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ABSTRACT
The alarmin IL-33 is an IL-1 family member that stimulates pleiotropic immune reactions depending on the target tissue and microenvironmental factors. In this study, we have investigated the role of IL-33/ST2 axis in antitumor response to melanoma. Injection of IL-33 in mice-bearing subcutaneous B16.F10 melanoma resulted in significant tumor growth delay. This effect was associated with intratumoral accumulation of CD8+ T cells and eosinophils, decrease of immunosuppressive myeloid cells, and a mixed Th1/Th2 cytokine expression pattern with local and systemic activation of CD8+ T and NK cells. Moreover, intranasal administration of IL-33 determined ST2-dependent eosinophil recruitment in the lung that prevented the onset of pulmonary metastasis after intravenous injection of melanoma cells. Accordingly, ST2-deficient mice developed pulmonary metastasis at higher extent than wild-type counterparts, associated with lower eosinophil frequencies in the lung. Of note, depletion of eosinophils by in vivo treatment with anti-Siglec-F antibody abolished the ability of IL-33 to both restrict primary tumor growth and metastasis formation. Finally, we show that IL-33 is able to activate eosinophils resulting in efficient killing of target melanoma cells, suggesting a direct antitumor activity of eosinophils following IL-33 treatment. Our results advocate for an eosinophil-mediated antitumoral function of IL-33 against melanoma, thus opening perspectives for novel cancer immunotherapy strategies.

INTRODUCTION
Interleukin-33 (IL-33) is an atypical epithelial-derived alarmin belonging to the IL-1 cytokine family that plays multiple roles in allergy, autoimmunity and inflammation. In the precursor form, IL-33 exists as a nuclear protein that regulates pro-inflammatory gene expression. In the biologically active form, IL-33 is released in the extracellular milieu and can act as an “alarmin,” when produced in response to cell damage or pathogen encounter. IL-33 signals through its specific receptor ST2, which is expressed by many hematopoietic cells, including Th2 cells, mast cells (MCs), regulatory T cells (Treg), group 2 innate lymphoid cells (ILC2s), myeloid cells, natural killer (NK) cells, activated Th1 and CD8+ T cells, affecting multiple arms of the immune response.

Although initially studied in the context of allergic reactions and other T helper-2 (Th2)-mediated responses, IL-33 is now known to stimulate also Th1-type of immune responses, depending on the target tissue and on the cytokine milieu. Hence, IL-33 can synergize with IL-12 to stimulate the secretion of IFNγ by NK and NKT cells and to promote the effector function of CD8+ T cells. A role for the axis IL-33/ST2 in antitumor immune responses has recently emerged and both pro-tumoral and antitumoral functions have been reported. High serum levels of IL-33 were found to correlate with poor prognosis in patients with breast, gastric, non-small cell lung cancer or hepatocellular carcinoma. In mice, the IL-33/ST2 axis was shown to promote the growth and metastases of breast and colon cancer facilitating the accumulation of immunosuppressive myeloid cells, such as myeloid-derived suppressor cells (MDSC) and F4/80+ macrophages within the tumor microenvironment. On the other hand, in a human papilloma virus (HPV)-associated model for cancer immunotherapy IL-33 was shown to act as a potent vaccine adjuvant augmenting Th1 and CD8+ T-cell responses, inducing antitumor immunity in vivo. Furthermore, transgenic host and tumoral expression of IL-33 was shown to inhibit tumor growth in mice, with two proposed mechanisms on the tumor microenvironment, namely by increasing NK and CD8+ T cell
IL-33 delays melanoma growth in mice and promotes antitumor immune responses

We evaluated the antitumor effects of IL-33 in a mouse model of transplantable melanoma. Mice implanted subcutaneously with B16.F10 metastatic melanoma cells received IL-33 injections (0.4 μg intraperitoneally) at various times after tumor implant, namely at the time of melanoma cells implant (T0), when tumor nodules reached the mean diameter of 3 mm or 6 mm (Fig. 1A). Treatment with IL-33 significantly inhibited the growth of melanoma nodules (Fig. 1B) and increased the survival rate of tumor-bearing mice (Fig. 1C) at all tumor growth stages. We next characterized the local immune infiltrate in melanoma-bearing animals one day after the last cytokine administration. Notably, IL-33 treatment induced significant recruitment of immune cells compared with the control (Fig 2A and B). Multicolor flow cytometry analysis of immune cell populations revealed significant increase in CD8+ T cells and eosinophils in IL-33-treated tumors (Fig. 2B). In contrast, the frequency of tumor-infiltrating CD11b+ myeloid cells, particularly of suppressive monocytic (Mo)-MDSC, F4/80+ macrophages and myeloid dendritic cells (mDC) was markedly decreased in mice receiving IL-33 (Fig. 2B). No changes in macrophage polarization toward M1 or M2 phenotype in terms of CD206 expression were observed (data not shown). In addition, no significant differences were observed with CD4+ T and B lymphocytes, NK cells, plasmacytoid DC (pDC), CD8α-expressing DC (CD8α+ DC), or granulocytic (Gr)-MDSC (Fig. 2B).

