




IL-33 restricts tumor growth and inhibits pulmonary metastasis in melanoma-bearing mice through eosinophils

Valeria Lucarini, Giovanna Ziccheddu, Iole Macchia, Valentina La Sorsa, Francesca Peschiaroli, Carla Buccione, Antonella Sistigu, Massimo Sanchez, Sara Andreone, Maria Teresa D'Urso, Massimo Spada, Daniele Macchia, Claudia Afferni, Fabrizio Mattei & Giovanna Schiavoni


To cite this article: Valeria Lucarini, Giovanna Ziccheddu, Iole Macchia, Valentina La Sorsa, Francesca Peschiaroli, Carla Buccione, Antonella Sistigu, Massimo Sanchez, Sara Andreone, Maria Teresa D'Urso, Massimo Spada, Daniele Macchia, Claudia Afferni, Fabrizio Mattei & Giovanna Schiavoni (2017) IL-33 restricts tumor growth and inhibits pulmonary metastasis in melanoma-bearing mice through eosinophils, *Oncoimmunology*, 6:6, e1317420, DOI: [10.1080/2162402X.2017.1317420](https://doi.org/10.1080/2162402X.2017.1317420)



To link to this article: <https://doi.org/10.1080/2162402X.2017.1317420>

 View supplementary material 

 Accepted author version posted online: 20 Apr 2017.
Published online: 19 May 2017.

 Submit your article to this journal 

 Article views: 1093

 View related articles 

 View Crossmark data 

 Citing articles: 34 View citing articles 

ORIGINAL RESEARCH



IL-33 restricts tumor growth and inhibits pulmonary metastasis in melanoma-bearing mice through eosinophils

Valeria Lucarini^{a,*}, Giovanna Ziccheddu^{a,*}, Iole Macchia^a, Valentina La Sorsa^a, Francesca Peschiaroli^a, Carla Buccione^a, Antonella Sistigu^b, Massimo Sanchez^c, Sara Andreone^a, Maria Teresa D'Urso^a, Massimo Spada^a, Daniele Macchia^a, Claudia Afferni^{d,**}, Fabrizio Mattei^{a,**}, and Giovanna Schiavoni^{a,**}

^aDepartment of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy; ^bUnit of Tumor Immunology and Immunotherapy, Department of Research, Advanced Diagnostics and Technological Innovation, Regina Elena National Cancer Institute, Rome, Italy; ^cCore Facilities Services Cytometry Unit, Istituto Superiore di Sanità, Rome, Italy; ^dDepartment of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

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.

ARTICLE HISTORY

Received 19 January 2017
Revised 31 March 2017
Accepted 4 April 2017

KEYWORDS

Eosinophils; interleukin (IL)-33; melanoma; mouse models; pulmonary metastasis


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.¹ 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.² 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.³

Although initially studied in the context of allergic reactions and other T helper-2 (Th2)-mediated responses,^{4,5} IL-33 is now known to stimulate also Th1-type of immune responses, depending on the target tissue and on the cytokine milieu.⁶ Hence, IL-33 can synergize with IL-12 to stimulate the

secretion of IFN γ by NK and NKT cells^{7,8} and to promote the effector function of CD8⁺ T cells.⁹ 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.^{10,11} 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.¹²⁻¹⁵ 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.¹⁶⁻¹⁹ 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*.²⁰ 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

CONTACT Dr. Giovanna Schiavoni ✉ giovanna.schiavoni@iss.it Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.

 Supplemental data for this article can be accessed on the [publisher's website](#).

*Equal contribution.

**Share senior co-authorship.

© 2017 Taylor & Francis Group, LLC

responses^{21,22} and through ILC2-mediated tumor apoptosis.²³ Overall, these findings suggest that IL-33 expression in tumor cells may increase immunogenicity promoting antitumor immune responses, whereas IL-33 in tumor stroma and serum may facilitate immune suppression, although the mechanisms involved remain to be determined.

IL-33 drives substantial recruitment of eosinophils to inflamed sites, such as the lung, likely by promoting eosinophilopoiesis in the bone marrow (BM)^{5,24} as well as induces degranulation of eosinophils.^{25,26} Some recent experimental evidences indicate that eosinophils play a prominent role in anticancer immunity and tumor-infiltrating eosinophils have been correlated with tumor regression in mouse models of melanoma.²⁷⁻²⁹ In addition, elevated counts of blood eosinophils are 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, underlying the important function of eosinophils in these processes.

Results

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 μ g 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 DC (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), CD8 α -expressing DC (CD8 α ⁺ DC), or granulocytic (Gr)-MDSC (Fig. 2B). Next, we investigated the intratumoral expression of Th1 and Th2-related cytokines and chemokines related to immune cell trafficking. Quantitative PCR of tumor tissues from IL-33 treated mice revealed increased expression levels of IL-33 pathway-related cytokines, such as thymic stromal lymphopoietin (TSLP) and IL-25, ST2 receptor and the chemokines CCL17, CCL20 and CCL22, with respect to controls (Fig 2C). Consistent with the immune infiltrate profile, mice receiving IL-33 exhibited higher expression levels of CCL2, CXCL10, CX3CL1, known to be chemoattractants for CD8⁺ T cells³³ and of CCL5, CCL11, CCL13, CCL24, all involved in eosinophil recruitment.³⁴ Of note, both Th2-related cytokines (IL-10 and IL-13) and the Th1-related effector molecule Granzyme B were highly expressed in tumors from IL-33-treated mice compared with the controls (Fig. 2C). No significant modulation of IL-4 and IL-17 genes was observed (Fig. S1A). Since IL-33 is known to stimulate both NK and CD8⁺ T cell-mediated effector responses,^{7,21,22,35} we further analyzed the effects of IL-33 on the activation of these immune effectors in our model. Compared to controls, mice receiving IL-33 showed increased percentages of CD8⁺ T and NK cells co-expressing the effector molecules CD107a and IFN γ both in the tumor (Fig. 2D and E) and in the spleen (Fig. 2F), suggesting local and systemic activation of CD8⁺ and NK cells following treatment with IL-33. To verify tumor-specificity of T cell response, we transplanted mice with B16.OVA cells and found that IL-33 treatment induced significant increase in the proliferative response of spleen cells following re-stimulation with MHC-I and MHC-II-restricted peptides of OVA, with respect to controls (Fig. 2G). Taken together, these results indicate that IL-33 hampers melanoma *in vivo* growth and generates a tumor microenvironment favorable for tumor control through the modulation of both Th1 and Th2 cytokines and chemokines inducing the homing of CD8⁺ T lymphocytes and eosinophils

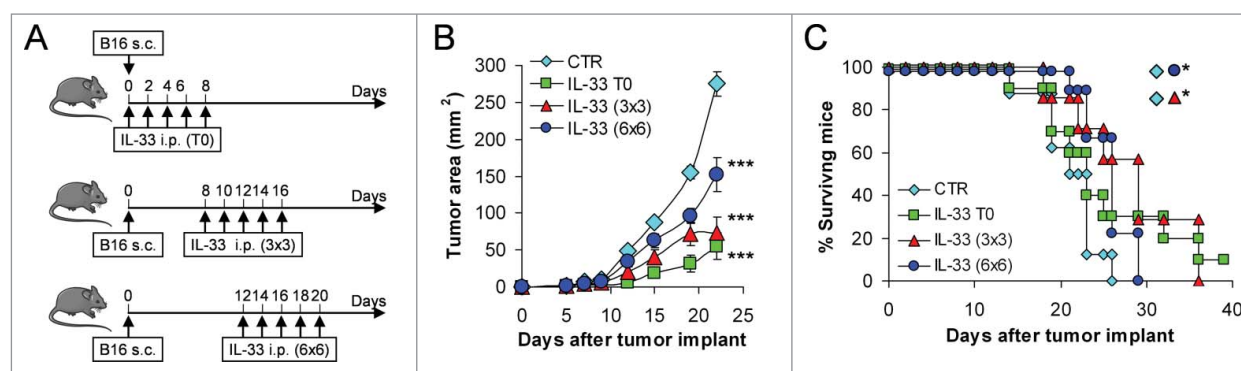


Figure 1. Antitumor activity of IL-33 in mice transplanted with B16.F10 melanoma cells. (A) Schematic representation of experimental protocol. C57BL/6 mice were injected subcutaneously with 0.8×10^6 B16.F10 melanoma cells and treated intraperitoneally with recombinant mouse IL-33 (0.4 μ g) at different tumor stages. (B) Tumor growth in the indicated groups. Mean tumor area in individual mice \pm SD is shown (n = 10). (C) Kaplan–Meier plot representing the percentage of surviving mice within each group. One representative experiment out of five is shown. * $p < 0.05$; *** $p < 0.001$. i.p.: intraperitoneal; CTR: control; s.c.: subcutaneous.

