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Dissecting Interleukin-33/ST2- dependent pathway in antitumor immune response to melanoma

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Riassunto

L'interleuchina-33 (IL-33) è una citochina appartenente alla famiglia dell'interleuchina-1 (IL-1), espressa dalle cellule endoteliali ed epiteliali e associata a disturbi allergici, infiammazioni ed infezioni. IL-33, nella forma di precursore, svolge la propria azione come regolatore trascrizionale dei geni proinfiammatori prevalentemente nel nucleo delle cellule, tuttavia può avere funzione di allarmina quando, a seguito di un danno tissutale, può essere rilasciata nello spazio extracellulare e convertita nella sua forma biologicamente attiva. L'attivazione del pathway della IL-33 avviene attraverso il legame con il suo recettore specifico ST2, espresso da molte cellule ematopoietiche, comprese le cellule Th2, mastociti (MCs), cellule T regolatorie (Treg), cellule linfoidi innate del gruppo 2 (ILC2s), cellule mieloidi, cellule natural killer (NK), e linfociti T $CD8^+$.

IL-33 è una citochina prevalentemente implicata nell'induzione delle risposte immunitarie di tipo 2, tuttavia studi recenti hanno evidenziato un ruolo nella promozione delle risposte immunitarie di tipo 1, essa agisce infatti stimolando la secrezione delle citochine e chemochine Th1, le quali promuovono la proliferazione ed l'attivazione dei linfociti T $CD8^+$ e delle cellule NK. Per il suo doppio ruolo, IL-33 può essere considerata una citochina pleiotropica, in grado perciò di stimolare sia una risposta Th1 che Th2. Recentemente molti studi hanno sottolineato un ruolo controverso dell'asse IL-33/ST2 nella tumorigenesi, mostrando sia effetti immunoprotettivi e che pro-tumorali, a seconda del sito di espressione e dell'istotipo del tumore. Nei modelli di tumore della mammella e tumore del colon-retto, è stato dimostrato che l'asse IL-33/ST2 favorisce la crescita tumorale e la formazione di metastasi, facilitando l'accumulo di cellule mieloidi soppressorie (MDSC) all'interno del microambiente tumorale. Contrariamente a quanto accade per questi modelli tumorali, in modelli di tumore alla prostata e carcinoma renale, numerosi studi hanno evidenziato un ruolo protettivo della IL-33. Lavori recenti hanno evidenziato inoltre un ruolo anti-tumorale della IL-33 in modelli di melanoma umano e murino, tuttavia i meccanismi attraverso i quali la IL-33 esplica questa funzione protettrice risultano ancora da chiarire. Il melanoma è da sempre considerato uno dei tumori più aggressivi, resistente alla maggior parte delle chemioterapie e immunoterapie correnti. Evidenze cliniche in pazienti affetti da melanoma indicano che molti parametri associati a una risposta immunitaria Th2 correlano con una prognosi favorevole e con la risposta alla

terapia. Per questo motivo IL-33, essendo in grado di stimolare sia una risposta Th1 che una risposta Th2, potrebbe avere un ruolo chiave in questo modello tumorale.

In questo studio, abbiamo analizzato il ruolo dell'asse IL-33/ST2 nella crescita tumorale e nel processo metastatico del melanoma. I nostri dati mostrano come la somministrazione di IL-33 esogena in topi C57Bl/6, trapiantati con cellule di melanoma B16.F10, induce un rallentamento significativo della crescita tumorale ed un aumento della sopravvivenza nei topi. Questo effetto è associato ad un aumento, nell'infiltrato tumorale, di linfociti T $CD8^+$ e di eosinofili, e ad una elevata espressione di citochine e chemochine Th1/Th2 responsabili del reclutamento di queste popolazioni immuni al sito tumorale. La deplezione *in vivo* di eosinofili (mediante somministrazione di anticorpo anti-Siglec-F) è in grado di inibire l'azione antitumorale della IL-33 e il reclutamento dei linfociti T $CD8^+$ al sito tumorale, con conseguente crescita in questi topi del tumore primario. Perciò in questo modello gli eosinofili hanno il ruolo di cellule accessorie, in grado di controllare la crescita tumorale inducendo il reclutamento dei linfociti T $CD8^+$ i quali hanno invece un'azione citotossica diretta nei confronti delle cellule tumorali.

Abbiamo esplorato inoltre il ruolo dell'asse IL-33/ST2 nel processo metastatico del melanoma. La somministrazione intranasale di IL-33, prima dell'iniezione endovenosa di cellule di melanoma B16.F10, determina una riduzione della formazione di metastasi in modo ST2-dipendente. A questo proposito topi deficienti per il recettore ST2 ($ST2^{-/-}$) sviluppano metastasi polmonari in maniera significativamente maggiore rispetto ai topi di controllo, suggerendo che in seguito all'inoculo del tumore vengono prodotti livelli endogeni di IL-33 che giocano un ruolo importante nell'immunosorveglianza contro l'insorgenza di metastasi polmonari. Nel modello metastatico, IL-33 induce un forte reclutamento di eosinofili nel polmone dei topi trattati. La deplezione degli eosinofili in questi topi inibisce l'azione protettiva della IL-33 ma non influenza il reclutamento dei linfociti T $CD8^+$ nel sito polmonare, indicando quindi che gli eosinofili svolgono un ruolo diretto nell'ostacolare la formazione delle metastasi polmonari.

Abbiamo inoltre osservato che eosinofili differenziati da midollo osseo in presenza di IL-33 mostrano un fenotipo più attivato ed una più elevata capacità citotossica nei confronti delle cellule di melanoma B16.F10 rispetto agli eosinofili differenziati senza IL-33.

Infine, abbiamo valutato il crosstalk tra eosinofili e cellule melanoma utilizzando dispositivi microfluidici. Dati ottenuti dopo 48h di co-coltura tra eosinofili e cellule di

melanoma mostrano che gli eosinofili migrano in maniera attiva verso le cellule di melanoma trattate con IL-33 e non verso le cellule tumorali non trattate. Inoltre la migrazione e l'infiltrazione degli eosinofili dipende esclusivamente dalla presenza delle cellule del melanoma trattate con IL-33, e non dalla sola IL-33, suggerendo quindi che essa non agisce da chemoattrattante diretto per gli eosinofili, ma stimola le cellule di melanoma a secernere fattori in grado di attrarre gli eosinofili al sito tumorale.

I nostri risultati quindi dimostrano un importante ruolo antitumorale dell'asse IL-33/ST2 nella progressione del melanoma e nella formazione di metastasi, sottolineando il ruolo chiave di eosinofili in questo processo e aprendo prospettive per la creazione di nuove immunoterapie nel melanoma.

Abstract

Interleukin-33 (IL-33) is a cytokine belonging to the IL-1 family that is expressed by endothelial and epithelial cells, playing multiple roles in allergic disorders, inflammation and infection. In the precursor form, IL-33 is usually found in the cell nucleus where it acts as a transcriptional regulator of pro-inflammatory genes. Upon tissue damage or cell stress, IL-33 is released in the extracellular space and converted in a biologically active form which functions as an alarmin. 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. IL-33 has been originally implicated in the induction of type-2 immune responses, however recently it was shown to have a role in promoting type-1 responses stimulating the secretion of Th1 cytokines and chemokines and promoting the function of CD8 T and NK and NKT cells. For its dual role, IL-33 may be considered a pleiotropic cytokine inducing or reducing inflammation depending on the target tissue. Recently, many studies have underlined a controversial role of the IL-33/ST2 axis in anti-tumor immunity, showing both immunoprotective and tumor-promoting effects, depending on the site of origin and the clinical stage of disease. In breast and colorectal cancer models IL-33/ST2 axis was shown to promote tumor growth and metastases formation facilitating the accumulation of myeloid-derived suppressor cells (MDSCs) within the tumor microenvironment. On the other hand, in human models of prostate cancer and kidney renal clear cell carcinoma IL-33 displayed an antitumoral role, since downregulation of IL-33 expression is associated with metastatic progression. Moreover in 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 anti-tumor immunity *in vivo*. Moreover, in melanoma recent studies have underlined an antitumoral role of IL-33 although the mechanisms of action are ill defined. Melanoma is considered one of the most aggressive types of cancer, resistant to conventional chemotherapy and immunotherapy. Clinical evidences in melanoma patients indicate that eosinophils and other parameter associated to Th2 immune response correlate with response to therapy. Given the peculiar properties of IL-33 in promoting both Th1 and Th2 immune response, this cytokine may represent a potential target to harness the melanoma microenvironment.

In the present study, we have investigated the role of IL-33/ST2 axis in anti-tumor response to melanoma. Injection of IL-33 in mice bearing subcutaneous B16.F10 melanoma resulted in significant tumor growth delay and increase of mice survival. 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. Depletion of eosinophils by *in vivo* treatment with anti-Siglec-F antibody abolished the ability of IL-33 to restrict primary tumor growth by inhibiting the recruitment and activation of $CD8^+$ T cells at tumor site.

Moreover, we have explored the role of the IL-33/ST2 axis in melanoma metastatic process. Intranasal administration of IL-33, before intravenous injection of B16.F10 melanoma cells, determined a reduction in pulmonary metastasis formation in a ST2-dependent manner. Analysis of the lung immune environment revealed an important recruitment of eosinophil, but not of other immune cell populations, following treatment with IL-33 that prevented the onset of pulmonary metastasis. Accordingly, ST2-deficient mice developed pulmonary metastasis at higher extent than wild-type counterparts, associated with lower eosinophil frequencies in the lung. The role of eosinophils in our metastasis model is crucial, since restriction of macro and micrometastasis formation in mice receiving IL-33 was completely abolished after eosinophils depletion. The absence of eosinophils in this model did not determine the decrease of other immune cell populations, demonstrating that IL-33 inhibits pulmonary melanoma metastasis through recruitment of activated eosinophils without involvement of $CD8^+$ T or NK cells-mediated responses. We also showed that IL-33 is able to activate eosinophils *in vitro* resulting in efficient killing of target melanoma cells, suggesting a direct anti-tumor activity of eosinophils following IL-33 treatment.

Finally, we evaluated the crosstalk between eosinophils and melanoma cells using an organ-on-chip approach based on the use of microfluidic devices for co-culture of two different cellular systems. Image analysis of cell migration underlined a strong ability of IL-33 to attract eosinophils at tumor site. Eosinophils migration and infiltration was observed only in response to melanoma cells treated with IL-33, but not to IL-33 alone, suggesting that IL-33 does not act as a direct chemoattractant for eosinophils, but rather acts stimulating melanoma cells to secrete factors that recruit eosinophils at tumor site.

On the whole, our results demonstrate an important antitumoral role of IL-33/ST2 axis against melanoma progression and metastasis formation, underlying the key role of

eosinophils in this process and opening perspectives for novel cancer immunotherapy strategies.

Contents

1. Introduction

Cancer progression is a complex and multistep phenomenon that includes proliferation, evasion of apoptosis, maintenance of vascularization and ability for invasion and metastasis [1] . The tumor cells are able to create interactions with non-malignant cells to promote tumor growth creating a tumor microenvironment (TM) that consists of cancer cells, immune cells, endothelial cells, pericytes, and fibroblasts [2] . The interactions between cells in TM are critical for disease initiation, development and ultimately metastasis formation. For this reason, understanding the role of the TM in disease progression and chemotherapy is now considered central for cancer management. The tumor and the surrounding microenvironment are closely related and interact constantly through the release of extracellular signals that promote tumor angiogenesis and induce immune tolerance in order to allow the tumor growth and invasiveness [3, 4]. Intercellular communication is driven by a complex and dynamic network of cytokines, chemokines and growth factors that recruit immune cells at the tumor site [5]. The TM is rich in a variety of immune cells composed by macrophages, granulocytes, leukocytes, mast cells, dendritic cells (DCs), and natural killer (NK) [6] [7]. The regulation of immune responses is mediated by cytokines secreted from tumor and immune cells that could induce tumor suppression or promote tumor progression.