Results

IL-33 delays melanoma growth in mice and promotes antitumor immune responses

We next characterized the local immune infiltrate in mouse models of melanoma. In addition, elevated counts of blood eosinophils were associated with positive response to ipilimumab therapy in advanced melanoma patients.30-32 In this work, we have investigated the antitumoral role of IL-33 in restricting melanoma growth and metastasis, underlaying the important function of eosinophils in these processes.
at the tumor site, leading to local and systemic activation of CD8\(^+\) T and NK cells.

**Depletion of eosinophils abolishes the antitumor effect of IL-33 and the recruitment and activation of CD8\(^+\) T cells**

Eosinophils were recently described to mediate tumor regression through recruitment of tumor-reactive CD8\(^+\) T cells.\(^{28}\) Accordingly, eosinophils sorted from tumors of IL-33-treated mice expressed high levels of CD8\(^+\) T cell-attracting chemokines (Fig. S2). To address the role of eosinophils in IL-33-induced antitumor effects, tumor-bearing mice receiving IL-33 were injected with an anti-Siglec-F monoclonal antibody (mAb) that selectively depletes eosinophils via induction of apoptosis\(^{26}\) (Fig. 3A, S3). Of note, eosinophil depletion abolished the antitumor efficacy of IL-33 resulting in rapid melanoma tumor growth (Fig. 3B). This effect was paralleled by abrogation of IL-33-induced CD8\(^+\) T cell recruitment within the tumor (Fig. 3C). In the absence of eosinophils, activation of both tumor-infiltrating NK and CD8\(^+\) T cells was also abrogated, as shown by reduced percentages of CD107a\(^+\)IFN\(\gamma\) NK and CD8\(^+\) T cells compared with the values found in IL-33-treated animals (Fig. 3D). These data indicate that eosinophils play an essential role in IL-33-induced antitumor effects and that their function may be linked to the recruitment and/or the activation of CD8\(^+\) T and NK cells.

**IL-33/ST2 axis conditions the pulmonary environment to restrict melanoma metastasis formation**

Since IL-33 is known to control the homing of immune cell populations at the pulmonary level,\(^{5,37}\) we investigated the role of this cytokine in the metastatic process of melanoma. To this end, mice were administered IL-33 intranasally and then transferred intravenously with B16.F10 melanoma cells to induce lung metastasis formation (Fig 4A). Of note, IL-33 significantly inhibited
pulmonary metastasis formation (Fig. 4B), as displayed by dose-dependent reduction of metastatic foci numbers (Fig. 4C) and micrometastases formation (Fig. 4D and E) in treated animals as compared with controls. To evaluate whether IL-33 caused remodeling of the pulmonary immune microenvironment, we analyzed the immune cell populations associated with metastasis reduction following IL-33 treatment. Flow cytometry analysis of pulmonary CD45+ infiltrates evidenced a marked increase of eosinophil frequency, but not of CD8+ T cells, in mice treated with IL-33 compared with control (Fig. 4F). No significant variations occurred in the other immune cell populations analyzed (Fig. 4F). In addition, IL-33 did not affect the activation of pulmonary NK and CD8+ T cells as shown by analysis of CD107a and IFNγ expression (Fig. S4). This immune profile correlated with upregulation of eosinophil-attracting chemokines CCL5, CCL11, CCL13, CCL24, but not of the CD8+ T cell-attracting CXCL10, (Fig. 4G) in lungs of mice receiving IL-33 with respect to control animals. Of note, Granzyme B and IFNγ were also markedly expressed in lungs of mice exposed to IL-33 (Fig. 4G). In addition, IL-33 treated lungs displayed augmented expression of IL-33 pathway-related ST2, TSLP, IL-25, CCL17, CCL20 and CCL22, Th2 cytokines IL-10 and IL-13 and Th1 cytokines IL-2 and IL-12 (Fig. 4G). IL-4 and IL-17 genes were not modulated in the lungs following IL-33 exposure (Fig. S1B). These findings suggest that pulmonary conditioning with IL-33 prevents the onset of pulmonary metastasis after intravenous injection of melanoma cells by promoting the recruitment of eosinophils without increasing CD8+ T and NK cell responses. IL-33 reduced lung metastasis in a ST2-dependent manner, since ST2-deficient (ST2−/−) mice did not respond to IL-33 and developed metastasis to the same extent as untreated ST2−/− mice, indicating that IL-33 signals through host-expressed ST2 (Fig. 5A). Of note, ST2−/− mice developed metastasis at greater extent compared with WT mice (Fig. 5B). In fact, hematoxylin/eosin staining of lung tissue sections revealed earlier appearance of metastatic foci in ST2−/− lungs, visible at day 8 from tumor injection and larger numbers of metastatic nodules at day 19 in comparison to the WT counterparts (Fig. 5B). Flow cytometry analysis of the lung infiltrate evidenced significant reductions in eosinophils and NK cells in ST2−/− mice with respect to WT mice (Fig. 5C and D). However, CD107a/IFNγ staining underscored greater activation of pulmonary NK cells from ST2−/− mice compared with WT mice, indicating competent functionality of these cells in ST2−/− mice (Fig. S5). These results demonstrate that IL-33/ST2 axis protects from pulmonary melanoma metastasis and suggest that IL-33-triggered infiltration of eosinophils within the lungs may represent a crucial event in this phenomenon.