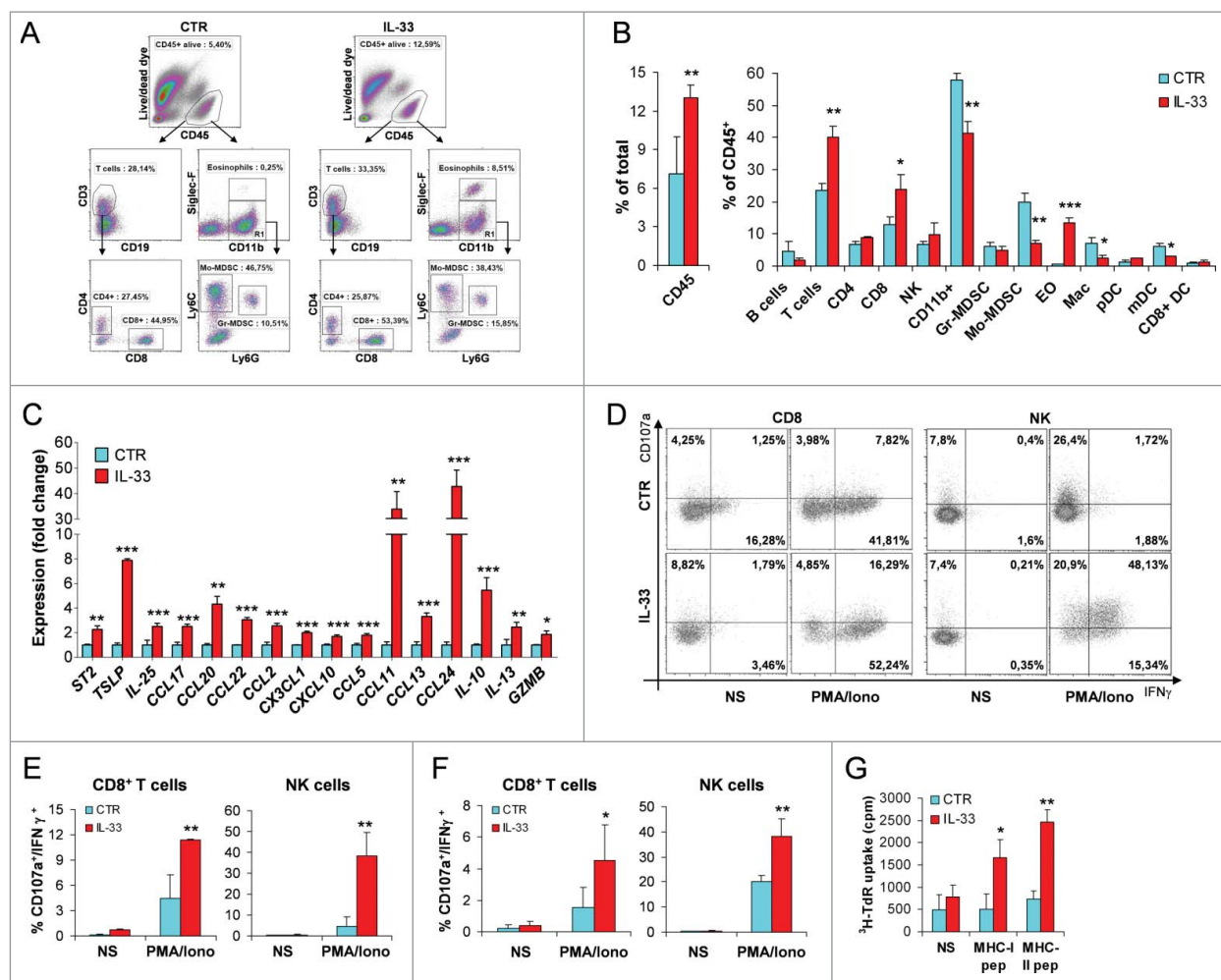


Figure 2. Effects of IL-33 on the local tumor immune microenvironment and on systemic response. C57BL/6 mice transplanted subcutaneously with 0.8×10^6 B16.F10 melanoma cells were treated with $0.4 \mu\text{g}$ of IL-33, starting from 3 mm mean diameter. One day after the last IL-33 injection, tumors and spleens were harvested. (A and B) Flow cytometry analysis of tumor-infiltrating cells. (B) Data show mean values of individual mice ($n = 3-5$) from four separate experiments \pm SD. (C) Expression of Th1/Th2 cytokines and chemokines in melanoma tumors by qPCR. Data are expressed as fold change of mRNA expression vs control. (D-F) Flow cytometry analysis of IFN γ and CD107a expression in CD8⁺ T and NK cells from tumors (D-E) and spleen (F) after *in vitro* stimulation with PMA/Ionomycin. Histograms represent the frequencies of the indicated populations. Mean from at least three independent experiments is shown \pm SD. (G) Antigen-specific proliferative response of spleen cells from mice bearing B16.OVA tumors, treated or not with IL-33, following *in vitro* re-stimulation with class-I or class-II-restricted OVA peptides. Data show ³H-thymidine incorporation after 4 d of culture, mean \pm SD of culture triplicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CTR: control; NS: not-stimulated; GZMB: Granzyme B.

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 apoptosis³⁶ (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 γ ⁺ 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