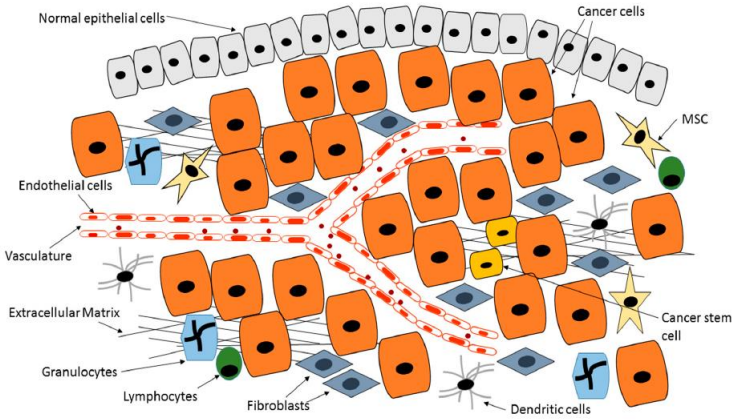


Figure 1: Tumor microenvironment. Tumor cells interact with several cells including mesenchymal stem cells (MSC), endothelial cells, fibroblasts, immune cells, cancer stem cells as well as with the extracellular matrix (ECM). *Senthebane et al. "The role of tumor*

microenvironment in chemoresistance", International Journal of Molecular Science, 2017.

1.1 IL-33

Interleukin-33 (IL-33) is a cytokine belonging to IL-1 family and expressed by endothelial cells, epithelial cells and fibroblast [8, 9]. It was first identified in 1999 as the protein DVS27 expressed in canine arteries [10], and later in 2003 as "nuclear factor from high endothelial venules" (NF-HEV) expressed in human endothelial cells [11]. Finally, IL-33 was rediscovered in 2005 by a computational sequence search, which identified it as an IL-1 family member [8]. In the precursor form, IL-33 exists as a nuclear protein that acts as a transcriptional regulator of pro inflammatory genes [12]. In the biologically active form, IL-33 is released, upon tissue damage, in the extracellular space where it functions as an alarmin [13]. IL-33 signals through its specific receptor ST2, which is expressed by many hematopoietic cells, including T helper 2 (Th2) cells, mast cells, regulatory T cells (Tregs), group 2 innate lymphoid cells (ILC2s), myeloid cells, natural killer (NK) cells and activated Th1 and $CD8^+$ T cells [14]. IL-33 has been originally implicated in the induction of type-2 immune responses, however recently it was shown to have a role in promoting type-1 responses stimulating the secretion of Th1 cytokines and chemokines and promoting the function of $CD8^+$ T, NK and NKT cells [15, 16]. For its dual role, IL-33 may be considered as a pleiotropic cytokine inducing or reducing inflammation depending on the target tissue.

Many studies have associated IL-33 with a variety of biological responses such as the development and regulation of immune responses, maintenance of tissue homeostasis, repair, and remodelling. The role of IL-33 has also been established in several diseases such as allergies, inflammation, infection and cancer where it can exert both immunoprotective or tumor-promoting effects, depending on the tissue of origin and on the clinical stage of disease [17-19].

1.1.1 Expression and release of IL-33

Under basal conditions, IL33 mRNA and protein are constitutively and abundantly expressed in many tissues in mice and humans [20]. It is stored and released quickly in response to cellular damage or tissue injury, therefore IL-33

has been considered as a danger-associated molecular pattern (DAMP) or an "alarmin" [21]. IL-33 is produced as a precursor or in full-length form (270 amino acids in humans and 266 in mice) with the N-terminus containing a non classical nuclear localization sequence and a homeodomain-like helix-turn-helix DNA-binding domain as well as a chromatin-binding domain (residues 1–75 in human). N-terminal domain is necessary for nuclear localization [12]. Indeed, transgenic mice, lacking the IL-33 nuclear localization sequence and expressing IL-33 under the endogenous promoter, exhibit a constitutive release of bioactive IL-33 which leads the development of Th2-based inflammation, elevated eosinophilia and fatal multiorgan failure [22]. These data suggest that nuclear IL-33 controls self-production and release in an autoregulatory circuit. IL-33 can operate in at least two spaces, nuclear and extracellular, and in at least two forms which are: full-length IL- 33 (proIL-33) and mature IL-33 (mtrIL-33). The nuclear space is the exclusive domain of proIL-33, it is composed by an amino-terminal nuclear domain that is necessary and sufficient for nuclear localization and chromatin association [12]. As previously described, nuclear localization and binding to histones are important for IL-33 function and regulation, thus proIL-33 acts as a transcriptional regulator of pro-inflammatory genes expression [23]. Nucleus is where IL-33 is usually found, however proIL-33 can translocate to the cytoplasm and processed by caspases which inactivate its pro-inflammatory function [24]. Since proIL-33 is an active protein, it can be released in the extracellular milieu, following tissue damage, where it acts as an alarmin. ProIL-33 is often digested into mtrIL-33 (Fig. 2), a form with a lower molecular weight (18-20 kDa), by neutrophil cathepsin G or elastase that produce a major bioactive form of IL-33 [25]. Many studies have observed that the generation of highly active mtrIL-33 may be crucial for the induction of ST2-dependent responses [26, 27]. This suggests that IL-33 activity could be amplified by the inflammatory environment thanks to the cleavage of the full-length form by protease from innate immune cells recruited to the injured tissue. This is an important advance in the understanding of IL-33 biology and related pathologies such as asthma, rheumatoid arthritis or intestinal inflammation. IL-33 has been shown to initiate a type 2 inflammatory response in these diseases, and neutrophils, which play a critical role, could lead to the generation of superactive forms of IL-33 which may exacerbate the immune response [25].

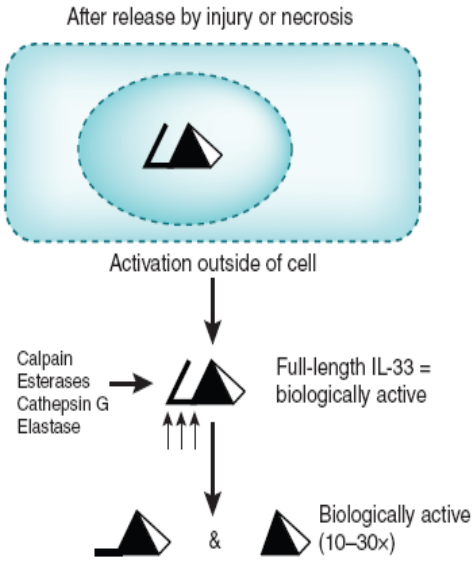


Figure 2: Processing of full-length IL-33. Full-length IL-33 is stored in the nucleus and released after a tissue damage. It can be processed by proteases in shorter forms that have an enhanced biological activity. *Martin N. T. "Interleukin 33 is a guardian of barriers and a local alarmin", Nature Immunology, 2015.*

1.1.2 IL-33/ST2 signalling pathway

IL-33 released outside the cells can mediate its biological effects by interacting with its specific receptor ST2 [28, 29]. IL-33 signalling through ST2 has profound implications for the immune system and therefore must be regulated at multiple levels. Indeed ST2 exists in two isoforms: the membrane-bound form (ST2L) and the soluble form (sST2). The availability of IL-33 is tightly regulated by soluble sST2, a decoy receptor which prevents productive interaction of IL-33 with ST2L [30, 31] .

IL-33 binding to ST2L induces a conformational change that allows ST2 to interact with IL-1RAcP leading to the dimerization of the TIR domain [32]. This allows the recruitment of adaptor myeloid differentiation primary response protein 88 (MyD88) and subsequently the activation of kinases IRAK1 and IRAK4 and the adaptor protein TNF receptor associated factor 6 (TRAF6). Finally these events determine the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-kB (NF-κB) pathways. The activation of the pathways drive the proliferation, cell survival and Th1/Th2 cytokines secretion by $ST2^{+}$ cells [33-35].

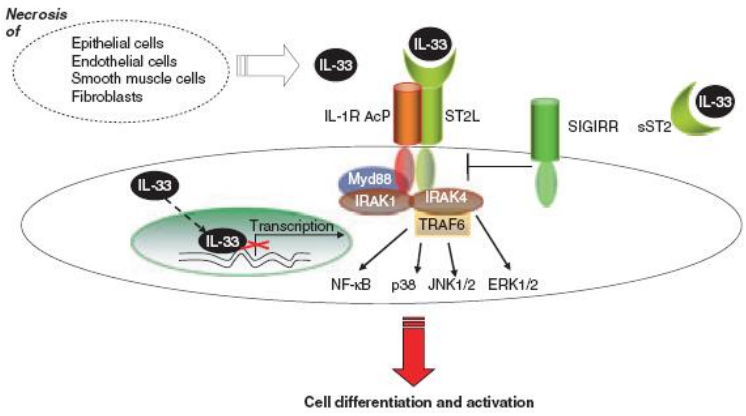


Figure 3: IL-33 pathway. IL-33 binds to ST2 receptor that interact with IL1RAcP for the recruitment of MyD88 and TRAF6. These events allow the activation of NF-kB pathway and differentiation and activation of $ST2^+$ cells. *Kurowska-Stolarska M "Interleukin-33: a novel mediator with a role in distinct disease Pathologies", Journal of Internal Medicine, 2010.*

1.1.3 Immune regulation by IL-33

Tissue damage and mechanical stress to epithelial, endothelial, and stromal cells lead to the release of IL-33 from these cells. IL-33 then signals through ST2 expressed by many different immune cells, enhancing their function [36]. Although initially viewed as a key cytokine in allergic reactions and other Th2-mediated responses[37], IL-33 is now known to stimulate also Th1-type of immune responses [38-40]. Indeed IL-33 not only induces helper T cells, mast cells, eosinophils and basophils to produce type 2 cytokines but can also regulate macrophages, dendritic cells (DCs), NKs, $CD8^+$ T cells under certain pathophysiological conditions [38, 41, 42]. In Th2 cells, ST2 expression depends on the Th2 lineage-specifying transcription factor GATA-3 and signal transducer and activator of transcription 5 (STAT5) [43]. GATA-3 promotes the up-regulation of ST2 in Th2 cells, although a GATA-3-independent pathway has been recently described in Th1 cells. In these cells, ST2 expression depends on the expression of Th1 lineage-specifying transcription factor T-bet and STAT4, but not GATA-3 [43].