**Eosinophils mediate the ability of IL-33 to restrict melanoma metastasis formation**

To define the role of eosinophils in IL-33-mediated suppression of lung metastasis, C57Bl/6 mice treated intranasally with IL-33 were subjected to eosinophils depletion by Siglec-F mAb injection and then intravenously transferred with B16.F10 cells. Nineteen days after melanoma cell transfer, mice were killed and lungs assayed for the presence of metastasis (Fig. 6A). Injection of Siglec-F mAb caused efficient eosinophil depletion.
in the lung (Fig. S6). Remarkably, restriction of macro and micrometastases formation in mice receiving IL-33 was completely abolished in the absence of eosinophils (Fig. 6B and C). To elucidate the underlying mechanisms of this phenomenon, we evaluated the effect of eosinophils depletion on the lung immunoenvironment. Flow cytometry analysis of lung immune infiltrates evidenced selective decrease of eosinophils, but not of other immune cell subtypes, in mice treated with IL-33 plus anti-Siglec-F mAb with respect to animals administered with IL-33 alone (Fig. 6D). Moreover, the percentages of CD107a^+INFNγ^+ NK and CD8^+ T cells in IL-33-treated mice were not modified upon eosinophil depletion (Fig. 6E). Thus, these results indicate that IL-33 inhibits pulmonary melanoma metastasis through recruitment of eosinophils and without involvement of CD8^+ T or NK cells-mediated responses. Of interest, transcriptional profiling of lung tissues revealed that eosinophil depletion reduced the expression of IL-33-induced Th1-related genes (INFNγ, IL-12), but not that of Th2-related cytokines (IL-5, IL-13) nor of IL-33 pathway-related molecules (ST2, IL-25, CCL17, CCL20, CCL22; Fig. 6F).

**IL-33 activates eosinophils and promotes cytotoxic activity against melanoma cells**

To evaluate the phenotype of IL-33-recruited lung eosinophils, we analyzed the expression of T cell-attracting chemokines and effector molecules in sorted pulmonary eosinophils from IL-33-treated mice. In accordance with the lack of pulmonary infiltration of CD8^+ T cells, IL-33-recruited lung eosinophils did not express higher levels of CXCL9, CXCL10 or CCL5 compared with resting controls (Fig. 7A). In contrast, they expressed and produced significant levels of INFNγ and Granzyme B (Fig. 7A; S7), suggesting that these cells display an activated phenotype without expressing T cell-attracting chemokines.
Since eosinophils are known to exert direct cytotoxic effects against various targets, including cancer cells, we asked whether IL-33-recruited eosinophils in the lung could halt melanoma metastasis formation by directly inducing tumor cell killing. To address this issue, we used BM-derived eosinophils. Since IL-33 was shown to inhibit eosinophilopoiesis in BM progenitors when given at early culturing times, we developed a protocol for eosinophils differentiation by culturing BM cells in presence of IL-5 for the first 10 d of culture followed by IL-33 for the last 6 d of culture (Fig. S8A and B). This IL-33-based protocol generated similar numbers of eosinophils as the IL-5-based protocol (Fig. S8C). Of note, the eosinophils terminally differentiated with IL-33 (IL-33 EO) exhibited a much more activated phenotype with respect to eosinophils differentiated with IL-5 for the whole culturing time (IL-5 EO), as shown by higher side scatter and by upregulation of CD69 and CD11b expression (Fig. 7B and S8D). Furthermore, IL-33 EO expressed increased levels of Granzyme B and IFNγ, with respect to IL-5 EO (Fig. 7C), in resemblance with IL-33-recruited lung eosinophils. IL-33 EO also exhibited increased expression of TNF-α, IL-12p40, IL-10 and IL-13, but decreased levels of IL-4, as compared with IL-5 EO (Fig. 7C). Next, we assessed the cytotoxic activity of these eosinophils against B16 melanoma cells. PKH26-labeled B16 cells were cultured in the presence or absence of IL-33 EO or IL-5 EO at different E:T ratios for 5 h, and cell death was measured by Annexin V staining. Remarkably, IL-33 EO were able to efficiently induce target cell death, starting from 1:25 ratio (31% cell killing), reaching 55% Annexin-V+ B16 cells at 1:50 ratio, compared with 9.5% and 13% apoptosis observed with the respective amounts of IL-5 EO (Fig. 7D). In conclusion, these results demonstrate that IL-33 can activate eosinophils increasing their cytotoxic functions against melanoma cells and suggest that this mechanism may underlie, at least in part, the eosinophil-dependent antitumoral function of IL-33.