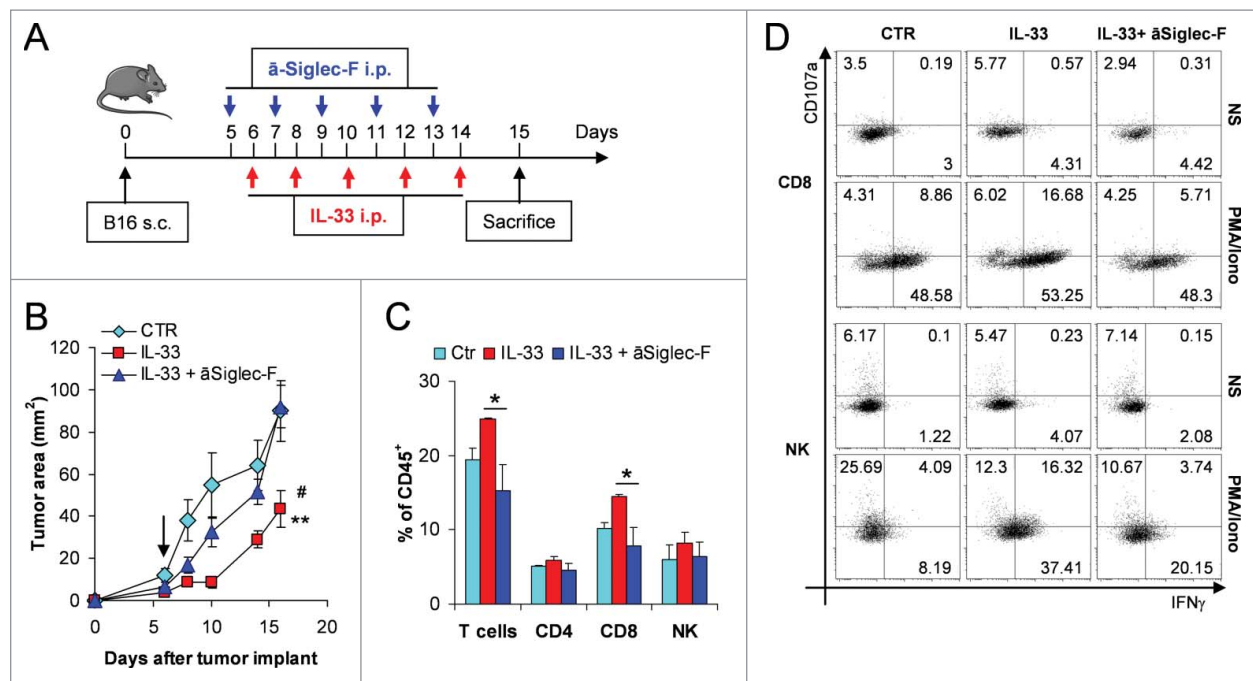


Figure 3. Eosinophil depletion abolishes the antitumor effect of IL-33 in melanoma-bearing mice. (A) Schematic representation of experimental protocol. C57BL/6 mice were injected subcutaneously with 0.8×10^6 B16.F10 melanoma cells and treated intraperitoneally with recombinant mouse IL-33 ($0.4 \mu\text{g}$). For the depletion of eosinophils, a group of mice received repeated intraperitoneal injections of anti-Siglec-F mAb ($15 \mu\text{g}$ per mouse). (B) Tumor growth in the indicated groups. Mean tumor area in individual mice \pm SD is shown ($n = 10$). $\#p < 0.05$, IL-33 vs control; $**p < 0.01$, IL-33 vs IL-33 + anti-Siglec-F. (C) Flow cytometry analysis of tumor-infiltrating cells. Data show mean values of individual mice ($n = 3$ – 5) from three separate experiments \pm SD. $*p < 0.05$. (D) Flow cytometry analysis of IFN γ and CD107a expression in tumor-infiltrating CD8⁺ T and NK cells after *in vitro* stimulation with PMA/Ionomycin. One representative experiment out of two is shown. CTR: control; \bar{a} Siglec-F: anti-Siglec-F mAb; NS: not-stimulated; i.p.: intraperitoneal; s.c.: subcutaneous.

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 IFN γ 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 IFN γ 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/IFN γ staining underscored greater activation of pulmonary NK cells from ST2^{-/-} with respect to 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

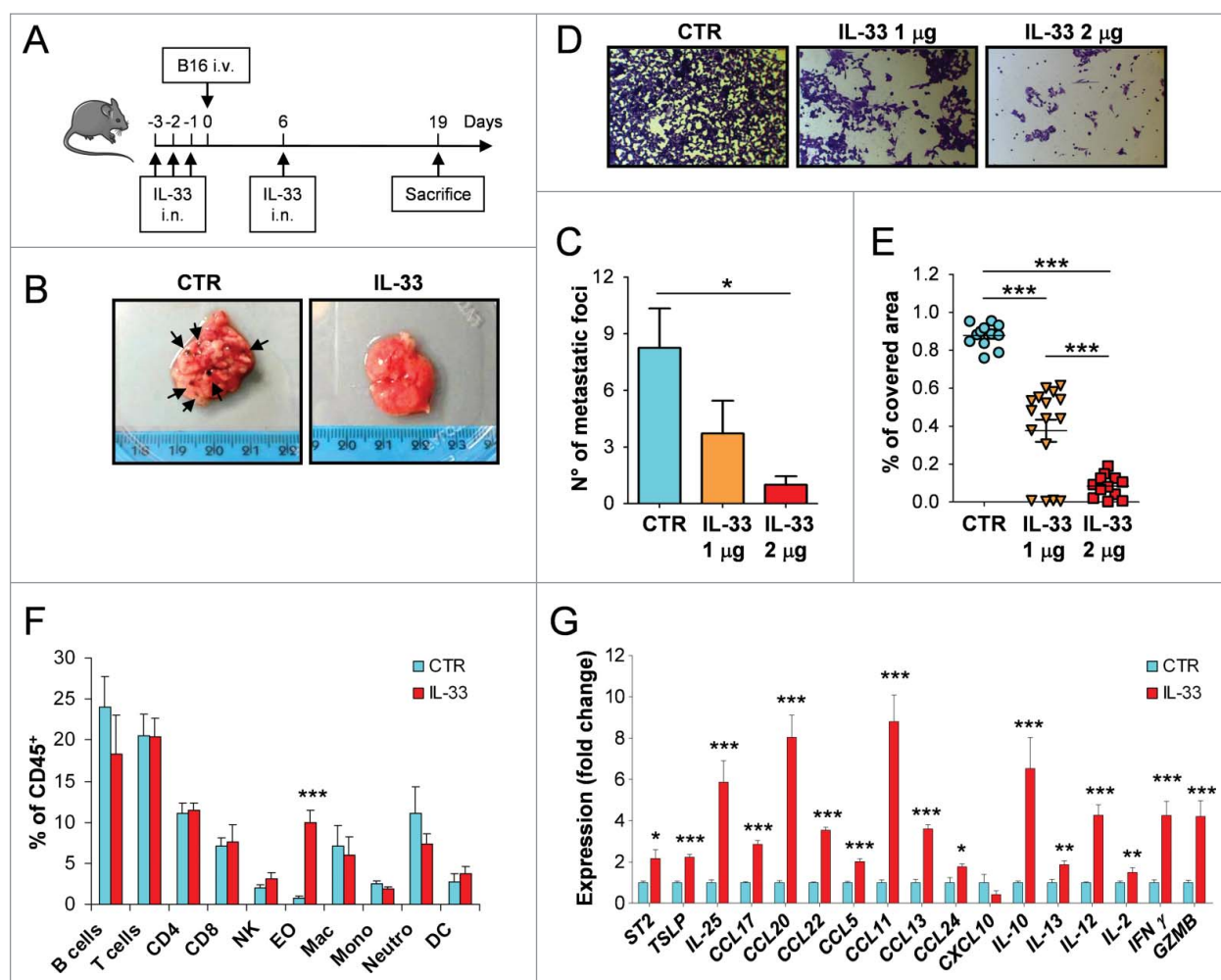


Figure 4. IL-33 restricts the formation of melanoma pulmonary metastasis. (A) Schedule of experimental metastasis induction and IL-33 treatment. C57BL/6 mice were treated intranasally with IL-33 (1 μ g or 2 μ g per mouse) and then injected intravenously with 0.3×10^6 B16.F10 melanoma cells. Mice were killed at day 19 post B16 inoculation and lung metastatic foci, were enumerated. (B) Representative microphotographs of lungs from mice receiving or not IL-33 (2 μ g per mouse). Arrows point to visible metastatic foci. (C) Enumeration of metastatic foci in lungs of B16 melanoma-injected mice treated or not IL-33 at the indicated doses. Bars represent the mean counts in individual mice \pm SD ($n = 6$) from three independent experiments. (D) Clonogenic assay *in vitro* to determine lung micrometastases. Representative micrograph of melanoma cell clones upon culture of lung cells from mice treated as indicated. (E) Quantitative analysis of lung micrometastases. Dots represent the percentage of covered area of melanoma cell clones from lungs of single mice. One experiment out of three is shown. (F) Flow cytometry analysis of pulmonary immune-infiltrating cells. Data show the mean frequencies of the indicated CD45⁺-gated immune cell populations in individual mice \pm SD ($n = 6$) from 4 separate experiments. (G) Quantitative PCR analysis of expression of indicated cytokines and chemokines in lung tissues. Data are expressed as fold change of mRNA expression vs control. * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$. CTR: control; GZMB: Granzyme B; i.n.: intranasal; i.v.: intravenous.