Extracellular IL-33 promotes the proliferation and the activation of group ILC2s stimulating the production of cytokines like IL-13 and IL-5 that are important for

the response to helminths and allergens [44]. IL-33-activated ILC2s can promote eosinophils expansion and survival, as well as the maintenance of M2-polarized macrophages [45, 46]. Activated ILC2s have also been shown to license DCs to produce CCL17, thereby recruiting eosinophils and Th2 cells during memory responses [47]. This suggests that early interactions between $ST2^+$ ILC2s, DCs, and Th2 cells shape the memory response against both pathogen-associated and environmental antigens. As mentioned above, macrophages, mast cells, basophils, eosinophils, and DCs have been shown to express ST2, implying these cells as targets for IL-33. IL-33 induces the proliferation, activation and migration of eosinophils, moreover it stimulates their degranulation and the production of IL-13, TGF-β, CCL3, CCL17, and CCL24 in the lungs during airway inflammation [48-50]. Macrophages can respond to IL-33 by upregulating IL-13 mRNA, creating an autocrine M2 polarization loop and increasing Th2 polarization *in vivo* [51]. Basophils respond to IL-33 by producing GM-CSF, which stimulates the expansion of dendritic cells, as well as IL-4 and IL-13, which promote Th2 responses [52, 53]. The expression of ST2 is relatively low in DCs but the stimulation with IL-33 induces the production of a number of cytokines and chemokines, such as IL-6, TNF, IL-1β, and CCL17, by bone marrow-derived DCs and upregulates the expression of class II major histocompatibility complex (MHC) and co-stimulatory molecules, such as CD80, CD86, CD40 [42, 54]. Moreover, IL-33 has a crucial role in sustaining the proliferation of a Treg cell subset expressing ST2, contributing to establish tolerance in models of autoimmunity and transplantation [55]. Several studies have reported that administration of recombinant IL- 33 leads to significant increase in the frequency and total number of splenic Treg cells [56, 57]. IL-33 induced $ST2^+$ Treg cell proliferation and expression of the epidermal growth factor-like molecule amphiregulin (AREG), which enhances immune regulatory functions and supports tissue repair [58].

IL-33 drives Th2 immune response particularly during an allergen exposure or parasite infection in the lung. In these settings, IL-33 increases the expression of ST2 on basophils, mast cells and ILC2 that produce cytokines for eosinophils recruitment at lung. This condition mediates on the one hand the parasite expulsion and, on the other hand, causes the allergic pathology and tissue fibrosis, therefore IL-33 stimulate myeloid cells to produce IL-2 which support Treg cells

proliferation and differentiation [59, 60]. The ability of IL-33 to stimulate Treg cells following the elimination of pathogens would allow the repair of tissue that are damaged as a result of the infection [58, 61].

IL-33 can stimulate cytotoxic T lymphocytes (CTLs) and Th1 cells to promote a protective antiviral response. When tissue damage is accompanied with pathogens infiltration, IL-33 operates developing Th1 immune responses to remove the pathogens [62]. IL-12 secreted by antigen presenting cells (APCs) after binding with pathogen-associated molecular patter (PAMP) upregulates ST2 expression on CD8 T cells that respond to IL-33 and secrete IFN-γ, TNFα and other Th1 cytokines and chemokines [39, 41, 62] [16]. Moreover, IL-33 enhances the differentiation of T cells and also augments T cells functionality, particularly the capacity to co-express several cytokines and chemokines [41, 63]. Notably, ST2 is constitutively expressed by NK cells and the triggering of IL-33/ST2 pathway causes expansion and activation of NK cells and increases the production of IFN-γ [15, 38]. Since IL-33 can affect several type of immune cells, it is considered a pleiotropic cytokine that is able to stimulate both Th1 and Th2 immune responses.

1.1.4 IL-33 and cancer

IL-33 has been studied mainly in the context of allergic disorders or parasite infection. However, recent studies have addressed its role in cancer, revealing a controversial role of IL-33/ST2 axis in antitumor immune responses [64-66]. Depending on the site of origin and on the clinical stage of disease, IL-33 can exert both pro-tumoral and anti-tumoral functions, increasing cancer cell proliferation and migration or otherwise preventing tumor growth and metastasis formation.

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 [67, 68]. In mice, the IL-33/ST2 axis promotes intratumoral cell proliferation, vascularization and epithelial cell trasformation of breast and colorectal cancer [69, 70]. Indeed in these tumor models, IL-33 administration *in vivo* operates a pro-tumoral role stimulating tumor growth and promoting metastasis formation at lung and liver. In a murine 4T1 breast cancer model, IL-33 treatment is associated with accumulation of $CD11b^+$ $Gr1^+$ $TGFb^+$ myeloid-derived suppressor cells (MDSCs), ILC2 and Treg cells which promote the

generation of immune tolerance resulting in increased tumor growth [71, 72]. Recent studies further supported the crucial role of IL-33 in the expansion of MDSCs in breast cancer model, by reducing apoptosis and promoting proliferation of MDSCs within the tumor microenvironment [69]. MDSCs show a strong ability to suppress adaptive immune responses facilitating tumor cell invasion and metastasis, thus promoting tumor growth [73]. Mice lacking a functional ST2 receptor ($ST2^{-/-}$) exhibit a decrease in the percentage of MDSCs in tumor tissue, blood and spleen [69]. In human MCF7 and MDA-MB231 breast cancer models, IL-33 show a direct action on malignant cancer cells. Stimulation with IL-33 enhances proliferation as well as colony formation of a ST2-expressing breast cancer cells *in vitro* [74]. Thus, IL-33/ST2 pathway can act directly on tumor cells, or indirectly, modulating the immune response and for this reason is considered an important target for breast cancer therapy.

In non-small cell lung cancer and hepatocellular carcinoma IL-33 also plays a pro-tumoral role, since IL-33 and ST2 expression is increased in tumor tissue compared with normal tissue [75, 76]. In addition, in patients with lung cancer the transcript level of IL-33 is positively correlated with tumoral stages and overall survival [76]. In gastric cancer IL-33/ST2 axis exerts a pro-tumorigenic role increasing the proliferation and invasion of tumor cells through the activation of ERK1/2 pathway which is crucial for invasion and metastasis [77]. In colorectal cancer the activation of IL-33/ST2 axis promotes tumorigenesis increasing tumor growth *in vivo* while the administration of IL-33 stimulates the upregulation of stem cell genes, the recruitment of macrophages at tumor site and the secretion of CXCR4 and metalloproteinases (MMP-2 and MMP-9) that contribute increasing the invasive potential of tumor cells resulting in faster tumor development [78] . Other studies have shown that the expression of IL-33 and ST2 is increased in colon carcinoma compared with normal tissue because the stimulation of IL-33/ST2 pathway increases the intestine inflammation and the expression level of IL-6, a cytokine correlated with tumorigenesis [70]. On the contrary, the expression of soluble ST2 (sST2) is inversely correlated with colorectal cancer progression, since sST2 inhibits tumor angiogenesis and modifies tumor microenvironment reducing macrophages infiltration [70].

On the other hand, IL-33 can also exert an anti-tumoral role. In a human papilloma virus (HPV)-associated model, IL-33 was shown to act as a potent

vaccine adjuvant augmenting Th1 and $CD8^+$ T-cell responses and inducing anti-tumor immunity *in vivo* [79]. Moreover a research group has demonstrated the anti-tumoral role of IL-33 in prostate carcinoma. This study shows that IL-33 expression is higher in primary tumors with respect to metastases of murine prostate carcinoma suggesting an inverse correlation between IL-33 expression and disease progression [80]. Indeed, in human prostate and kidney renal clear cell carcinoma, down-regulation of IL-33 is associated with the transition from primary to metastatic tumors and represents a new form of tumor immune escape, implying IL-33 expression as a potential immune biomarker [80].

In recent years, an anti-tumoral role of IL-33 in melanoma development has also emerged. Transgenic host and tumoral expression of IL-33 was shown to inhibit tumor growth in melanoma-bearing mice, with two proposed mechanisms on the tumor microenvironment, namely by increasing NK and $CD8^+$ T cell responses [81, 82] and through ILC2-mediated tumor apoptosis [83]. In mice transplanted with B16 melanoma, IL-33 was shown to decrease the accumulation, the differentiation and suppressive capacity of granulocytic MDSCs in the spleen and tumor microenvironment [84]. Moreover, MDSCs treated *in vitro* with IL-33 show a reduced capacity to induce the proliferation and differentiation of Treg cells as well as decreased reactive oxygen species production and T-cell suppressive functions. These features result in subversion of immunosuppressive environment, thus leading to tumor growth reduction following IL-33 exposure *in vivo* [84]. Overall, these findings suggest that IL-33 in melanoma may increase immunogenicity promoting antitumor immune responses, although the mechanisms of action remain to be better defined.

1.2 Metastatic melanoma

Metastatic melanoma is one of the most aggressive types of cancer characterized by a rapid progression, metastasis to regional lymph nodes and distant organs and high resistance to conventional chemotherapy, immunotherapy and targeted therapy. The median survival time for patients with metastatic melanoma is 8-9 months and the 3-year overall survival rate is less than 15% [85]. For this reason new approaches to melanoma treatment are urgently needed.

Melanoma is a heterogeneous disease, which suggests a richly complex etiology. The classification of melanoma is based on histological parameters of the lesions, or considering the characteristics of genetic mutations which are useful for targeted therapies. Molecular analyses have revealed consistent genetic patterns among different melanoma subtypes. The most common genetic mutation in melanoma is in the BRAF (V600E) gene, observed in approximately 50-60% of skin melanomas [86]. BRAF mutation involves the synthesis of abnormal BRAF protein that induces rapid growth and cell division [87]. In tumors, where this mutation is detected, it is possible to use active drugs which are able to act against this melanoma subtype. In addition, NRAS mutations are observed in 15–30% of cutaneous melanomas and are mutually exclusive of BRAF mutations. The mutated NRAS protein supports the growth and survival of tumor cells and renders melanoma particularly aggressive [88]. Loss of tumor suppressor genes (TSGs) is also been described in melanoma, often accompanying mutated oncogenes within the same tumor. Several studies have shown that the cell cycle regulators p16 and p14ARF are frequently inactivated in melanoma tumors [89, 90]. Finally, a small percentage of melanoma tumors (1-3%) display mutations in the c-KIT gene, which is found in melanoma of the skin chronically damaged by the sun [91]. Although targeted therapies are available for melanoma patients carrying certain mutations, the clinical response is often unsatisfactory and the management of patients not displaying specific mutations remains an issue. Therefore, the search for alternative and/or combined therapeutic approaches against this deadly disease is becoming crucial.

A modern method for melanoma treatment is based on cancer immunotherapy, given the importance of immune system in the control of tumor cell proliferation and invasiveness. Immunotherapeutic approaches have underscored the potential of manipulating immune responses and laid the foundations for further elucidating complex underlying immunological mechanisms. Melanoma is one of the most immunogenic tumors and its immunogenicity has made this disease a preferred target for application of different therapeutic strategies based on the stimulation of tumor-specific T-cell-mediated immune responses or on the adoptive transfer of activated tumor-specific T cells [92-94]. However, the results of such immunotherapeutic approaches in clinical trials are mostly unsatisfactory. This could be due to a profound immunosuppression in melanoma microenvironment.

In fact, melanoma cells secrete soluble factors that shape a tumor microenvironment that favours tumor escape from host immunosurveillance [95]. From this point of view, melanoma may be considered as a representative model for cross-talk between malignant cells and the immune system [96, 97].