**Discussion**

Despite initial emphasis on the function of IL-33 in the context of Th2 immunity and related immunopathologies, accumulating evidence demonstrates that IL-33 can also stimulate Th1 immunity and its role in antitumor immune responses is coming to light. However, the current literature suggests that IL-33 may have either a beneficial or detrimental role depending on the tumor histotype and the site of expression. In melanoma, limited in vivo studies have been produced, most of
which used transgenic overexpression of IL-33 in tumor cells or in the host immune system. In these reports, IL-33 was shown to exert an antitumoral effect through a mechanism depending on induction of CD8$^+$ T cell and NK cell-mediated immunity.\textsuperscript{21,22} In a study using mice transplanted with IL-33-expressing tumors or intratumorally infected with adenovirus containing the IL33 gene, abundant recruitment of eosinophils in the tumor microenvironment has been observed, although the antitumor role of these cells was not directly assessed.\textsuperscript{23} In the present study, we report that IL-33 restricts both primary tumor growth and metastasis formation through the active recruitment of eosinophils, though our data suggest that different antitumor mechanisms may be operated by eosinophils at the two sites. Within the primary tumor microenvironment, the antitumor effect of IL-33 was associated with accumulation of both CD8$^+$ T cells and eosinophils, accompanied by upregulation of chemokines that drive the recruitment of these cells. Of note, tumor-infiltrating eosinophils themselves expressed high levels of CD8$^+$ T cell-attracting chemokines following IL-33 exposure and their selective depletion significantly inhibited CD8$^+$ T cell homing and activation resulting in abrogation of IL-33 antitumor efficacy. These findings suggest that within the primary tumor site IL-33-recruited eosinophils play an accessory role in mediating tumor rejection through recruitment of tumor-reactive CD8$^+$ T cells, in accordance with a previous report.\textsuperscript{28} Despite IL-33 did not increase the frequencies of NK cells, it induced local and systemic activation of both NK and CD8$^+$ T cells, in keeping with recent studies showing a potent function of IL-33 in driving type 1 immune response in tumor through the activation of CD8$^+$ T and NK cells.\textsuperscript{20-22,41} Since IL-33-stimulated NK cell effector responses were also abrogated upon removal of eosinophils, it is possible that the latter contribute to NK cell activation either directly or indirectly through secretion of activating cytokines and/or chemokines, such as CCL5.\textsuperscript{42}

Figure 6. Eosinophils mediate the anti-metastatic effect of IL-33 against melanoma. (A) C57BL/6 mice were treated with intranasal instillations of IL-33 (2 μg per mouse) and then intravenously transferred with B16.F10 cells (0.3 × 10$^6$). For the depletion of eosinophils, a group of mice received repeated intraperitoneal injections of anti-Siglec-F mAb (15 μg per mouse). (B) Hematoxylin/Eosin staining of lung tissues harvested at day 19 post-tumor implant. Scale bar = 200 μm (top row) or 40 μm (bottom row). (C) Clonogenic assay in pulmonary cells. Dots represent individual mice from two separate experiments. (D) Flow cytometry analysis of pulmonary infiltrating leukocytes at day 19. Data show the mean frequencies of the indicated immune cell populations (CD45$^+$-gated) in individual mice (n = 6) from two separate experiments ± SD. *p < 0.05. (E) Flow cytometry analysis of pulmonary CD8$^+$ T lymphocytes and NK cells expressing CD107a/IFNγ following in vitro stimulation with PMA/Ionomycin. Data represent the mean frequencies from 3 separate experiments. (F) Expression of the indicated cytokines and chemokines in lung tissues by qPCR. Data are expressed as fold change of mRNA expression vs control. **p < 0.01; ***p < 0.001. CTR: control; aSiglec-F: anti-Siglec-F mAb; NS: not-stimulated; GZMB: Granzyme B. ns: not significant.
At the tumor site, besides inducing the expression of cytokines and chemokines related to the IL-33/ST2 axis (IL-25, TSLP, CCL17, CCL20, CCL22), IL-33 stimulated a mixed Th2 (IL-5, IL-10, IL-13, but not IL-4) and Th1 (IL-12 and Granzyme B) expression pattern, which possibly contributes to its antitumor efficacy. Of interest, tumor-infiltrating immunosuppressive myeloid cells, particularly monocytic MDSC and F4/80+ macrophages, were decreased upon IL-33 treatment, contributing to create a tumor immune environment favorable to antitumor responses. This observation is in contrast with previous studies showing that IL-33/ST2 axis promotes the intratumoral accumulation of suppressive MDSC and/or macrophages in mouse models of breast and colorectal cancer. On the other hand, another study showed that administration of IL-33 inhibits the in vitro differentiation and in vivo accumulation of MDSC in the tumor and spleen of B16F1 melanoma-bearing mice. It has been recently reported that IL-33 promotes antitumor responses through the accumulation and the activation of tumor-infiltrating mDC increasing Ag cross-presentation to CD8+ T cells. Although we have not specifically assessed the effects of IL-33 on DC activation in our models, we report decreased frequencies of intratumoral mDC in IL-33-treated mice, with respect to controls. This discrepancy may be attributable to different IL-33 dosage (0.4 μg vs 1 μg) and timing of administration (every other day vs daily) used in our study with respect to the report of Dominguez and colleagues.