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⁺IFN γ ⁺ 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 (IFN γ , 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 IFN γ and Granzyme B (Fig. 7A; S7), suggesting that these cells display an activated phenotype without expressing T cell-attracting chemokines.

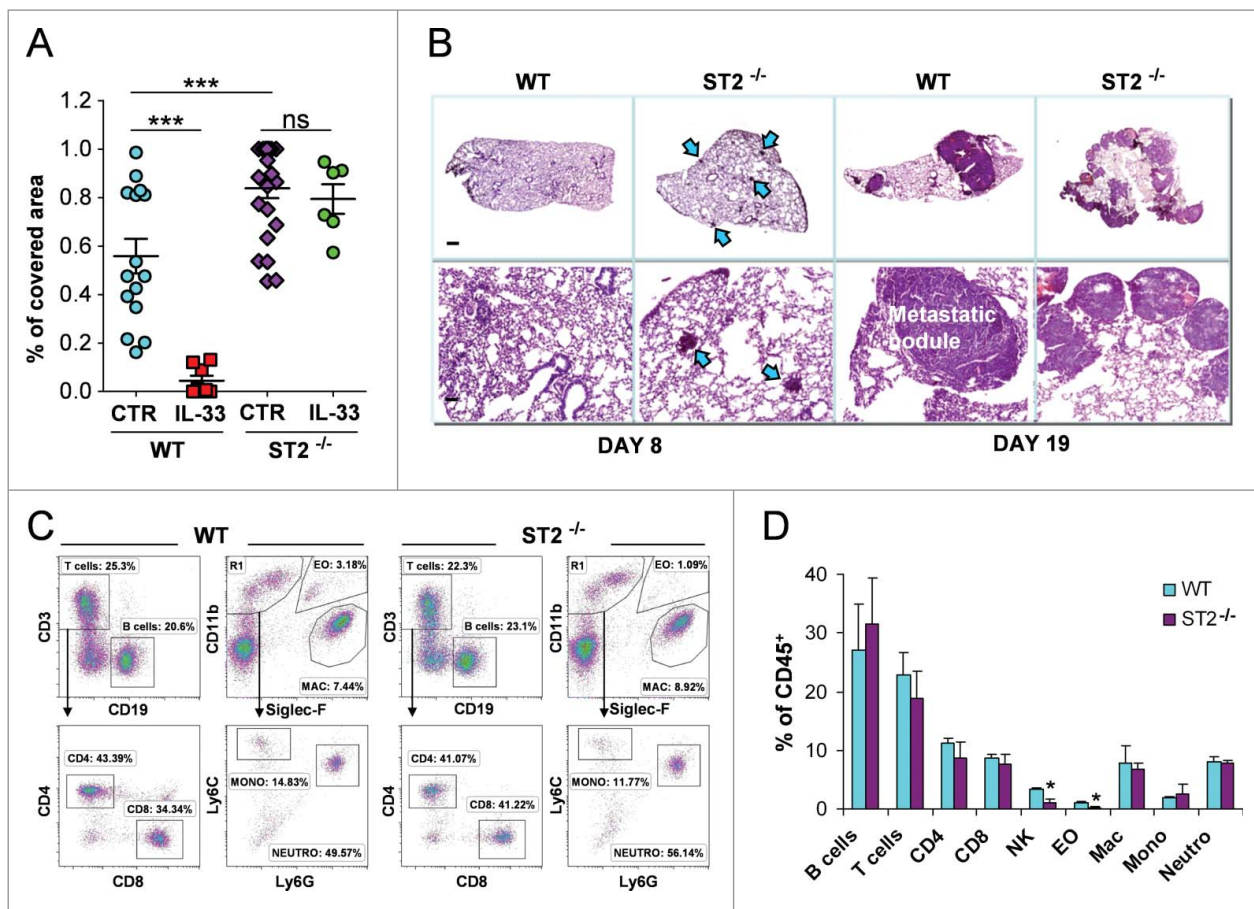


Figure 5. IL-33/ST2 axis controls the formation of melanoma pulmonary metastasis. ST2^{-/-} and WT mice were treated or not with intranasal IL-33 (2 μ g per mouse) and then intravenously injected with B16 melanoma cells (0.3×10^6). (A) Quantitative analysis of lung micrometastases determined by clonogenic assay. Dots represent the percentage of covered area of melanoma cell clones from lungs of single mice. One experiment out of three is shown. *** $p < 0.001$. (B) Hematoxylin/Eosin staining of lung tissues harvested on days 8 and 19 after tumor implant. Scale bar = 200 μ m (top row) or 40 μ m (bottom row). Arrows show metastatic foci inside the lungs. (C and D) Flow cytometry analysis of pulmonary immune-infiltrating cells. (D) Data show the mean frequencies of the indicated CD45⁺-gated immune cell populations in individual mice \pm SD ($n = 6$) from three separate experiments. * $p < 0.01$. CTR: control. ns: not significant.

Since eosinophils are known to exert direct cytotoxic effects against various targets, including cancer cells,^{34,38} 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.³⁹ Since IL-33 was shown to inhibit eosinophilopoiesis in BM progenitors when given at early culturing times,⁴⁰ 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 IFN γ , 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 antitumor function of IL-33.

Discussion

Despite initial emphasis on the function of IL-33 in the context of Th2 immunity and related immunopathologies,² accumulating evidence demonstrates that IL-33 can also stimulate Th1 immunity^{20,35} and its role in antitumor immune responses is coming to light.¹⁰ 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