During melanoma carcinogenesis the activity of effector immune infiltrating cells, such as DCs, $CD4^+$ and $CD8^+$ Tcells, may be influenced by the prevalence of MDSC or Treg cells [98]. These immunosuppressive cells are enriched and activated within the melanoma microenvironment and were found to induce a profound impairment of anti-tumor immune responses, thus leading to melanoma progression. The Th1-type of immune response has a key role in the anti-tumor immunity due to a potent tumor-specific CTL activity [99]. $CD8^+$ T cells are thought to be the principal effectors in anti-tumor immunity due to their ability to directly lyse tumor cells. However, there is increasing evidence supporting a direct role for $CD4^+$ T cells in anti-tumor immunity, independent of $CD8^+$ T cells [100]. Indeed, melanoma is known to be particularly resistant to CTL activity, since it can evade $CD8^+$ T cell attack by losing or down-regulating surface MHC class I molecules [101]. Several studies have demonstrated that $CD4^+$ T cell can overcome this mechanism, since $CD4^+$ T cells can eliminate MHC-negative tumors which are resistant to $CD8^+$ T cells [100, 102]. Tumor-specific $CD4^+$ T cells may induce indirect anti-tumor mechanisms contributing to tumor destruction. These include the secretion of either Th1 (IFN-γ) or Th2 (IL-4) cytokines both of which display anti-angiogenic properties and are able to recruit immune effectors, like NK cells, macrophages and eosinophils to the tumor site [100, 103, 104]. Of interest, adoptive transfer of tumor-specific Th2, but not Th1, $CD4^+$ T cells was shown to induce the clearance of established lung melanoma metastasis in mice [102]. Accordingly, some evidences indicate that eosinophilia and other parameters associated to Th2-type of immunity may also correlate with response to therapy and favourable outcome [105].

Recently, a key role of eosinophils in anti-tumor response has emerged, particularly in solid cancers, such as melanoma. Although eosinophilia is generally considered as a good prognostic value for tumor growth and metastasis reduction [106, 107], the mechanisms underlying this phenomenon are unknown. In a prospective study in patients with metastatic

melanoma undergoing immunotherapy with Ipilimumab it was recently reported that high early eosinophilic counts in the peripheral blood was associated with an improved clinical response [108]. In responding patients an increase in the percentage of eosinophils, compared with their number before the beginning of Ipilimumab therapy, was observed. On the contrary, non-responding patients showed fewer eosinophils and a significant decrease of Eotaxin-1 concentration in the serum, indicating poor condition for eosinophils accumulation [108].

It has been recently reported that in a mouse model of melanoma, eosinophils play a crucial role recruiting $CD8^+$ T cells at tumor site [109]. Activated eosinophils migrate at tumor site after the release by tumor cells of damage-associated molecular patterns (DAMPs), wich are known to be chemoattractants for eosinophils. After migration, eosinophils produced a large amounts of chemokines, such as CXCL9, CXCL10, CCL5 that attracted $CD8^+$ T cells to the tumor, resulting in tumor eradication and improved mice survival [109].

Although eosinophils can have an indirect role in anti-tumor activity, other studies underline a direct mechanism of tumor cell killing. Human eosinophils are able to induce apoptotic cell death in colon carcinoma cells through direct contact between eosinophils and tumor cells and the upregulation of CD11a and ICAM-1 adhesion molecules [110]. Some studies underline possible mechanisms for eosinophils anti-tumor response based on the release of their cytotoxic granules, by presentation of tumor antigens to T cells, or by conditioning the tumor microenvironment through the release of immunomodulatory factors [111, 112]. In this respect, since IL-33 is known to promote eosinophilopoiesis, eosinophil recruitment to inflamed tissues and degranulation, we envisage that this cytokine may also play a role in the anti-tumoral activities of eosinophils [113-115].

2. Aims

As described above, local interactions between melanoma cells and immune infiltrating cells play a key role in melanoma development and metastatic spread. Although protective antitumor immunity is classically thought to require a Th1 type of immune responses, clinical evidence in melanoma patients indicate that eosinophilia and other parameter associated to Th2 immune responses may also correlate with response to therapy and favourable outcome. Given the peculiar ability of IL-33 in stimulating both Th1 and Th2 immune responses, we hypothesize that this cytokine may condition the local immune environment promoting anti-tumor responses against melanoma. Therefore, the main objective of this study is to investigate whether IL-33 plays a beneficial role in anti-tumor immune responses against melanoma growth and metastasis *in vivo*. To this end, we have investigated on the role of IL-33/ST2 axis in melanoma growth, focusing the attention on the tumor microenvironment, in particular on immune cell populations (i.e. eosinophils) involved in the immune response and the cytokines and chemokines produced. In a second step, the role of IL-33/ST2 axis was evaluated in melanoma metastasis formation at pulmonary level. The modification of immune cell subsets and activity was analysed both at local and systemic level. Finally we investigated the role of IL-33/ST2 axis in the crosstalk between cancer and immune cells using the microfluidic-based cell-on-chip technology, an innovative tool that reconstructs complex *in vivo* microenvironments on a microscale level, allowing for the study of cancer-immune cells interactions. Our results will provide important insights on the impact of IL-33-mediated immune pathways for future therapeutic strategies against clinical melanoma and other cancers.

3. Materials and methods

3.1 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 eight-week-old female mice were used for experiments. The 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 (Institut Gustave Roussy, Villejuif, France). The cells were routinely tested for morphology, growth curve and absence of mycoplasma and passaged for no more than 4 times from thawing.

3.2 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) was dissolved in 200 μl of phosphate-buffered saline (PBS) and injected intraperitoneally in mice 5 times, every other day, at different stages of tumor growth, starting from either T0 (0 mm mean diameter), 3x3 (3 mm mean diameter) or 6x6 (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 4 intranasal instillations (1 μg or 2 μg in 10 μl per mouse): 3 consecutive days before and on day 6 after tumor injection. Control groups received intranasal PBS. Mice were sacrificed on days 8-19 from tumor inoculation for evaluation of lung metastasis formation.

3.3 *In vivo* depletion of eosinophils

For the depletion of eosinophils in the subcutaneous melanoma model, mice received 5 repeated intraperitoneal injections of rat IgG2A anti-Siglec-F

monoclonal antibody (mAb, Clone #238047; 15 μg per mouse). For metastasis model, a group of mice received 6 intraperitoneal injections, every other day, and 2 every three days, of anti-Siglec-F mAb (15 μg per mouse). Control groups consisted of mice injected intraperitoneally with Isotype-matched control Ab.

3.4 Histological analysis of lung tissues

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

3.5 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) and type III collagenase (1 mg/ml) for 30 minutes at room temperature in agitation, followed by EDTA (0.1 M, pH 7.2) for additional 5 minutes. The homogenate was then passed through a cell sieve and the resulting cell suspension was treated with a lysis buffer (140 mM NH_4Cl, 17 mM Tris HCl, pH 7.2) to eliminate red blood cells.

3.6 Clonogenic assay for micrometastasis determination

Pulmonary cell suspensions were resuspended in DMEM supplemented with 10% FBS, plated in 6 well-plates at different dilutions in triplicates and cultured for 14 days. Medium was changed every other day in order to remove dead or unattached cells, allowing for 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 (http://imagej.nih.gov/ij/, 1997-2016). The presence of micrometastasis was represented as a fraction of area covered by microscope image compared to the total area of the image itself.

3.7 Analysis of immune cell infiltrate in tumor and lung tissues

For flow cytometry analysis cells were stained with the fluorescently labeled mAbs (Table I). Biotinylated antibodies were detected by streptavidin eFluor 450. Samples were run on a Gallios flow cytometer and analyzed with the Kaluza Analysis Software. 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); $CD45^+CD11b^{hi}Siglec\text{-}F^{hi}Ly6G^-$ (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); $CD45^+CD8\text{-}mPDCA1^-CD11b^+CD11c^{hi}$ (myeloid DC); $CD45^+mPDCA1^+CD11b^-CD11c^{+/low}$ (plasmacytoid DC); $CD45^+CD8a^+CD11b^-CD11c^{hi}$ ($CD8\alpha^+$ DC).

Table I. List of mAbs used in flow cytometry analyses.

Marker	Clone	Company
CD45	30 F11	Biolegend
CD3	145-2C11	Biolegend
CD8a	53-6.7	eBioscience
CD4	GK1.5	BD Pharmingen
F4/80	BM8	Biolegend
CD11b	M1/70	EBioscience
CD11c	N418	eBioscience
IFNγ	XMG1.2	eBioscience
CD69	H1.2F3	BD Pharmingen
Ly6C	AL-21	BD Pharmingen
Ly6G	1A8	BD Pharmingen
NK 1.1	PK136	BD Pharmingen
CD19	6D5	Miltenyi Biotec
CD49b	DX5	Miltenyi Biotec
CD107a	LAMP-1	Miltenyi Biotec
CD335/NKp46	29A1,4,9	Miltenyi Biotec
PDCA1	JF05-1C2.4.1	Miltenyi Biotec
Siglec-F	E50-2440	BD Pharmingen

3.8 Intracellular staining

Spleen, lung and tumor cells were seeded (0.3-1 x 10^6 cells per well) in 96-well U-bottomed plates in complete RPMI medium in the presence of Brefeldin A (1 μl/ml), Monensin (2 μl/ml) and anti-CD107a mAb and stimulated with PMA (100 ng/ml) and Ionomycin (1 μg/ml) for 5 hours at 37°C. After incubation, cells were first surface stained with anti-CD45, anti-CD3, anti-CD8, and anti-NK 1.1 mAbs and then intracellularly labelled with and anti-IFNγ mAb. Expression of CD107a and IFNγ in $CD8^+$ T and NK cells was analysed by flow cytometry. For detection of Granzyme B and IFNγ in pulmonary eosinophils, lung cells (1 x 10^6 cells per well) were incubated in 96-well U-bottomed plates in complete DMEM medium in the presence of Brefeldin A (1 μl/ml) and Monensin (2 μl/ml) for 5 hours 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 labelled with anti-IFNγ and anti-Granzyme B mAbs. Expression of Granzyme B and IFNγ in eosinophils ($CD45^+Siglec\text{-}F^+Ly6G^-CD11c^-CD3^-$) was evaluated by flow cytometry.

3.9 *In vitro* proliferation assay

Spleen cells from B16.OVA tumor-bearing mice treated with IL-33 or with PBS were seeded (2 x 10^5) in 96 round-bottomed well plates in the presence or absence of class-I restricted peptide SIINFEKL (1 μM) or class-II restricted peptide 323-339 (20 ng/ml). Cell cultures were incubated in complete medium for 4 days at 37°C, in 5% CO2 and then pulsed with ^{3}H-thymidine (1 μCi/well) for 16 h. Incorporation of ^{3}H-thymidine was analyzed by liquid scintillation counting.

3.10 Quantitative PCR

Total RNA was extracted from melanoma and lung tissues explanted from mice and from eosinophils isolated from peritoneum or lung, by using TRIsure reagent. Messenger RNA was reverse transcribed by means of Tetro cDNA Synthesis Kit. Quantitative reverse transcription-PCR (qPCR) was performed using Sensimix Plus SYBR Kit containing the fluorescent dye SYBR Green. Forward and reverse primers were purchased from Eurofin Genomix (Table II). The conditions of real-time PCR reaction were given as follows: 15 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C (45 cycles). PCR products were continuously measured by means of an ABI 7500 Real-Time PCR System. 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. Data were normalized to HPRT housekeeping gene ($2^{-\Delta Ct}$ method) and presented as fold change expression vs control.

Table II. List of forward and reverse primers used for real-time quantitative PCR.

