with PMA (10^{-7} M)-stimulated OVA-activated eosinophils at an E:T ratio of 25:1; *iv*, fibrosarcoma with PMA (10^{-7} M)-stimulated OVA-activated eosinophils at an E:T ratio of 50:1. *B*, Fibrosarcoma cell viability was quantified using [^{3}H]thymidine incorporation following fibrosarcoma treatment with unstimulated or PMA-stimulated OVA-activated eosinophils at E:T ratios of 12.5:1, 25:1, 50:1, and 100:1.

Discussion

The data reported in this study provides in vivo and in vitro evidence that eosinophils play an important role in limiting carcinogenesis and/or the growth of chemically induced tumors. We hypothesize that the initial tissue damage incurred by the MCA injection in BALB/c WT, *CCL11*$^{-/-}$, IL-5 Tg, *IL-5*/*CCL11*$^{-/-}$, and Δ*dblGATA* mice alike, induces a wound-healing inflammatory response, inducing the deposition of extracellular matrix to form the fibrotic capsule and supporting a strong influx of innate effector cells (23). In WT, *CCL11*$^{-/-}$, *IL-5*/*CCL11*$^{-/-}$, and Δ*dblGATA* mice, this initial response is not sufficient to contain malignant transformation, nor for the induction of an appropriate immune surveillance response, and mutating cells rapidly overwhelm the host. However, in the IL-5 Tg mice, this initial inflammatory response favors the establishment and maintenance of an appropriate eosinophil-mediated antitumor response. The fibrotic capsule surrounding the carcinogen, containing the extracellular matrix and associated glycosaminoglycans, provides the scaffold for the formation of a strong IL-5 chemotactic gradient and maintenance of eosinophil-mediated tumor eradication (24). Hence, eosinophils may also play a role in enhancing the MCA-encapsulation process, restricting the contact of surrounding subepithelial tissue with MCA and resulting in a subsequent reduction in cellular mutations, as well as playing an active role in the ongoing immune surveillance process.

Hence, the relationship between eosinophils and other immune cells known to be involved in tumor immune surveillance is of considerable interest. In addition, the signals required for the recruitment of eosinophils into the tumor environment and the subsequent mechanisms used by eosinophils to induce tumor cell death warrant further investigation. Although eosinophil recruitment into the tumor may be partially dependent on the basal expression of eosinophil-specific chemokines, including CCL11 (8), from the fibrosarcoma itself, both the innate and adaptive immune responses are likely to be involved in eosinophil recruitment and activation. The potential cooperation between eosinophils and other innate cells, namely NK cells, NKT cells, and macrophages in eosinophil recruitment, is of particular interest. The role of NK cells in tumor immune surveillance, including MCA-induced fibrosarcomas, is well characterized (2, 25). NK cells mediate spontaneous cytotoxicity against MHC class I-deficient tumor cells and are known to produce the Th2-mediated cytokines IL-4 and IL-5 (26), thus providing strong eosinophil-specific chemoattractants and activation signals within the tumor environment. In the absence of a concurrent influx of mast cells into MCA-induced tumors in IL-5 Tg mice (data not shown), CD2^{+} cells (27), potentially including T and NKT cells, and also subsets of NK cells, monocytes, and B cells, are the most likely sources of IL-5.

Once recruited into the tumor, eosinophils have the ability to harness both direct and indirect mechanisms of tumor lysis. It has been well characterized in allergic disease, some parasitic infections, and tumor models that the potential exists for eosinophils to mediate direct killing via the release of granule-associated cytotoxic proteins, including major basic protein, eosinophil peroxidase, eosinophil cationic protein, and eosinophil-derived neurotoxin, which has been linked to tumor cell apoptosis (28). In addition, tumor-specific Abs may earmark tumor cells for killing by eosinophils via mechanisms similar to those demonstrated in studies of immunity to helminthic parasites (29). Our recent studies have demonstrated that migration of parasitic helminths in vivo can be impeded by eosinophils, and eosinophil attachment to larvae occurs rapidly and early in primary infections via complement C3 (30, 31). However, at least in the *Nippostrongylus brasiliensis* parasitic model, Ab is not the main mechanism of recognition and attachment of eosinophils to helminth larvae (L. A. Dent, unpublished data).

In vivo experiments using either an eotaxin-expressing melanoma (L. Simson and C. R. Parish, unpublished data) or an eotaxin-expressing hepatocellular carcinoma (32) have revealed that although eosinophils are successfully recruited into the tumors, there is little affect on tumor growth unless the tumors are grown in IL-5 Tg recipients, confirming the potential importance of IL-5 in tumor eradication. In addition, our preliminary in vitro results with eosinophils confirm the obligatory role of IL-5 and suggest other myeloid cells may be involved in tumor eradication (J. I. Ellyard, L. Simson, and C. R. Parish, unpublished data). It is likely that eosinophils act in concert with other immune cells in the immune surveillance process. For example, it is known that NKT cells suppress MCA-induced fibrosarcoma formation (2), and recently NKT cells, in association with eosinophils, have been shown to contribute to allergen-induced airway inflammation in an IL-4- and eotaxin-dependent manner (33, 34).

In conclusion, our results expand considerably on previous findings showing the ability of Th2-mediated immunity and eosinophils to induce tumor suppression. Clarifying the role of eosinophils in tumor immune surveillance has the potential for elucidating the general immune function of these cells as well as establishing their role in tumor development and cancer immunotherapy.

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Disclosures

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