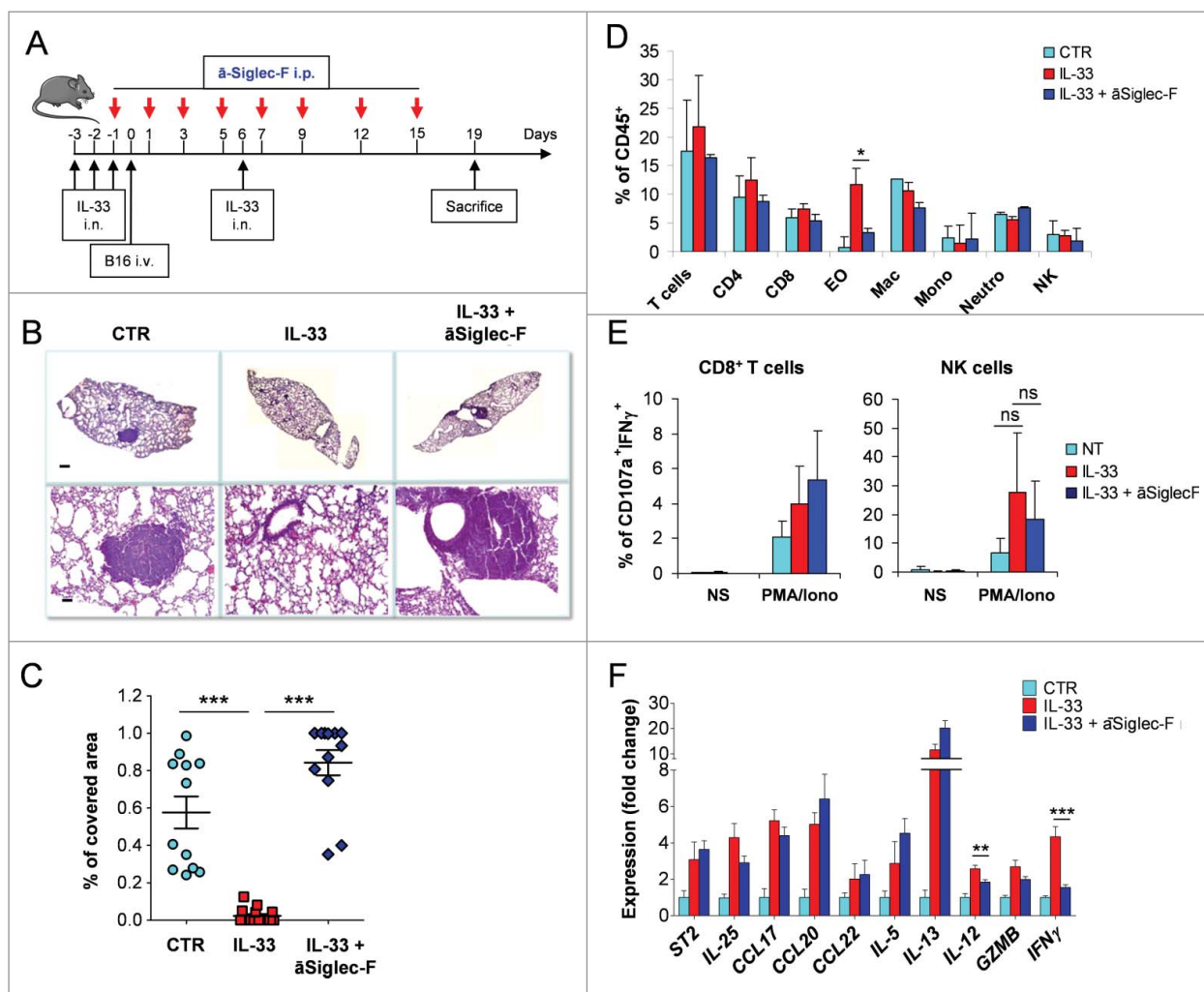


Figure 6. Eosinophils mediate the anti-metastatic effect of IL-33 against melanoma. (A) C57BL/6 mice were treated with intranasal instillations of IL-33 (2 μ g per mouse) and then intravenously transferred with B16.F10 cells (0.3×10^6). For the depletion of eosinophils, a group of mice received repeated intraperitoneal injections of anti-Siglec-F mAb (15 μ g per mouse). (B) Hematoxylin/Eosin staining of lung tissues harvested at day 19 post-tumor implant. Scale bar = 200 μ m (top row) or 40 μ m (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 \pm SD. * $p < 0.05$. (E) Flow cytometry analysis of pulmonary CD8⁺ T lymphocytes and NK cells expressing CD107a/IFN γ following *in vitro* stimulation with PMA/ionomycin. Data represent the mean percentages 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; \bar{a} Siglec-F: anti-Siglec-F mAb; NS: not-stimulated; GZMB: Granzyme B. ns: not significant.

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.^{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.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.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.^{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 CCL5.⁴²

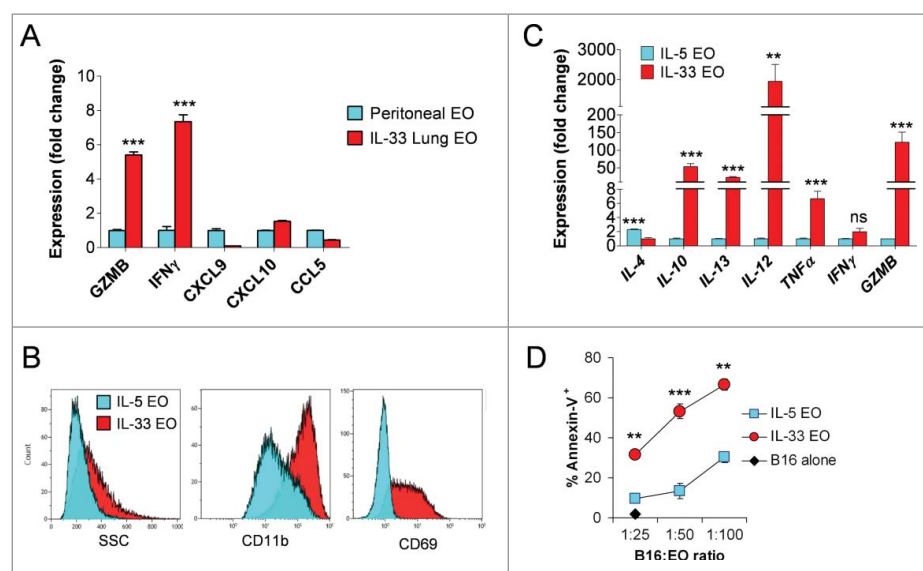


Figure 7. IL-33 activates eosinophils to mediate B16.F10 melanoma cell death. (A) qPCR analysis of indicated cytokines and chemokines in pulmonary-infiltrating eosinophils from IL-33-treated mice vs control peritoneal eosinophils. Data are expressed as fold change of mRNA expression vs peritoneal EO. *** $p < 0.001$. (B) Flow cytometry analysis of CD69, CD11b and side scatter in BM-derived eosinophils differentiated in presence of IL-5 (IL-5 EO) or IL-5 followed by IL-33 (IL-33 EO) from BM of naïve mice. (C) qPCR analysis of indicated cytokines in BM-derived IL-33 EO vs IL-5 EO. Data are expressed as fold change of mRNA expression vs IL-5 EO. ** $p < 0.001$ *** $p < 0.001$. (D) Flow cytometry analysis of B16.F10 melanoma cell death following co-culture for 5 hours with IL-33 EO vs IL-5 EO at the indicated ratios. Bars correspond to mean Annexin-V⁺ PKH-labeled melanoma cells from several samples ($n = 5-8$ replicates per condition). ** $p < 0.001$. 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.^{11,29,43} 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^{17,18} and colorectal cancer.^{19,44} 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.⁴⁵ 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.⁴¹ 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.⁴¹

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*.^{25,46} 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.²⁷ 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.^{47,48} 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 IFN γ) 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.³⁴ 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 IFN γ 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 IFN γ) 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.^{29,49,50} Alternatively, through secretion of Granzyme B eosinophils may contribute to amplify pro-inflammatory responses, as also described for other immune cell types.⁵¹

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,⁵² confirming that IL-33 can directly activate eosinophils.^{26,53} 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).^{34,38,54-56} 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.²⁸ 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.⁵⁷ 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^{28,29,54} and have been associated with a good prognosis in several clinical solid cancers, including colorectal, oral squamous and prostate carcinomas.³⁴ 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.⁵⁸ 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.⁵⁹