Gene	Forward and Reverse Primers (5'-3')	NCBI Accession Number	Amplicon Size (Base Pairs)
CCL2	AGGTGGTCCCAAAGAAGCTGTA ATGTCTGGACCCATTCCTTCT	NM_011333.3	85 bp
CCL5	ATATGGCTCGGACACCACTC GTGACAAACACGACTGCAAGA	NM_013653.3	123 bp
CCL11	CACCCTGAAAGCCATAGT GTCAAGAGAGGAGGTTG	NM_011330.3	142 bp
CCL13	GGGCTGGAGCTGAGGAGATT CTTCTGCTGTGTGGATTCTGTC	NM_010779.2	224 bp
CCL17	TGGCTGCTCTGCTTCTGGGG TGCACAGATGAGCTTGCCCTGG	NM_011332.3	197 bp
CCL20	GACAGATGGCCGATGAAGCTT TCACAGCCCTTTTCACCCAGT	NM_016360.1	108 bp
CCL22	TGGCTACCCTGCGTGTCCCA CGTGATGGCAGAGGGTGACGG	NM_009137.2	142 bp
CCL24	CTCCTTCTCCTGGTAGCCTG GATGAAGATGACCCCTGCCT	NM_019577.4	168 bp
CX3CL1	ACGAAATGCGAAATCATGTGC CTGTGTCGTCTCCAGGACAA	NM_009142	120 bp
CXCL9	TCTGCCATGAAGTCCGCTGTTCT TGGTGCTGATGCAGGAGCATCG	NM_008599.4	112 bp
CXCL10	CTCTCGCAAGGACGGTCCGC TCCGGATTCAGACATCTCTGCTCAT	NM_021274.1	166 bp
IFN-γ	TCAAGTGGCATAGATGTGGAAGAA TGGCTCTGCAGGATTTTCATG	NM_008337.4	92 bp
IL-2	CCTGAGCAGGATGGAGAATTACA TCCAGAACATGCCGCAGAG	NM_008366.3	141 bp
IL-4	ACAGGAGAAGGGACGCCAT GAAGCCCTACAGACGAGCTCA	NM_021283.2	95 bp
IL-5	CACAGCTGTCCGCTCACCGA CCACAGTACCCCCACGGACAGT	NM_010558.1	150 bp
IL-10	GGTTGCCAAGCCTTATCGGA ACCTGCTCCACTGCCTTGCT	NM_010548.2	191 bp
IL-12	GGAAGCACGGCAGCAGAATA AACTTGAGGGAGAAGTAGGAATGG	NM_001303244.1	180 bp
IL-13	AGACCAGACTCCCCTGTGCA TGGGTCCTGTAGATGGCATTG	NM_008355.3	123 bp
IL-17	GCTCCAGAAGGCCCTCAGA AGCTTTCCCTCCGCATTGA	NM_010552.3	142 bp
IL-25	GGCATTTCTACTCAGGAACGGA GGTGGAGAAAGTGCCTGTGC	NM_080729.3	109 bp
ST2 (IL1RL1)	GAATGGGACTTTGGGCTTTG CAGGACGATTTACTGCCCTCC	NM_001025602.3	76 bp
TNFα	CATCTTCTCAAAATTCGAGTGACAA TGGGAGTAGACAAGGTACAACCC	NM_013693.3	175 bp
TSLP	CGACAGCATGGTTCTTCTCA CGATTTGCTCGAACTTAGCC	NM_021367.2	170 bp
GZMB	GATCGGGAGTGTGAGTCCTAC GAAAGCACGTGGAGGTGAAC	NM_013542.2	183 bp
HPRT	CTGGTGAAAAGGACCTCTCG TGAAGTACTCATTATAGTCAAGGGCA	NM_013556.2	109 bp

3.11 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. 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 employed. Briefly, peritoneal cells were harvested from naïve mice 3 days after intraperitoneal injection of 3 ml of 3% thioglycollate broth medium, 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 ($CD45^+CD11b^+Siglec\text{-}F^+Ly6G^-CD11c^-$) were purified by cell sorting on a FACSAria. Cell purity was checked on the same instrument immediately after sorting and ranged >98%.

3.12 Bone marrow-eosinophils differentiation

Eosinophils were generated from cultures of bone marrow (BM) cells following a modified protocol previously described [116]. 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. As illustrated in Fig. 4, BM cells were cultured at 1 x 10^6/ml in RPMI 1640 containing 20% FBS, 1% glutamine, 25mM Hepes, 1X NEAA, 1 mM sodium pyruvate, supplemented with 100 ng/ml SCF and 100 ng/ml FLT3-L. On day 4, 10 ng/ml rmIL-5 was 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 in order 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. Eosinophils purity was determined by flow cytometry ($CD11b^+Siglec\text{-}F^+Ly6G^-CD11c^-$) and resulted >80%. Activation of

eosinophils was further determined by up-regulation of CD69, CD11b and side scatter.

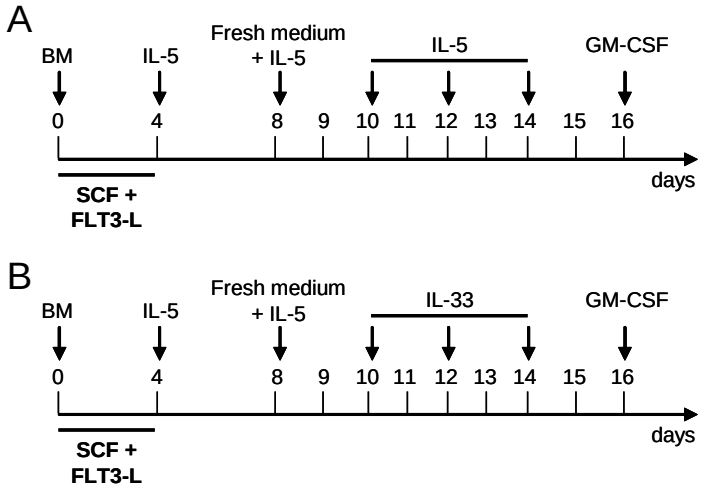


Figure 4: Generation of BM-derived eosinophils.

A) Schematic representation of protocols employed to generate IL-5-EO. BM cells were cultured with Flt3-L and SCF for 4 days followed by IL-5. B) Generation of IL-33-EO. BM cells were cultured with SCF, FLT3-L and IL-5 as previously described. At day 10 eosinophils received IL-33 till day 16. GM-CSF was then added, in both cell cultures, for 24 h before cell harvesting.

3.13 Cytotoxicity assay

Eosinophil-mediated cytotoxicity against tumor cells was evaluated by flow cytometry. B16.F10 melanoma cells were labeled with the PKH26 Red fluorescent Cell Linker and then seeded in 96 wells U-bottomed plates (1 x 10^4 cells per well) in the presence of BM-derived eosinophils (IL-5 EO or IL-33 EO) at E:T ratio of 25:1, 50:1 or 100:1. Co-cultures were incubated for 5 hours at 37°C. Cells were then stained with Annexin-V and then analyzed by flow cytometry. Apoptosis of target melanoma cells was calculated as percentage of Annexin-V^+ cells among gated $PKH26^+$ population.

3.14 Microfluidic devices for co-culture of B16.F10 melanoma cells and immune cells.

Microfluidic devices were fabricated in PDMS, a biocompatible silicone elastomer, following well-established replica molding procedures [97, 99, 117]. The master molds were created by a two-layer microfabrication process, using the negative photoresist SU-8 and carried out according to parameters specified in the manufacturer's datasheets. Briefly, patterns for standard photolithography were designed with CAD software and transferred onto two chrome masks by electron-beam lithography. A schematic picture of the microfluidic device used is shown in Fig. 5. Prior to cell loading, the devices were sterilized under ultraviolet light in a laminar flow hood for 15 min and put on ice to contrast gel solidification. B16.F10 melanoma cells were stained with live-compatible PKH67 Green Fluorescent Cell Linker and resuspended in Matrigel (2 mg/ml). Where indicated, IL-33 (100 ng/ml) was added to the cell-Matrigel mixture. The melanoma cell-Matrigel mixtures (2×10^4 cells in 3 μl) were then loaded in the narrow chambers of the devices (Fig. 5). After melanoma cell-Matrigel loading, the device was placed at 37°C for 30 minutes to allow for gel solidification. Subsequently, naïve spleen cells or BM eosinophils were labeled with live-compatible PKH26 Red Fluorescent Cell Linker, resuspended in DMEM complete medium and loaded (1×10^6 cells in 200 μl) in the central chamber of the device. The lateral reservoir chambers were then filled with fresh medium. Phase-contrast, visible and fluorescence microphotographs of devices were generated over a period of 48 hours by using an EVOS-FL fluorescence microscope, provided with built-in imaging software for image overlays. The profile of eosinophils migration were analyzed with ImageJ software after 48 h of co-culture and calculated as fluorescence intensity. The slope of a straight line, expressed by the coefficient of x, indicates eosinophils migration toward IL-33 treated melanoma cells (slope>0), or on the contrary, towards untreated melanoma cells (slope<0).

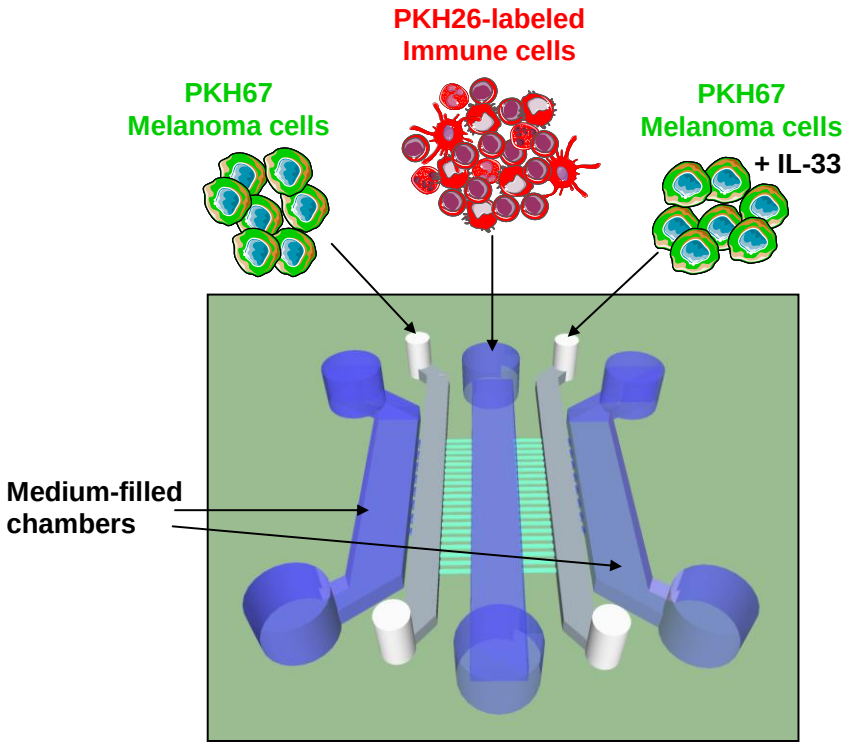


Figure 5: Schematic representation of gel-based microfluidic device. Lateral channels are filled through the appropriate inlet wells with medium. The central channel is loaded with red-fluorescent labelled spleen cells or eosinophils. The two grey chambers are loaded with green-fluorescent labelled B16.F10 melanoma cells resuspended in Matrigel with or without 100 ng/ml IL-33. Microchannels connect the gel chambers with the central chamber. The height of the loading wells is 1000 μm.