In contrast to what observed at the primary tumor site, pulmonary conditioning with IL-33 via ST2 protected mice from the onset of experimental pulmonary melanoma metastasis via selective recruitment in the lung of eosinophils, but not of CD8+ T cells. Accordingly, while eosinophil-recruiting chemokines were upregulated in the lungs of IL-33-treated mice, we did not observe induction of CD8+ T cell-attracting chemokines neither in the lung nor in sorted pulmonary eosinophils. Remarkably, depletion of eosinophils abolished the efficacy of IL-33 to condition the pulmonary microenvironment, which resulted in the increase of metastasis without producing significant variations in frequency or activation of CD8+ T cells or NK cells in the lung. These data suggest that IL-33-triggered pulmonary eosinophils do not serve as accessory cells, as observed within the primary tumor site, but may rather play a direct role in halting pulmonary metastasis. IL-33 is known to both promote degranulation of eosinophils and to elicit eosinophilic lung recruitment in vivo. In addition, IL-33 was shown to promote lung recruitment of an innate immune IL-5-producing population that increased pulmonary eosinophil infiltration and suppressed experimental melanoma metastasis in mice. In the same study, an increased IL-33 expression was observed in the lungs of mice early after melanoma tumor injection, suggesting a possible involvement of the endogenous cytokine in cancer immune surveillance. Consistently, our finding that ST2−/− mice displayed earlier appearance of metastases and developed larger numbers of metastatic nodules with respect to WT mice suggests that endogenous levels of IL-33 are produced following tumor inoculation and play a relevant role in cancer immune surveillance preventing pulmonary metastasis. Notably, increased metastasis correlated with reduced frequencies of pulmonary eosinophils in deficient mice. Since eosinophils are recruited in response to tumor necrosis it is tempting to speculate that IL-33 may function as a natural alarmin released by dying tumor cells as part of an early inflammatory reaction. However, it remains to be determined whether and how basal levels of eosinophils may contribute to endogenous IL-33-mediated protective effect.

Of note, depletion of eosinophils in IL-33-treated mice suppressed the upregulation of pulmonary Th1, but not Th2, cytokine transcripts. This observation suggests that IL-33-recruited...
eosinophils (directly or indirectly) play a crucial role for the Th1 arm of immune response induced by IL-33. Eosinophils are known to secrete various cytokines, including those associated with Th1 response (such as TNF-α and IFNγ) and Th2 response (such as IL-4, IL-5, IL-13). In this regard, it has been proposed that eosinophils may be classified into E1 or E2 based on the cytokine secretome skewing toward Th1 or Th2, type of immunity, respectively. Our BM-derived eosinophils terminally differentiated with IL-33 (IL-33 EO) expressed higher levels of Th1 cytokines (TNF-α, Granzyme B and IL-12) and lower IL-4, with respect to IL-5 EO. We might therefore hypothesize that IL-33 stimulates eosinophils to display an E1-like phenotype, as also indicated by Granzyme B and IFNγ expression in IL-33-recruited pulmonary eosinophils. Since CD8+ T cells were not affected at the pulmonary level by IL-33, we may assume that expression of Th1 cytokines (particularly IFNγ) by IL-33-recruited pulmonary eosinophils may serve for boosting CD4+ T cell responses. This hypothesis is worth considering, since CD4+ T cells were shown to reject cytotoxic T lymphocyte (CTL)-resistant MHC class-II negative melanoma tumors. Alternatively, through secretion of Granzyme B eosinophils may contribute to amplify pro-inflammatory responses, as also described for other immune cell types.

Of note, terminal differentiation of BM-EO with IL-33 also resulted in phenotypic activation of eosinophils, as denoted by marked upregulation of CD69 and CD11b and by high side scatter, indicative of presence of cytotoxic granules, confirming that IL-33 can directly activate eosinophils. These phenotypic features correlated with a strong ability of IL-33 EO to kill target melanoma cells. Induction of tumor cell death by mouse and human eosinophils has already been reported and relies on the capacity of these cells to release a wide range of cytotoxic granules, such as eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). The antitumor mechanisms by which eosinophils operate in vivo remain to be elucidated. We might hypothesize that at the primary tumor site IL-33-recruited eosinophils may function mainly as accessory cells attracting tumor-reactive CD8+ T cells. At the pulmonary site, our data strongly suggest that IL-33-recruited lung eosinophils do not influence CD8+ T cell recruitment or activation but rather prevalently play a direct antitumor role via tumor cell killing (as depicted in Fig. 8). The underlying molecular bases for such different cellular functions of IL-33-driven eosinophils need further investigation, although it is plausible that tissue-specific environmental factors may contribute shaping their phenotype. In this respect, it will be of relevance to determine the direct cellular targets for IL-33 regulating eosinophilic infiltration at the primary tumor site and within the lung.