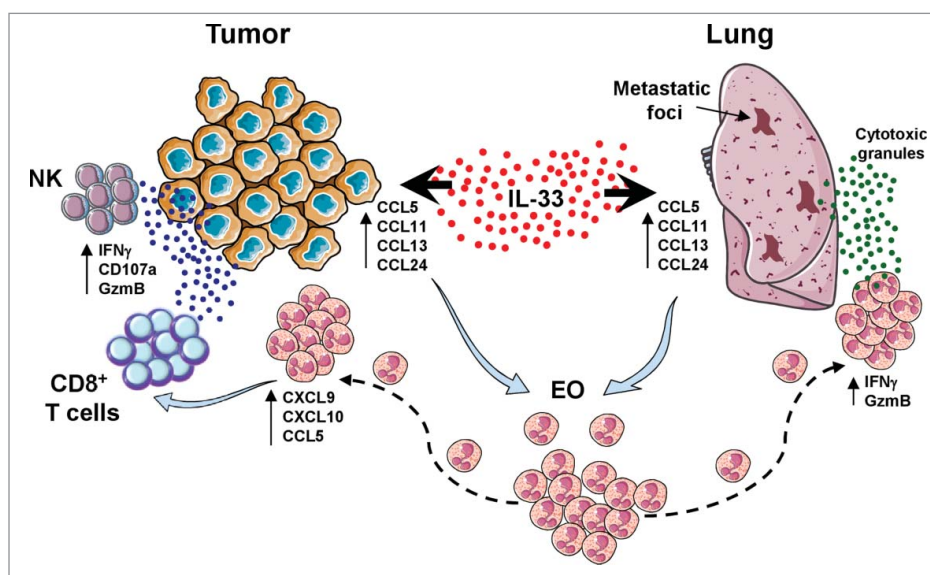


Figure 8. Hypothetic model for IL-33-mediated antitumor responses against melanoma. IL-33 through upregulation of CCL5, CCL11, CCL13 and CCL24 promotes eosinophil recruitment at tumor sites. At the primary tumor level, eosinophils (EO) play an accessory role promoting the recruitment of CD8⁺ T cells 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 passaged 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 μ g per mouse; Biolegend), dissolved in 200 μ L 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 \times 3 (3 mm mean diameter) or 6 \times 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 μ g or 2 μ g in 10 μ L 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 μ g Siglec-F antibody monoclonal rat IgG_{2A} (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 μ g 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- μ m thickness and stained with H&E. The metastatic nodules and distribution were examined by an Axioscop 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 (53–6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-IFN γ (XMG1.2), anti-Granzyme B (NGZB), all from eBioscience; anti-CD4 (GK1.5), anti-CD69 (H1.2F3), anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-NK1.1 (PK136) anti-Siglec-F (E50–2440), all from BD PharMingen; anti-CD19 (6D5); anti-CD49b (DX5), anti-CD107a (LAMP-1), anti-CD335/NKp46 (29A1,4,9), anti-mPDCA1 (JF05–1C2.4.1) all from Miltenyi Biotec. Biotinylated antibodies were detected by streptavidin eFluor 450 (eBiosciences). Samples were run on a Gallios flow cytometer and analyzed with the Kaluza Analysis Software (Beckman Coulter, Pasadena, CA). Cell populations were defined as follows: CD45⁺CD3⁺CD19⁻CD4⁺CD8⁻ (CD4⁺ 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^{hi}Siglec-F^{hi}Ly6G⁻ (Eosinophils); CD45⁺CD11b^{hi}Ly6C^{hi}Ly6G⁻ (Mo-MDSC in tumors, Monocytes in lungs); CD45⁺CD11b^{hi}Ly6C⁺Ly6G⁺ (Gr-MDSC in tumors, Neutrophils in lungs); CD45⁺CD11b^{low}F4/80⁺ (Macrophages); CD45⁺CD8 α ⁻mPDCA1⁻CD11b⁺CD11c^{hi} (myeloid DC); CD45⁺mPDCA1⁺CD11b⁻CD11c^{+/low} (plasmacytoid DC); CD45⁺CD8 α ⁺CD11b⁻CD11c^{hi} (CD8 α ⁺ DC).

Intracellular staining

Spleen, lung and tumor cells were seeded ($0.3\text{--}1 \times 10^6$ cells per well) in 96-well U-bottomed plates in complete RPMI medium in the presence of Brefeldin A (1 μ L/ml; Biolegend), Monensin (2 μ L/ml; Biolegend) and anti-CD107a

mAb and stimulated with PMA (100 ng/mL) and Ionomycin (1 μ g/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-IFN γ mAb. Expression of CD107a and IFN γ in CD8⁺ T and NK cells was analyzed by flow cytometry. For detection of Granzyme B and IFN γ in pulmonary eosinophils, lung cells (1×10^6 cells per well) were incubated in 96-well U-bottomed plates in complete DMEM medium in the presence of Brefeldin A (1 μ L/ml) and Monensin (2 μ L/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-IFN γ and anti-Granzyme B mAbs. Expression of Granzyme B and IFN γ 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 (2×10^5) in 96 U-bottomed well plates in the presence or absence of class-I restricted peptide SIINFEKL (1 μ M; 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 μ Ci/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 TRIsure reagent (BioLine). mRNA was reverse transcribed by means of Tetro cDNA Synthesis Kit (BioLine). Quantitative reverse transcription-PCR (qPCR) was performed using Sensimix 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 quantization, threshold cycle (CT) values were determined by the sequence detection system software (Applied Biosystems). Data were normalized to HPRT housekeeping gene ($2^{-\Delta Ct}$ 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 FACSAria (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 1×10^6 /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 (Milteny 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 (1×10^4 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.

Acknowledgments

The authors wish to thank Dr. Silvia Piconese (La Sapienza University, Rome) and Drs. Gianni Marone and Maria Rosaria Galdiero (University of Naples Federico II, Naples) for critical reading of the manuscript and useful scientific discussions. We are also grateful to Mrs. Anna Pacca for animal care support.

Funding

This study was supported by the Italian Association for Cancer Research (AIRC; IG 14297 to G. Schiavoni) and the Italian Ministry of Health (RF-2011–02347120 to F. Mattei).