3.15 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 ($P \leq 0.05$).

4. Results

4.1 IL-33 inhibits melanoma growth in mice

We investigated the effects of IL-33 treatment in a mouse model of transplantable melanoma. To this purpose, C57Bl/6 mice were implanted subcutaneously with B16.F10 metastatic melanoma cells and treated with 5 injections of IL-33 (0.4 µg intraperitoneally) at different tumor stages as schematized in Fig. 6A. Data revealed that treatment with IL-33 significantly inhibited melanoma progression (Fig. 6B) at all tumor growth stages, with higher efficiency when administered at earlier stages, and increased the survival rate (Fig. 6C) of tumor-bearing mice.

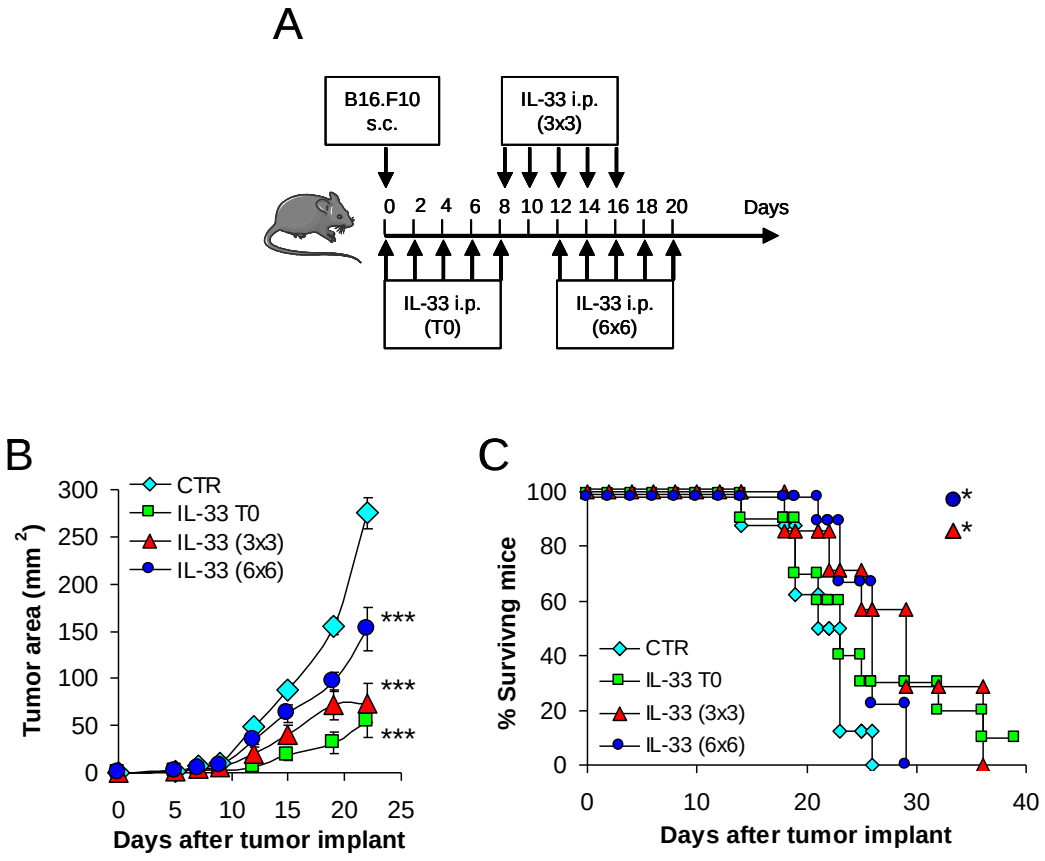
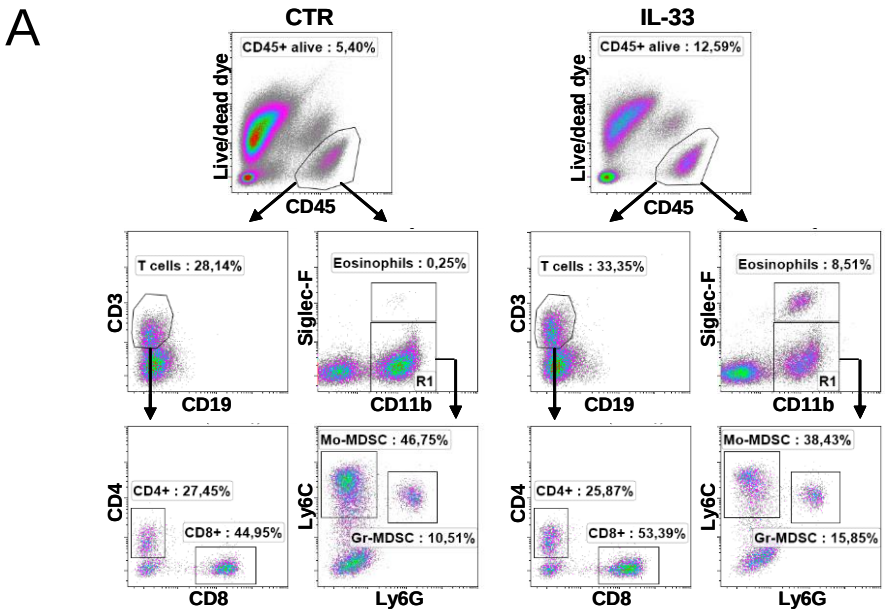


Figure 6: IL-33 delays melanoma growth and prolongs survival in mice (A) Schematic representation of experimental protocol. C57BL/6 mice were injected subcutaneously with 0.8 x 10^6 B16.F10 melanoma cells and treated intraperitoneally with recombinant mouse IL-33 (0.4 µg) at various times after tumor implant, namely at the time of melanoma cells injection (T0), when tumor nodules reached the mean diameter of 3 mm or 6 mm. (B) Tumor growth in the indicated groups. Mean tumor area in individual mice ±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<0.01; ***P<0.001. i.p.: intraperitoneal; CTR: control; s.c.: subcutaneous.

4.2 IL-33 promotes tumor immune infiltration, the upregulation of Th1/Th2 cytokines and chemokines and the activation of CD8$^+$ T and NK cells.

To investigate the effects of IL-33 on the local tumor microenvironment and identify the immune cell components regulated by IL-33/ST2 axis relevant for antitumor response, we characterized the local immune infiltrate in melanoma-bearing animals following IL-33 treatment. Melanoma tumors were explanted one day after the last cytokine administration and multicolour flow cytometry analysis was performed. The data showed an important recruitment of immune cells in IL-33 treated mice compared to controls and, in particular, an increase in CD8$^+$ T cells and eosinophils in IL-33-treated tumors (Fig. 7A-B). In contrast, the frequency of tumor-infiltrating CD11b$^+$ myeloid cells, particularly of suppressive monocytic (Mo)-MDSC, F4/80$^+$ macrophages and myeloid DC (mDC) was markedly decreased in mice receiving IL-33 (Fig. 7B). In addition, no significant differences were observed with CD4$^+$ T and B lymphocytes, NK cells, plasmacytoid DC (pDC), CD8alpha-expressing DC (CD8α$^+$ DC), or granulocytic (Gr)-MDSC (Fig. 7B).



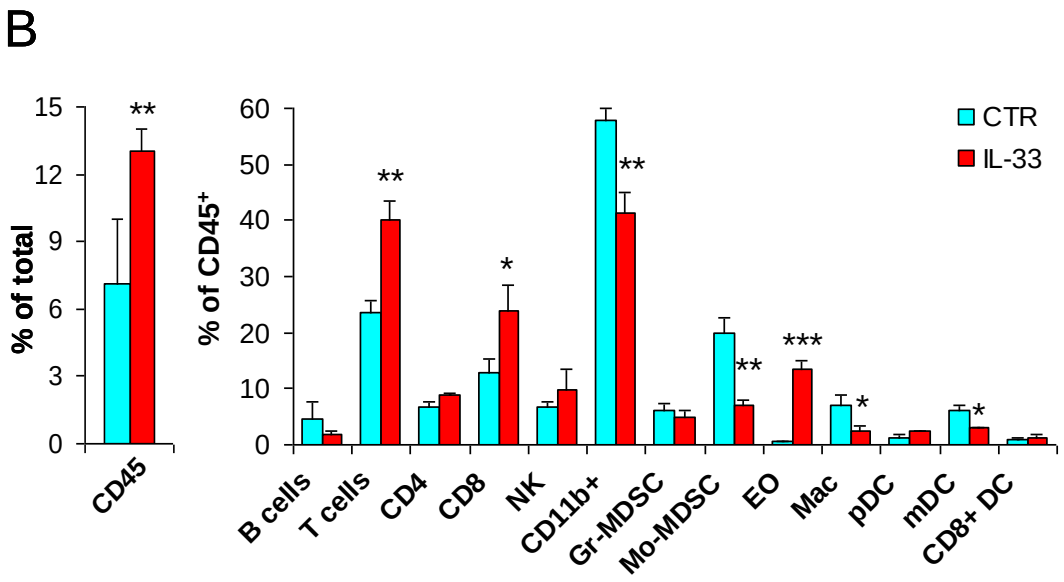


Figure 7: Effects of IL-33 on the local tumor immune microenvironment. C57BL/6 mice received subcutaneously 0.8 x 10^6 B16.F10 melanoma cells and then were treated with 0.4 µg of IL-33, starting from 3 mm mean diameter. Tumors were harvested the day after the last IL-33 injection. (A) Gating strategy of immune cell populations analyzed. (B) Flow cytometry analysis of tumor-infiltrating cells. Data show mean values of individual mice (n=5) from 4 separate experiments ± SD. *P< 0.05; **P<0.01; ***P<0.001.

Next, we investigated the intratumoral expression of Th1 and Th2-related cytokines and chemokines involved in immune cell trafficking. Quantitative PCR of tumor tissues from IL-33 treated mice revealed increased expression levels of IL-33 pathway-related cytokines thymic stromal lymphopoietin (TSLP) and IL-25, ST2 receptor and the chemokines CCL17, CCL20 and CCL22, with respect to controls (Fig. 8). 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 cells [118] and of CCL5, CCL11, CCL13, CCL24, all involved in eosinophils recruitment [119] (Fig. 8). 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 to the controls (Fig. 8).

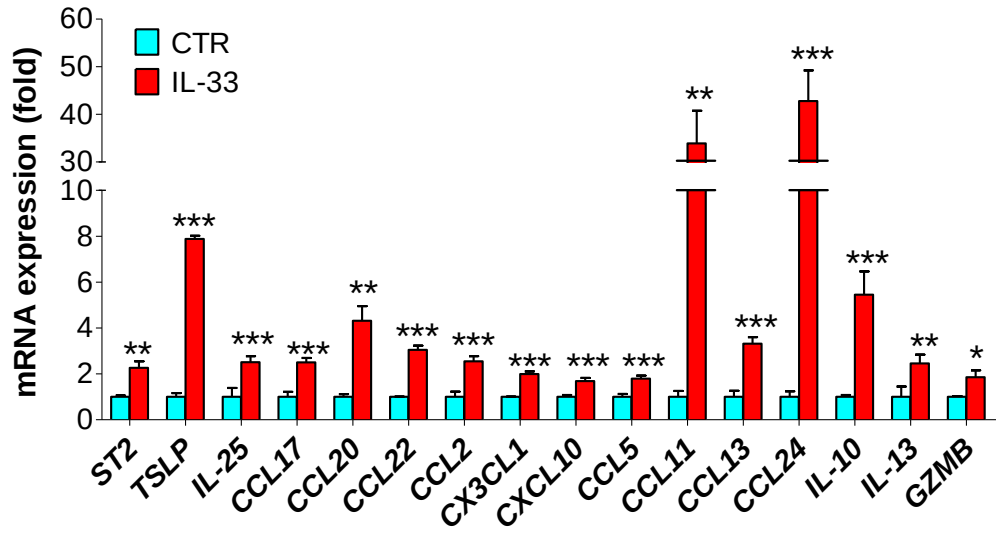


Figure 8: Cytokines and chemokines expression in tumor microenvironment. Evaluation of Th1/Th2 cytokines and chemokines in melanoma tumors by real-time qPCR. Data are expressed as fold change of mRNA expression *vs* control. *P< 0.05; **P<0.01; ***P<0.001.