Tumor-infiltrating eosinophils have been correlated with tumor regression in mouse models of melanoma and other solid cancers and have been associated with a good prognosis in several clinical solid cancers, including colorectal, oral squamous and prostate carcinomas. Our results identify a previously unrecognized eosinophil-dependent antitumor mechanism triggered by IL-33/ST2 axis that restricts melanoma growth and metastasis, which may open to novel therapeutic options against melanoma. In this respect, it would be of interest to test if eosinophils in the context of inflammatory scenario triggered by IL-33 would enhance the therapeutic response to immune checkpoint inhibitors directed against CTLA-4 and/or PD-1, as reported in a murine acute myeloid leukemia model. Furthermore, given the capacity of IL-33 to expand also Treg cells, it is plausible that targeting Treg with mAbs would increase IL-33 antitumor immunity. Lastly, given the association of IL-33-mediated eosinophilia with allergic diseases, our findings hold epidemiologic implications on tumor and pulmonary metastasis incidence in allergic subjects, such as asthmatics.

![Figure 8](image-url). Hypothetic model for IL-33-mediated antitumor responses against melanoma. IL-33 through upregulation of CCL5, CCL11, CCL13 and CCL24 promotes eosinophil recruitment at tumor sites. At the primary tumor level, eosinophils (EO) play an accessory role promoting the recruitment of CD8+ T cells via CXCL9, CXCL10, CCL5 expression and the activation of tumor-reactive CD8+ T and NK cell responses. At the pulmonary level, IL-33-recruited eosinophils display an activated phenotype and control metastasis insurgence likely through direct tumor cell killing.
**Materials and methods**

**Mice and cell lines**

C57BL/6 (Charles River Laboratories) and ST2 mice (kindly provided by Dr Andrew N. McKenzie, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) were housed in the animal facility at the Istituto Superiore di Sanità (Rome, Italy) and manipulated in accordance with the local Ethical Committee guidelines.

Six to 8-week-old female mice were used for experiments. B16.F10 murine metastatic melanoma cell line was purchased from American Type Culture Collection (ATCC, CRL-6475). Ovalbumin (OVA)–expressing B16 melanoma cells (B16.OVA) were kindly provided by Dr Laurence Zitvogel (Gustave Roussy Cancer Campus, Villejuif, France). The cells were routinely tested for morphology, growth curve and absence of mycoplasma and passed for no more than four times from thawing.

**Murine tumor models and IL-33 treatments**

For the primary tumor model, C57BL/6 mice were injected subcutaneously with 0.8 × 10^6 B16.F10 or B16.OVA melanoma cells. Recombinant mouse IL-33 (0.4 μg per mouse; Biolegend), dissolved in 200 μL of phosphate-buffered saline (PBS), was injected intraperitoneally in mice five times, every other day, at different stages of tumor growth, starting from either T0 (0 mm mean diameter), 3 × 3 (3 mm mean diameter) or 6 × 6 (6 mm mean diameter). Control groups consisted of mice injected with PBS. Mice survival was monitored daily and tumor growth was measured twice per week using a caliper. For the metastasis model C57BL/6 and ST2 mice were injected intravenously with 0.3 × 10^6 B16.F10 melanoma cells. IL-33 treatment consisted of four intranasal instillations (1 μg or 2 μg in 10 μL per mouse): three consecutive days before and on day 6 after tumor injection. Control groups received intranasal PBS. Mice were killed on days 8–19 from tumor inoculation for evaluation of lung metastasis formation.

**In vivo depletion of eosinophils**

For the depletion of eosinophils in the subcutaneous melanoma model, mice received five intraperitoneal injections of 15 μg Siglec-F antibody monoclonal rat IgG2A (Clone #238047, R&D Systems). In the metastasis model, a group of mice received six intraperitoneal injections, every other day, and 2 every 3 d, of Siglec-F mAb (15 μg per mouse). Control groups consisted of mice injected intraperitoneally with Isotype-matched control Ab (R&D Systems).

**Histological analysis of lung tissues**

Lung tissues were excised and processed for staining with hematoxylin and eosin (H&E). Briefly, formalin-fixed paraffin-embedded tissues were sectioned at 5-μm thickness and stained with H&E. The metastatic nodules and distribution were examined by an Axioskop 2 plus microscope (Carl Zeiss).