References

- Milovanovic M, Volarevic V, Radosavljevic G, Jovanovic I, Pejnovic N, Arsenijevic N, Lukic ML. IL-33/ST2 axis in inflammation and immunopathology. *Immunol Res* 2012; 52(1–2):89–99; PMID:22392053; <https://doi.org/10.1007/s12026-012-8283-9>
- Liew FY, Girard JP, Turnquist HR. Interleukin-33 in health and disease. *Nat Rev Immunol* 2016; 16(11):676–89; PMID:27640624; <https://doi.org/10.1038/nri.2016.95>
- Lu J, Kang J, Zhang C, Zhang X. The role of IL-33/ST2L signals in the immune cells. *Immunol Lett* 2015; 164(1):11–7; PMID:25662624; <https://doi.org/10.1016/j.imlet.2015.01.008>
- Oboki K, Nakae S, Matsumoto K, Saito H. IL-33 and airway inflammation. *Allergy Asthma Immunol Res* 2011; 3(2):81–8; PMID:21461246; <https://doi.org/10.4168/aaair.2011.3.2.81>
- Gabriele L, Schiavoni G, Mattei F, Sanchez M, Sestili P, Butcheroni C, Businaro R, Mirchandani A, Niedbala W, Liew FY et al. Novel allergic asthma model demonstrates ST2-dependent dendritic cell targeting by cypress pollen. *J Allergy Clin Immunol* 2013; 132(3):686–95 e687; PMID:23608732; <https://doi.org/10.1016/j.jaci.2013.02.037>
- Gajardo Carrasco T, Morales RA, Perez F, Terraza C, Yanez L, Campos-Mora M, Pino-Lagos K. Alarmin immunologists: IL-33 as a putative target for modulating t cell-dependent responses. *Front Immunol* 2015; 6:232; PMID:26082774; <https://doi.org/10.3389/fimmu.2015.00232>
- Bourgeois E, Van LP, Samson M, Diem S, Barra A, Roga S, Gombert JM, Schneider E, Dy M, Gourdy P et al. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur J Immunol* 2009; 39(4):1046–55; PMID:19266498; <https://doi.org/10.1002/eji.200838575>
- Smithgall MD, Comeau MR, Yoon BR, Kaufman D, Armitage R, Smith DE. IL-33 Alifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* 2008; 20(8):1019–30; PMID:18550585; <https://doi.org/10.1093/intimm/dxn060>
- Yang Q, Li G, Zhu Y, Liu L, Chen E, Turnquist H, Zhang X, Finn OJ, Chen X, Lu B. IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells. *Eur J Immunol* 2011; 41(11):3351–60; PMID:21887788; <https://doi.org/10.1002/eji.2011141629>
- Lu B, Yang M, Wang Q. Interleukin-33 in tumorigenesis, tumor immune evasion, and cancer immunotherapy. *J Mol Med (Berl)* 2016; 94(5):535–43; PMID:26922618; <https://doi.org/10.1007/s00109-016-1397-0>
- Villarreal DO, Weiner DB. Interleukin 33: A switch-hitting cytokine. *Curr Opin Immunol* 2014; 28:102–6; PMID:24762410; <https://doi.org/10.1016/j.coi.2014.03.004>
- Sun P, Ben Q, Tu S, Dong W, Qi X, Wu Y. Serum interleukin-33 levels in patients with gastric cancer. *Dig Dis Sci* 2011; 56(12):3596–601; PMID:21643739; <https://doi.org/10.1007/s10620-011-1760-5>
- Bergis D, Kassis V, Ranglack A, Koeberle V, Piiper A, Kronenberger B, Zeuzem S, Waidmann O, Radeke HH. High serum levels of the interleukin-33 receptor soluble ST2 as a negative prognostic factor in hepatocellular carcinoma. *Transl Oncol* 2013; 6(3):311–8; PMID:23730411; <https://doi.org/10.1593/tlo.12418>
- Yang ZP, Ling DY, Xie YH, Wu WX, Li JR, Jiang J, Zheng JL, Fan YH, Zhang Y. The association of serum IL-33 and sST2 with breast cancer. *Dis Markers* 2015; 2015:516895; PMID:26456994
- Hu LA, Fu Y, Zhang DN, Zhang J. Serum IL-33 as a diagnostic and prognostic marker in non-small cell lung cancer. *Asian Pac J Cancer Prev* 2013; 14(4):2563–6; PMID:23725175; <https://doi.org/10.7314/APJCP.2013.14.4.2563>
- Jovanovic I, Radosavljevic G, Mitrovic M, Juranic VL, McKenzie AN, Arsenijevic N, Jonjic S, Lukic ML. ST2 deletion enhances innate and acquired immunity to murine mammary carcinoma. *Eur J Immunol* 2011; 41(7):1902–12; PMID:21484786; <https://doi.org/10.1002/eji.2011141417>
- Jovanovic IP, Pejnovic NN, Radosavljevic GD, Pantic JM, Milovanovic MZ, Arsenijevic NN, Lukic ML. Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells. *Int J Cancer* 2014; 134(7):1669–82; PMID:24105680; <https://doi.org/10.1002/ijc.28481>
- Xiao P, Wan X, Cui B, Liu Y, Qiu C, Rong J, Zheng M, Song Y, Chen L, He J et al. Interleukin 33 in tumor microenvironment is crucial for the accumulation and function of myeloid-derived suppressor cells. *Oncoimmunology* 2015; 5(1):e1063772; PMID:26942079; <https://doi.org/10.1080/2162402X.2015.1063772>
- Zhang Y, Davis C, Shah S, Hughes D, Ryan JC, Altomare D, Peña MMO. IL-33 promotes growth and liver metastasis of colorectal cancer in mice by remodeling the tumor microenvironment and inducing angiogenesis. *Mol Carcinog* 2017; 56(1):272–87; PMID:27120577; <https://doi.org/10.1002/mc.22491>
- Villarreal DO, Wise MC, Walters JN, Reuschel EL, Choi MJ, Obeng-Adjei N, Yan J, Morrow MP, Weiner DB. Alarmin IL-33 acts as an immunoadjuvant to enhance antigen-specific tumor immunity. *Cancer Res* 2014; 74(6):1789–800; PMID:24448242; <https://doi.org/10.1158/0008-5472.CAN-13-2729>
- Gao K, Li X, Zhang L, Bai L, Dong W, Shi G, Xia X, Wu L. Transgenic expression of IL-33 activates CD8(+) T cells and NK cells and inhibits tumor growth and metastasis in mice. *Cancer Lett* 2013; 335(2):463–71; PMID:23499895; <https://doi.org/10.1016/j.canlet.2013.03.002>
- Gao X, Wang X, Yang Q, Zhao X, Wen W, Li G, Lu J, Qin W, Qi Y, Xie F et al. Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *J Immunol* 2015; 194(1):438–45; PMID:25429071; <https://doi.org/10.4049/jimmunol.1401344>
- Kim J, Kim W, Moon UJ, Kim HJ, Choi HJ, Sin JI, Park NH, Cho HR, Kwon B. Intratumorally establishing type 2 innate lymphoid cells blocks tumor growth. *J Immunol* 2016; 196(5):2410–23; PMID:26829987; <https://doi.org/10.4049/jimmunol.1501730>
- Anderson EL, Kobayashi T, Iijima K, Bartemes KR, Chen CC, Kita H. IL-33 mediates reactive eosinophilopoiesis in response to airborne allergen exposure. *Allergy* 2016; 71(7):977–88; PMID:26864308; <https://doi.org/10.1111/all.12861>
- Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potentially activates human eosinophils. *J Allergy Clin Immunol* 2008; 121(6):1484–90; PMID:18539196; <https://doi.org/10.1016/j.jaci.2008.04.005>
- Hashiguchi M, Kashiwakura Y, Kojima H, Kobayashi A, Kanno Y, Kobata T. IL-33 activates eosinophils of visceral adipose