Since IL-33 is known to stimulate both NK and $CD8^+$ T cell-mediated effector response [81, 82], we further analysed the effects of IL-33 on the activation of these immune effectors in our model. Spleen and tumor cells were stimulated with PMA/Ionomycin for 5 hours at 37°C in the presence of brefeldin/monensin and CD107a and subsequently labeled with IFNγ for flow cytometry analysis. Compared to controls, mice receiving IL-33 showed increased percentages of $CD8^+$ T and NK cells co-expressing the effector molecules CD107a and IFNγ both in the tumor (Fig. 9 A-B) and in the spleen (Fig. 9C), suggesting local and systemic activation of $CD8^+$ T and NK cells following treatment with IL-33.

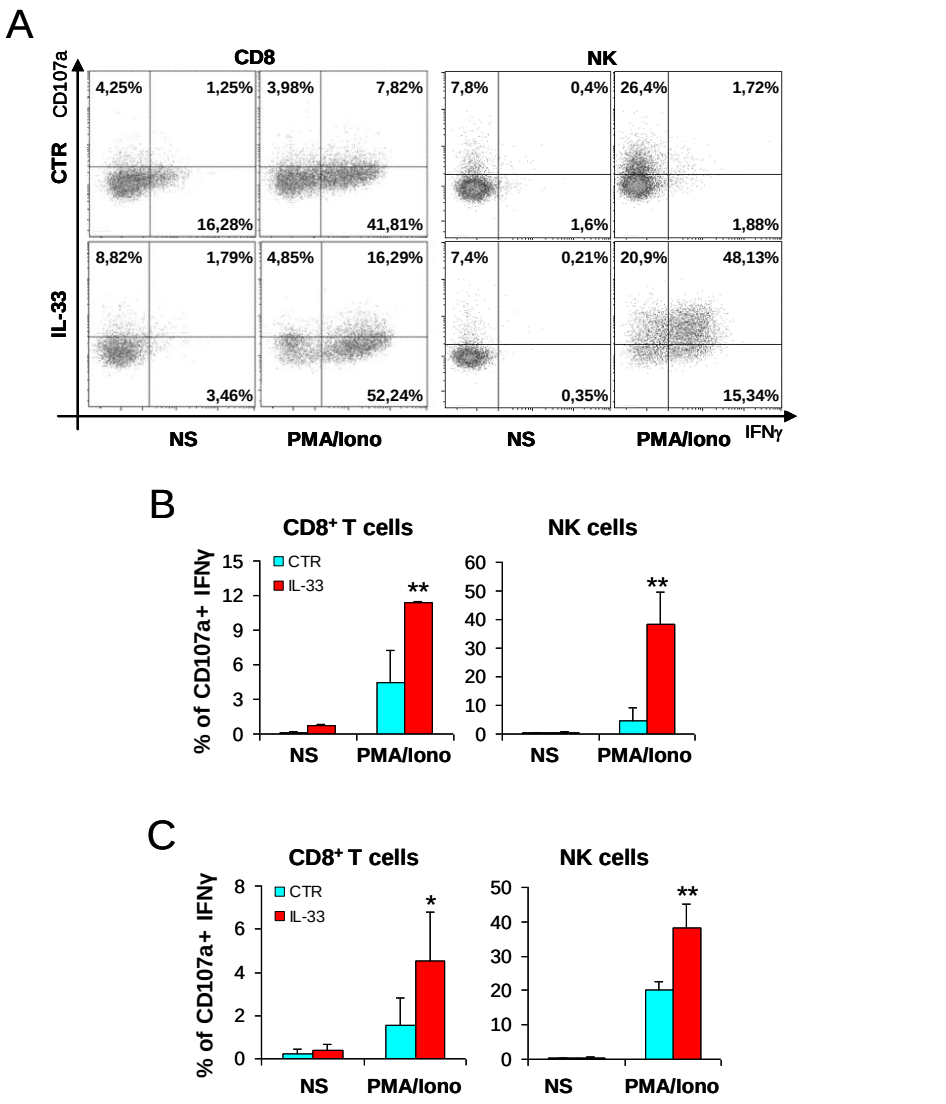


Figure 9: IL-33 effect on the activation of CD8+T and NK cells. Tumors and spleen were explanted from melanoma-bearing mice treated or not with IL-33 for flow cytometry analysis. Flow cytometry analysis of IFNγ and CD107a expression in CD8+ T and NK cells from tumors (B) and spleen (C) after *in vitro* stimulation with PMA/Ionomycin. Histograms represent the frequencies of the indicated populations. Mean from at least 3 experiments is shown ± SD. *P< 0.05; **P<0.01.

Finally, 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. 10).

Taken together, these results indicate that IL-33 hampers melanoma growth *in vivo* generating an immune microenvironment favourable for tumor growth control, through

the up-regulation of both Th1 and Th2 cytokines and chemokines, inducing the homing of $CD8^+$ T lymphocytes and eosinophils at the tumor site and leading to local and systemic activation of $CD8^+$ T and NK cells.

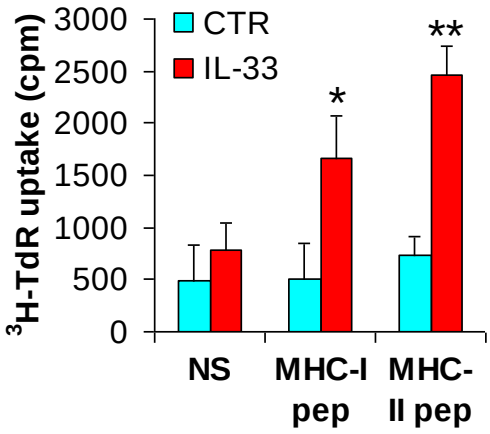


Figure 10: Antigen-specific proliferative response of spleen cells. Mice were injected subcutaneously with 0,8 x 10^6 B16.OVA cells and treated with IL-33. Spleen was removed to obtain splenocytes for *in vitro* proliferation assay. Spleen cells were in *vitro* re-stimulated with class-I or class-II-restricted OVA peptides. Data show ^{3}H-thymidine incorporation after 4 days of culture, mean ± SD of culture triplicates.*P< 0.05; **P<0.01. CTR: control; NS: not-stimulated.

4.3 Depletion of eosinophils abolishes the anti-tumor effect of IL-33 and the recruitment and activation of $CD8^+$ T cells

Eosinophils were recently described to have a key role in antitumor immune response and, in particular, to mediate tumor regression through recruitment of tumor-reactive $CD8^+$ T cells [109]. Accordingly, we sought to evaluate whether IL-33-induced eosinophils were able to promote the homing of $CD8^+$ T cells at tumor site. Eosinophils were sorted from melanoma tumors and analyzed for the expression of chemokines connected with $CD8^+$ T cells recruitment. Data showed that eosinophils sorted from tumors of IL-33-treated mice expressed high levels of the $CD8^+$ T cell-attracting chemokines CCL5, CXCL9, CXCL10 (Fig. 11).

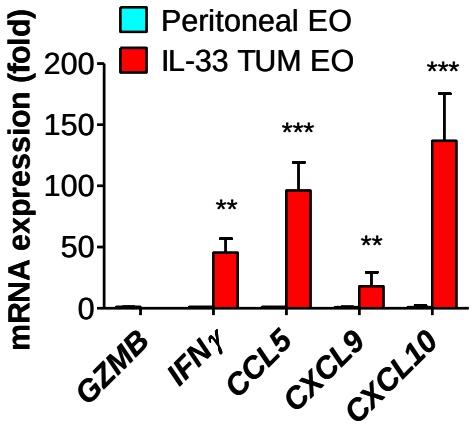


Figure 11: Expression of cytokines and chemokines in tumor-infiltrating eosinophils from IL-33-treated mice. C57BL/6 mice transplanted subcutaneously with 0,8 x 10^6 B16.F10 melanoma cells were treated with 0.4 μg of IL-33, starting from 3 mm mean diameter. One day after the last IL-33 injection, tumors were explanted and eosinophils were FACS-sorted. Quantitative PCR analysis of expression of indicated cytokines was performed in comparison with peritoneal eosinophils as controls. Data are expressed as fold change of mRNA expression vs peritoneal eosinophils. **P<0.01; ***P<0.001. TUM: tumor; EO: eosinophils.

To address the role of eosinophils in IL-33-induced anti-tumor effects, tumor-bearing mice receiving IL-33 were injected with an anti-Siglec-F monoclonal antibody (mAb, Fig. 12A) that selectively depletes eosinophils inducing apoptotic cell death. The depletion of eosinophils was verified in spleen, tumor and lymph nodes (Fig. 12B). We observed that eosinophil depletion abolished the anti-tumor efficacy of IL-33 resulting in rapid melanoma tumor growth (Fig. 12C).