**Tissue dissection**

Where indicated, tumor, spleen, and lung tissues from tumor-bearing mice were removed and cut into small fragments using scissors and then digested in medium containing DNase I (325 KU/ml; Sigma) and type III collagenase (1 mg/ml; Worthington Biochemicals) for 30 min at room temperature in agitation, followed by EDTA (0.1 M, pH 7.2) for additional 5 min. The homogenate was then passed through a cell sieve and the resulting cell suspension was treated with lysis buffer (140 mM NH4Cl, 17 mM Tris HCl, pH 7.2) to eliminate red blood cells.

**Clonogenic assay for micrometastases determination**

Pulmonary cell suspensions were resuspended in DMEM complete medium, plated in six well-plates at different dilutions in triplicates and cultured for 14 d. Medium was changed every other day to remove dead or unattached cells, allowing the growth of adherent melanoma cells, when present in the homogenate. Plates were then fixed with methanol and stained with 1% Crystal Violet. Melanoma colonies were examined under a microscope and quantified using ImageJ software. The presence of micrometastases was represented as a fraction of area covered by melanoma colonies with respect to the total area of the image.

**Analysis of immune cell infiltrate in tumor and lung tissues**

For flow cytometry analysis cells were stained with the following fluorescently labeled mAbs: anti-CD45 (30-F11), anti-CD3 (145-2C11), anti-F4/80 (BM8), all from Biolegend; anti-CD8a (53–67), anti-CD11b (M1/70), anti-CD11c (N418), anti-IFNγ (XMG1.2), anti-Granzyme B (NGZB), all from ebioscience; anti-CD4 (GK1.5), anti-CD69 (H1.2F3), anti-Ly6C (AL-21), anti-Ly6G (AK8), anti-NK1.1 (PK136) anti-Siglec-F (ES0–2440), all from BD PharMingen; anti-CD19 (6D5); anti-CD107a (GK1.5), anti-CD69 (H1.2F3), anti-Ly6C (AL-21), anti-Ly6G (AK8), anti-Granzyme B (NGZB), all from eBioscience; anti-CD4 (2C11), anti-F4/80 (BM8), all from Biolegend; anti-CD8a (53–67). For intracellular staining in lungs; CD45+CD3+CD19−CD4−CD8− (CD− T cells); CD45+CD3+CD19−CD4+CD8+ (CD8 T cells); CD45+CD3+CD19−CD4+CD8− (CD8 T cells); CD45+CD3−CD19+ (B cells); CD45−CD3−NK1.1−CD49b−NKP46− (NK cells); CD45−CD11b−Siglec-F−Ly6G− (Eosinophils); CD45−CD11b−Ly6C−Ly6G− (Mo-MDSC in tumors, Monocytes in lungs); CD45−CD11b−Ly6C−Ly6G+ (Gr-MDSC in tumors, Neutrophils in lungs); CD45−CD11b−F4/80− (Macrophages); CD45+CD8+−mPDCA1+CD11b+CD11c+h (myeloid DC); CD45+CD8a+ mPDCA1+CD11b+CD11c+h (plasmacytoid DC); CD45+CD8α+ mPDCA1+CD11b+CD11c+h (DC8α+ DC).

**Intracellular staining**

Spleen, lung and tumor cells were seeded (0.3–1 × 10^6 cells per well) in 96-well U-bottomed plates in complete RPMI medium in the presence of Brefeldin A (1 μL/ml; Biolegend), Monensin (2 μL/ml; Biolegend) and anti-CD107a
mAb and stimulated with PMA (100 ng/mL) and Ionomycin (1 μg/mL) for 5 h at 37 °C. After incubation, cells were first surface stained with anti-CD45, anti-CD3, anti-CD8, anti-NK 1.1 mAbs and then intracellularly labeled with anti-IFNγ mAb. Expression of CD107α and IFNγ in CD8+ T and NK cells was analyzed by flow cytometry. For detection of Granzyme B and IFNγ in pulmonary eosinophils, lung cells (1 x 10⁶ cells per well) were incubated in 96-well U-bottomed plates in complete DMEM medium in the presence of Brefaldin A (1 μL/ml) and Monensin (2 μL/ml) for 5 h at 37 °C. After incubation, cells were first surface stained with anti-CD45, anti-CD11b, anti-Siglec-F, anti-Ly6G, anti-CD11c, and anti-CD3 mAbs and then intracellularly labeled with anti-IFNγ and anti-Granzyme B mAbs. Expression of Granzyme B and IFNγ in eosinophils (CD45+ Siglec-F+ Ly6G+ CD11c+ CD3−) was evaluated by flow cytometry.

In vitro proliferation assay

Spleen cells from B16.OVA tumor-bearing mice treated with IL-33 or with PBS were seeded (2 x 10⁵) in 96 U-bottomed well plates in the presence or absence of class-I restricted peptide SIINFEKL (1 μM; Sigma) or class-II restricted peptide 323–339 (20 ng/mL; Sigma). Cell cultures were incubated in complete medium for 4 d at 37 °C, in 5% CO₂ and then pulsed with [³H]-thymidine (1 μCi/well) for 16 h. Incorporation of [³H]-thymidine was analyzed by liquid scintillation counting.