- tissue both directly and via innate lymphoid cells. *Euro J Immunol* 2015; 45(3):876-85; PMID:25504587; <https://doi.org/10.1002/eji.201444969>
27. Ikutani M, Yanagibashi T, Ogasawara M, Tsuneyama K, Yamamoto S, Hattori Y, Kouro T, Itakura A, Nagai Y, Takaki S et al. Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity. *J Immunol* 2012; 188(2):703-13; PMID:22174445; <https://doi.org/10.4049/jimmunol.1101270>
 28. Carretero R, Sektioglu IM, Garbi N, Salgado OC, Beckhove P, Hammerling GJ. Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8(+) T cells. *Nat Immunol* 2015; 16(6):609-17; PMID:25915731; <https://doi.org/10.1038/ni.3159>
 29. Mattes J, Hulett M, Xie W, Hogan S, Rothenberg ME, Foster P, Parish C. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: An eotaxin and STAT6-dependent process. *J Exp Med* 2003; 197(3):387-93; PMID:12566422; <https://doi.org/10.1084/jem.20021683>
 30. Delyon J, Mateus C, Lefevre D, Lanoy E, Zitvogel L, Chaput N, Roy S, Eggermont AM, Routier E, Robert C. Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: An early increase in lymphocyte and eosinophil counts is associated with improved survival. *Ann Oncol* 2013; 24(6):1697-703; PMID:23439861
 31. Gebhardt C, Sevko A, Jiang H, Lichtenberger R, Reith M, Tarnanidis K, Holland-Letz T, Umansky L, Beckhove P, Sucker A et al. Myeloid cells and related chronic inflammatory factors as novel predictive markers in melanoma treatment with ipilimumab. *Clin Cancer Res* 2015; 21(24):5453-9; PMID:26289067; <https://doi.org/10.1158/1078-0432.CCR-15-0676>
 32. Martens A, Wistuba-Hamprecht K, Geukes Foppen MH, Yuan J, Postow MA, Wong P, Romano E, Khammari A, Dreno B, Capone M et al. Baseline peripheral blood biomarkers associated with clinical outcome of advanced melanoma patients treated with ipilimumab. *Clin Cancer Res* 2016; 22(12):2908-18; PMID:26802161; <https://doi.org/10.1093/annonc/mdw016>
 33. Peske JD, Woods AB, Engelhard VH. Control of CD8 t-cell infiltration into tumors by vasculature and microenvironment. *Adv Cancer Res* 2015; 128:263-307; PMID:26216636; <https://doi.org/10.1016/bs.acr.2015.05.001>
 34. Sakkal S, Miller S, Apostolopoulos V, Nurgali K. Eosinophils in cancer: Favourable or unfavourable? *Curr Med Chem* 2016; 23(7):650-66; PMID:26785997; <https://doi.org/10.2174/0929867323666160119094313>
 35. Bonilla WV, Frohlich A, Senn K, Kallert S, Fernandez M, Johnson S, Kreuzfeldt M, Hegazy AN, Schrick C, Fallon PG et al. The alarmin Interleukin-33 drives protective antiviral CD8(+) T cell responses. *Science* 2012; 335(6071):984-9; PMID:22323740; <https://doi.org/10.1126/science.1215418>
 36. Zimmermann N, McBride ML, Yamada Y, Hudson SA, Jones C, Cromie KD, Crocker PR, Rothenberg ME, Bochner BS. Siglec-F antibody administration to mice selectively reduces blood and tissue eosinophils. *Allergy* 2008; 63(9):1156-63; PMID:18699932; <https://doi.org/10.1111/j.1398-9995.2008.01709.x>
 37. Hallstrand TS, Hackett TL, Altemeier WA, Matute-Bello G, Hansbro PM, Knight DA. Airway epithelial regulation of pulmonary immune homeostasis and inflammation. *Clin Immunol* 2014; 151(1):1-15; PMID:24503171; <https://doi.org/10.1016/j.clim.2013.12.003>
 38. Legrand F, Driss V, Delbeke M, Loiseau S, Hermann E, Dombrowicz D, Capron M. Human eosinophils exert TNF-alpha and granzyme A-mediated tumoricidal activity toward colon carcinoma cells. *J Immunol* 2010; 185(12):7443-51; PMID:21068403; <https://doi.org/10.4049/jimmunol.1000446>
 39. Dyer KD, Moser JM, Czapiga M, Siegel SJ, Percopo CM, Rosenberg HF. Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. *J Immunol* 2008; 181(6):4004-9; PMID:18768855; <https://doi.org/10.4049/jimmunol.181.6.4004>
 40. Dyer KD, Percopo CM, Rosenberg HF. IL-33 promotes eosinophilia in vivo and antagonizes IL-5-dependent eosinophil hematopoiesis ex vivo. *Immunol Lett* 2013; 150(1-2):41-7; PMID:23246474; <https://doi.org/10.1016/j.imlet.2012.12.002>
 41. Dominguez D, Ye C, Geng Z, Chen S, Fan J, Qin L, Long A, Wang L, Zhang Z, Zhang Y et al. Exogenous IL-33 restores dendritic cell activation and maturation in established cancer. *J Immunol* 2016; 198(3):1365-75; PMID:28011934; <https://doi.org/10.4049/jimmunol.1501399>
 42. Taub DD, Sayers TJ, Carter CR, Ortaldo JR. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytotoxicity. *J Immunol* 1995; 155(8):3877-88; PMID:7561094
 43. Eftimie R, Bramson JL, Earn DJ. Modeling anti-tumor Th1 and Th2 immunity in the rejection of melanoma. *J Theor Biol* 2010; 265(3):467-80; PMID:20450922; <https://doi.org/10.1016/j.jtbi.2010.04.030>
 44. Akimoto M, Maruyama R, Takamaru H, Ochiya T, Takenaga K. Soluble il-33 receptor sST2 inhibits colorectal cancer malignant growth by modifying the tumour microenvironment. *Nat Commun* 2016; 7:13589; PMID:27882929; <https://doi.org/10.1038/ncomms13589>
 45. Lim HX, Choi S, Cho D, Kim TS. IL-33 inhibits the differentiation and immunosuppressive activity of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *Immunol Cell Biol* 2016; 95(1):99-107; PMID:27507556; <https://doi.org/10.1038/icb.2016.72>
 46. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, Locksley RM. Systemically dispersed innate IL-13-expressing cells in Type 2 immunity. *Proc Natl Acad Sci USA* 2010; 107(25):11489-94; PMID:20534524; <https://doi.org/10.1073/pnas.1003988107>
 47. Cormier SA, Taranova AG, Bedient C, Nguyen T, Protheroe C, Pero R, Dimina D, Ochkur SI, O'Neill K, Colbert D et al. Pivotal advance: Eosinophil infiltration of solid tumors is an early and persistent inflammatory host response. *J Leukoc Biol* 2006; 79(6):1131-9; PMID:16617160; <https://doi.org/10.1189/jlb.0106027>
 48. Lotfi R, Lee JJ, Lotze MT. Eosinophilic granulocytes and damage-associated molecular pattern molecules (DAMPs): Role in the inflammatory response within tumors. *J Immunother* 2007; 30(1):16-28; PMID:17198080; <https://doi.org/10.1097/01.cji.0000211324.53396.f6>
 49. Zhang S, Bernard D, Khan WI, Kaplan MH, Bramson JL, Wan Y. CD4+ T-cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis. *Eur J Immunol* 2009; 39(5):1252-9; PMID:19338001; <https://doi.org/10.1002/eji.200839152>
 50. Zhang S, Li W, Xia Z, Mao Y. CD4 T cell dependent tumor immunity stimulated by dendritic cell based vaccine. *Biochem Biophys Res Commun* 2011; 413(2):294-8; PMID:21893031; <https://doi.org/10.1016/j.bbrc.2011.08.089>
 51. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. *Immunol Rev* 2010; 235(1):105-16; PMID:20536558; <https://doi.org/10.1111/j.0105-2896.2010.00908.x>
 52. Bochner BS. Systemic activation of basophils and eosinophils: Markers and consequences. *J Allergy Clin Immunol* 2000; 106(5):S292-302; PMID:11080745; <https://doi.org/10.1067/mai.2000.110164>
 53. Bouffi C, Rochman M, Zust CB, Stucke EM, Kartashov A, Fulkeron PC, Barski A, Rothenberg ME. IL-33 markedly activates murine eosinophils by an NF- κ B-dependent mechanism differentially dependent upon an IL-4-driven autoinflammatory loop. *J Immunol* 2013; 191(8):4317-25; PMID:24043894; <https://doi.org/10.4049/jimmunol.1301465>
 54. Simson L, Ellyard JI, Dent LA, Matthaei KI, Rothenberg ME, Foster PS, Smyth MJ, Parish CR. Regulation of carcinogenesis by IL-5 and CCL11: A potential role for eosinophils in tumor immune surveillance. *J Immunol* 2007; 178(7):4222-9; PMID:17371978; <https://doi.org/10.4049/jimmunol.178.7.4222>

55. Gatault S, Legrand F, Delbeke M, Loiseau S, Capron M. Involvement of eosinophils in the anti-tumor response. *Cancer Immunol Immunother* 2012; 61(9):1527-34; PMID:22706380; <https://doi.org/10.1007/s00262-012-1288-3>
56. Varricchi G, Bagnasco D, Borriello F, Heffler E, Canonica GW. Interleukin-5 pathway inhibition in the treatment of eosinophilic respiratory disorders: Evidence and unmet needs. *Curr Opin Allergy Clin Immunol* 2016; 16(2):186-200; PMID:26859368; <https://doi.org/10.1097/ACI.0000000000000251>
57. Long H, Liao W, Wang L, Lu Q. A player and coordinator: The versatile roles of eosinophils in the immune system. *Transfus Med Hemother* 2016; 43(2):96-108; PMID:27226792; <https://doi.org/10.1159/000445215>
58. Qin L, Dominguez D, Chen S, Fan J, Long A, Zhang M, Fang D, Zhang Y, Kuzel TM, Zhang B. Exogenous IL-33 overcomes T cell tolerance in murine acute myeloid leukemia. *Oncotarget* 2016; 7(38):61069-80; PMID:27517629; <https://doi.org/10.18632/oncotarget.11179>
59. Jensen-Jarolim E, Bax HJ, Bianchini R, Capron M, Corrigan C, Castells M, Dombrowicz D, Daniels-Wells TR, Fazekas J, Fiebiger E et al. Allergooncology – the impact of allergy in oncology. *Eaaci position paper. Allergy* 2017; <https://doi.org/10.1111/all.13119>.
60. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3(6):1101-8; PMID:18546601; <https://doi.org/10.1038/nprot.2008.73>