Quantitative PCR

Total RNA was extracted from melanoma and lung tissues explanted from mice and from eosinophils isolated from peritoneum, tumor or lung, by using TRIzol reagent (Bioline). mRNA was reverse transcribed by means of Tetro cDNA Synthesis Kit (Bioline). Quantitative reverse transcription-PCR (qPCR) was performed using Sensismix Plus SYBR Kit containing the fluorescent dye SYBR Green (Bioline). Forward and reverse primers (Table SI) were purchased from Eurofin Genomix. The conditions of real-time PCR reaction were given as follows: 15 sec at 95 °C, 30 sec at 60 °C, and 45 sec at 72 °C (45 cycles). PCR products were continuously measured by means of an ABI 7500 Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). Quality and specificity of amplicons in each sample were detected by dissociation curve analysis. Triplicates were performed for each experimental point. For quantification, threshold cycle (CT) values were determined by the sequence detection system software (Applied Biosystems). Data were normalized to HPRT housekeeping gene (2^−ΔΔCt method) and presented as fold change expression vs control.⁶⁰

Eosinophils isolation and sorting

Pulmonary eosinophils were isolated from lungs of mice treated intranasally with IL-33 and intravenously injected with B16.F10 melanoma cells at day 7 after tumor transfer (see Fig. 4A). Tumor-infiltrating eosinophils were isolated from IL-33-treated mice bearing subcutaneous melanoma one day after the last IL-33 administration. As control eosinophils, due to scarce numbers of cells obtained from tumors and lungs of untreated mice, peritoneal eosinophils were used.²⁸ Briefly, peritoneal cells were harvested from naïve mice 3 d after intraperitoneal injection of 3 mL of 3% thioglycollate broth medium (Difco), by washing the peritoneal cavity with 5 mL of PBS containing 2% FBS. Lung, tumor and peritoneal cells were stained with anti-CD45, anti-Ly6G, anti-Siglec-F, anti-CD11c, anti-CD11b and eosinophils (CD45+CD11b+Siglec-F+Ly6G−CD11c−) were purified by cell sorting on a FACS Aria (BD Biosciences). Cell purity was checked on the same instrument immediately after sorting and ranged >98% (see Fig. S9 for gating strategy).

Differentiation of bone marrow-eosinophils

Eosinophils were generated from cultures of BM cells following a modified protocol described previously.³⁹ Briefly, BM was extracted from the tibia and femur of naïve C57Bl/6 mice, and cell suspensions were treated with lysis buffer (140 mM NH₄Cl, 17 mM Tris HCl, pH 7.2) to eliminate red blood cells. Cells were cultured at 1 x 10⁶/mL in RPMI 1640 (EuroClone) containing 20% FBS, 1% glutamine, 25mM HEPES, 1X NEAA, 1 mM sodium pyruvate, supplemented with 100 ng/mL SCF (PeproTech) and 100 ng/mL FLT3-L (Milenyi Biotec). On day 4, 10 ng/mL rmIL-5 (PeproTech) were added to culture. On day 8, cells were transferred into a new flask containing fresh medium supplemented with rmIL-5. From day 10 to 16, 5 mL of fresh medium containing either rmIL-5 or rmIL-33 (100 ng/mL) was added every other day to generate IL-5 eosinophils (IL-5 EO) and IL-33 eosinophils (IL-33 EO). Cells were used for experiments on day 17, after 24 h incubation with 10 ng/mL of GM-CSF (PeproTech). Eosinophil purity was determined by flow cytometry (CD11b+Siglec-F+Ly6G−CD11c−) and resulted >80%.Activation of eosinophils was further determined by upregulation of CD69, CD11b and side scatter (Fig. S8).

Cytotoxicity assay

Eosinophil-mediated cytotoxicity against tumor cells was evaluated by flow cytometry as described previously.³⁸ Briefly, B16. F10 melanoma cells were labeled with the PKH26 Red fluorescent Cell Linker (Sigma) and then seeded in 96 wells U-bottomed plates (1 x 10⁴ cells per well) in the presence of BM-derived eosinophils (IL-5 EO or IL-33 EO) at different E:T ratios. Co-cultures were incubated for 5 h at 37 °C. Cells were stained with Annexin-V (e-Bioscience) and then analyzed by flow cytometry. Apoptosis of target melanoma cells was calculated as percentage of Annexin-V+ cells among gated PKH26+ population.

Statistical analysis

One-way ANOVA analysis of variance was performed to compare means among multiple groups, followed by post
hoc testing (Tukey). Log-rank Mantel–Cox test was used for the analysis of survival curves. Values were considered as significant when the probability was below the 5% confidence level \( (p \leq 0.05) \).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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