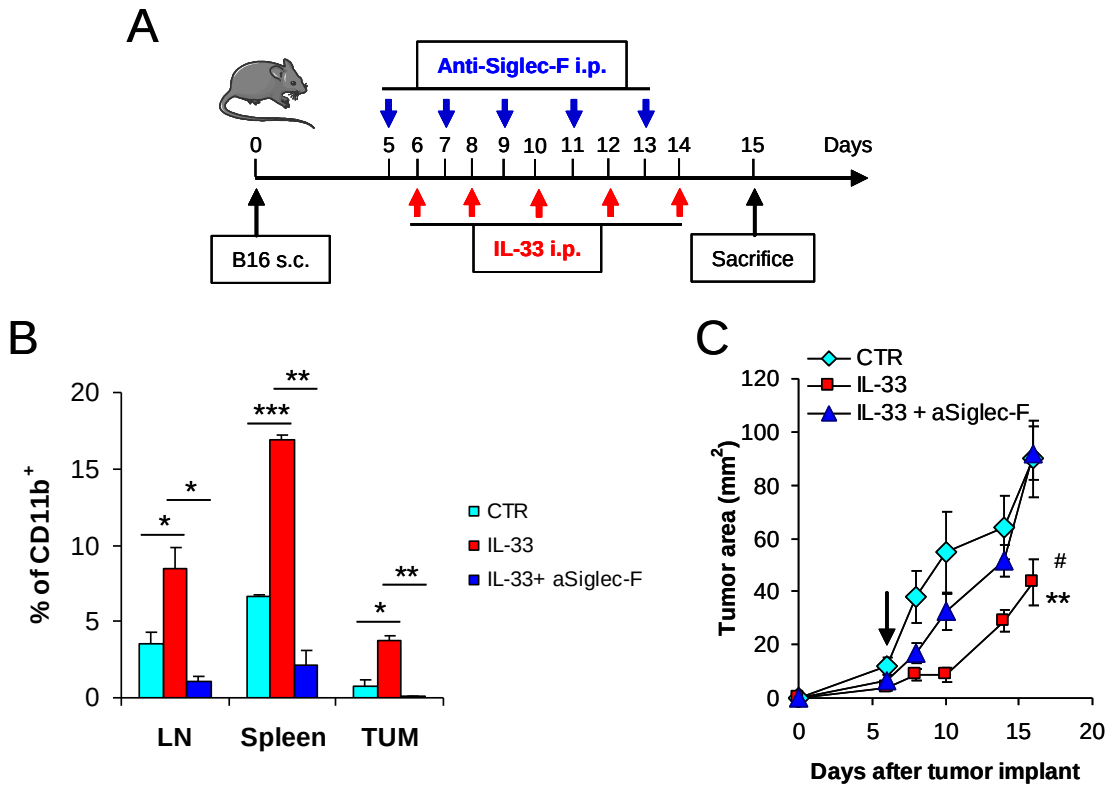


Figure 12: Eosinophil depletion abolishes the anti-tumor effect of IL-33 in melanoma-bearing mice. (A) Schematic representation of experimental protocol. C57BL/6 mice were injected subcutaneously with 0.8 x 10^6 B16.F10 melanoma cells and treated intraperitoneally with recombinant mouse IL-33 (0.4 mg). For the depletion of eosinophils, a group of mice received repeated intraperitoneal injections of anti-Siglec-F mAb (15 µg per mouse). (B) Efficient *in vivo* depletion of eosinophils in IL-33-treated mice following repeated anti-Siglec-F mAb injections. On the sixth day following treatment start, LN, spleen and tumors were harvested and analysed for presence of eosinophils by flow cytometry. Histograms represent mean percentages of eosinophils in individual mice (n=3). *P< 0.05; **P<0.01; ***P<0.001. (C) Tumor growth in the indicated groups. Mean tumor area in individual mice ± SD is shown (n=10). #P< 0.05, IL-33 vs control; **P< 0.01, IL-33 vs IL-33 + anti-Siglec-F. CTR: control; āSiglec-F: anti-Siglec-F mAb; i.p.: intraperitoneal; s.c.: subcutaneous.

In addition, our results showed that the depletion of eosinophils decreased the $CD8^+$ T cells recruitment at tumor site (Fig. 13A). Moreover, the 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 to the values found in IL-33-treated animals (Fig. 13B). These data indicate that eosinophils play an essential role in IL-33-induced anti-tumor effects and that their function at the primary tumor site may be linked to recruitment and/or activation of $CD8^+$ T and NK cells.

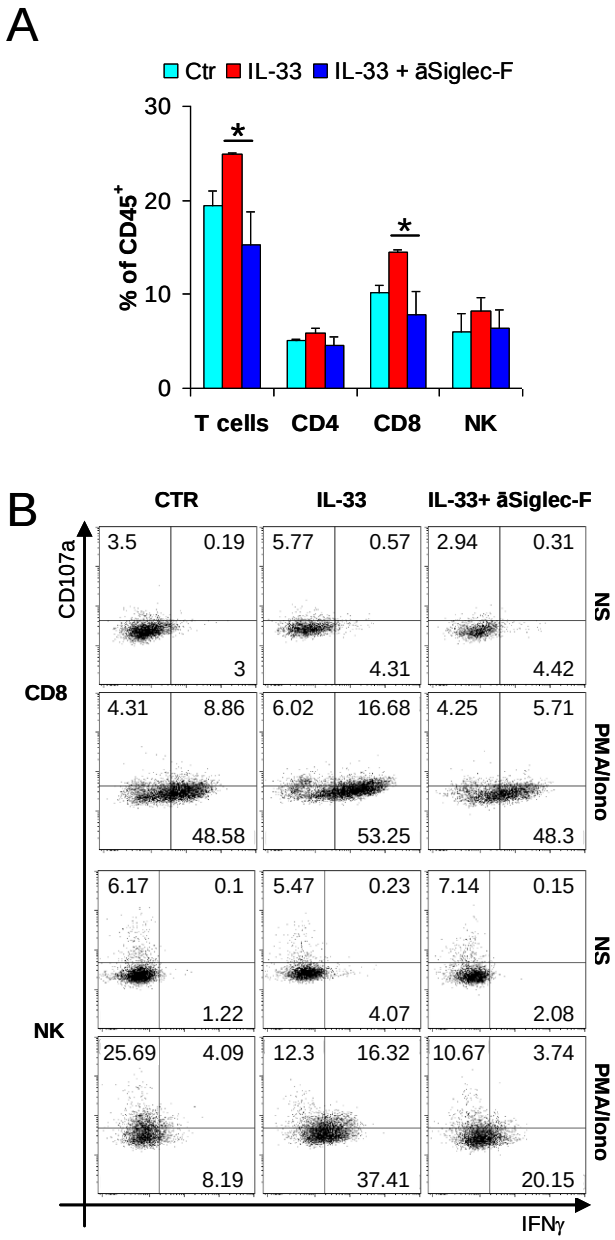


Figure 13: Effect of eosinophils depletion on tumor infiltrating CD8$^+$ T and NK cells. Melanoma-bearing mice were treated with IL-33 (0,4µg) and anti-Siglec-F mAb (15µg). The day after the last injection of IL-33 mice were sacrificed for the analysis of immune cell infiltrates. (A) Flow cytometry analysis of tumor-infiltrating cells. Data show mean values of individual mice (n=3-5) from 3 separate experiments ± SD. *P< 0.05. (B) Flow cytometry analysis of IFNγ 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; āSiglec-F: anti-Siglec-F mAb; NS: not-stimulated.

4.4 IL-33/ST2 axis conditions the pulmonary environment to restrict melanoma metastasis formation through eosinophils recruitment

Since IL-33 is known to control the homing of immune cell populations at the pulmonary level [37], we investigated the role of IL-33/ST2 axis in the protection against melanoma lung metastasis. To this end, mice were administered IL-33 intranasally and then transferred intravenously with B16.F10 melanoma cells to induce lung metastasis formation as schematized in Fig. 14A. Mice were sacrificed at day 19 and metastatic foci, in the lung, were enumerated. Our data revealed that IL-33 significantly inhibited pulmonary metastasis formation (Fig. 14B) as displayed by dose-dependent reduction of metastatic foci numbers in treated animals as compared to controls (Fig. 14C). To assess the presence of micrometastasis, lungs were homogenized and the cells subjected to a clonogenic assay *in vitro*. Cells were seeded at different dilutions to form melanoma colonies in 2 weeks. The percentage of covered area was calculated as the area covered by cells colonies with respect to the total area. The data showed that IL-33 inhibits micrometastasis formation maintaining the dose-dependent effect (Fig. 14D, E).

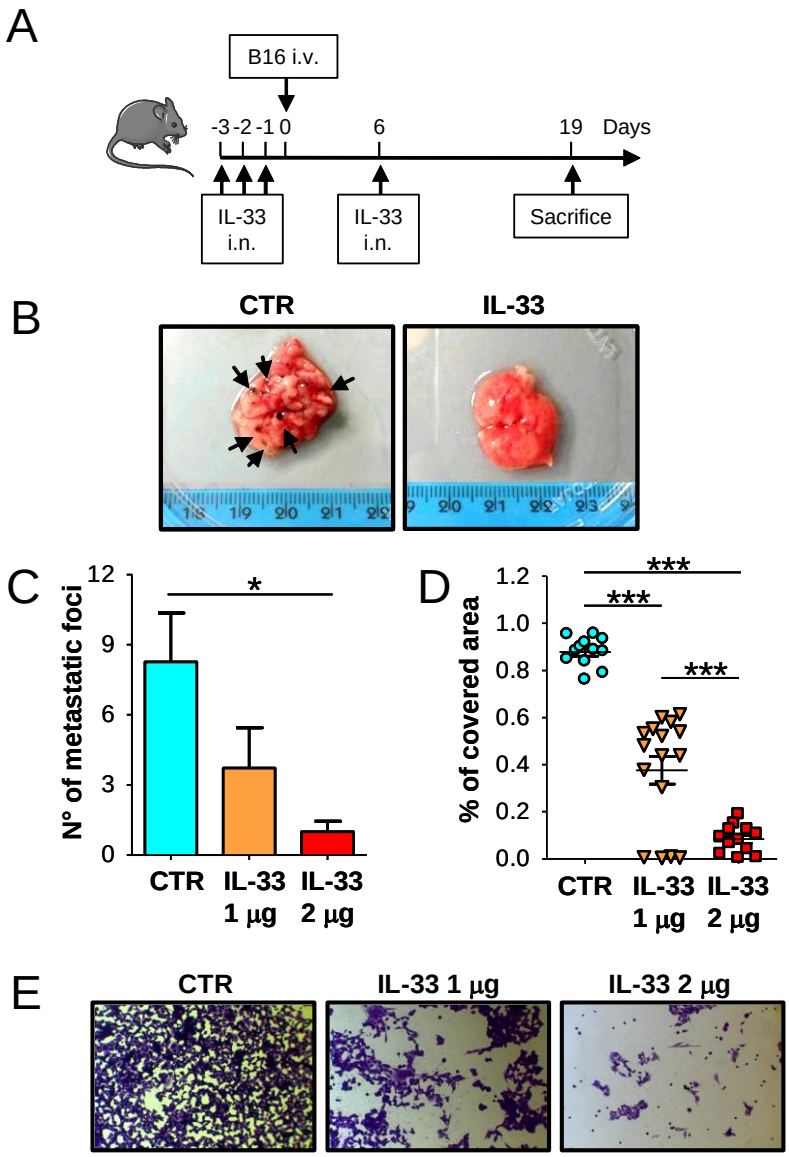


Figure 14: Role of IL-33 in anti-metastatic process. Schematic representation of experimental protocol of metastasis induction and IL-33 treatment. C57BL/6 mice were treated intranasally with IL-33 (1µg or 2 µg per mouse) and then injected intravenously with 0.3 x 10^6 B16.F10 melanoma cells. Mice were sacrificed at day 19 for the evaluation of metastasis. (B) Representative microphotographs of lungs from mice receiving or not 2 µg IL-33. 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 ± SD (n=6) from three independent experiments. (D) Quantitative analysis of clonogenic assay *in vitro* to determine lung micrometastasis. Dots represent the percentage of covered area of melanoma cell clones from lungs of single mice. (E) Representative micrograph of melanoma cell clones in clonogenic assay of lung cells from mice treated as indicated. One experiment out of three is shown. *P<0.01; ***P<0.001. CTR: control; i.n.: intranasal; i.v.: intravenous.

To evaluate whether the pulmonary immune microenvironment might be modified by IL33 administration, we analysed the immune cell populations in the lung associated with metastasis reduction following IL-33 treatment. Flow cytometry analysis of pulmonary $CD45^+$ infiltrates evidenced a marked increase of eosinophils frequency, but not of $CD8^+$ T cells, in mice treated with IL-33 compared to control (Fig. 15A). No significant variations occurred with other immune cell populations analysed. 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. 15B). This immune profile correlated with mRNA upregulation of eosinophils-attracting chemokines CCL5, CCL11, CCL13, CCL24, but not of the $CD8^+$ T cell-attracting CXCL10 (Fig. 15C), 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. 15C). 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. 15C).

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.

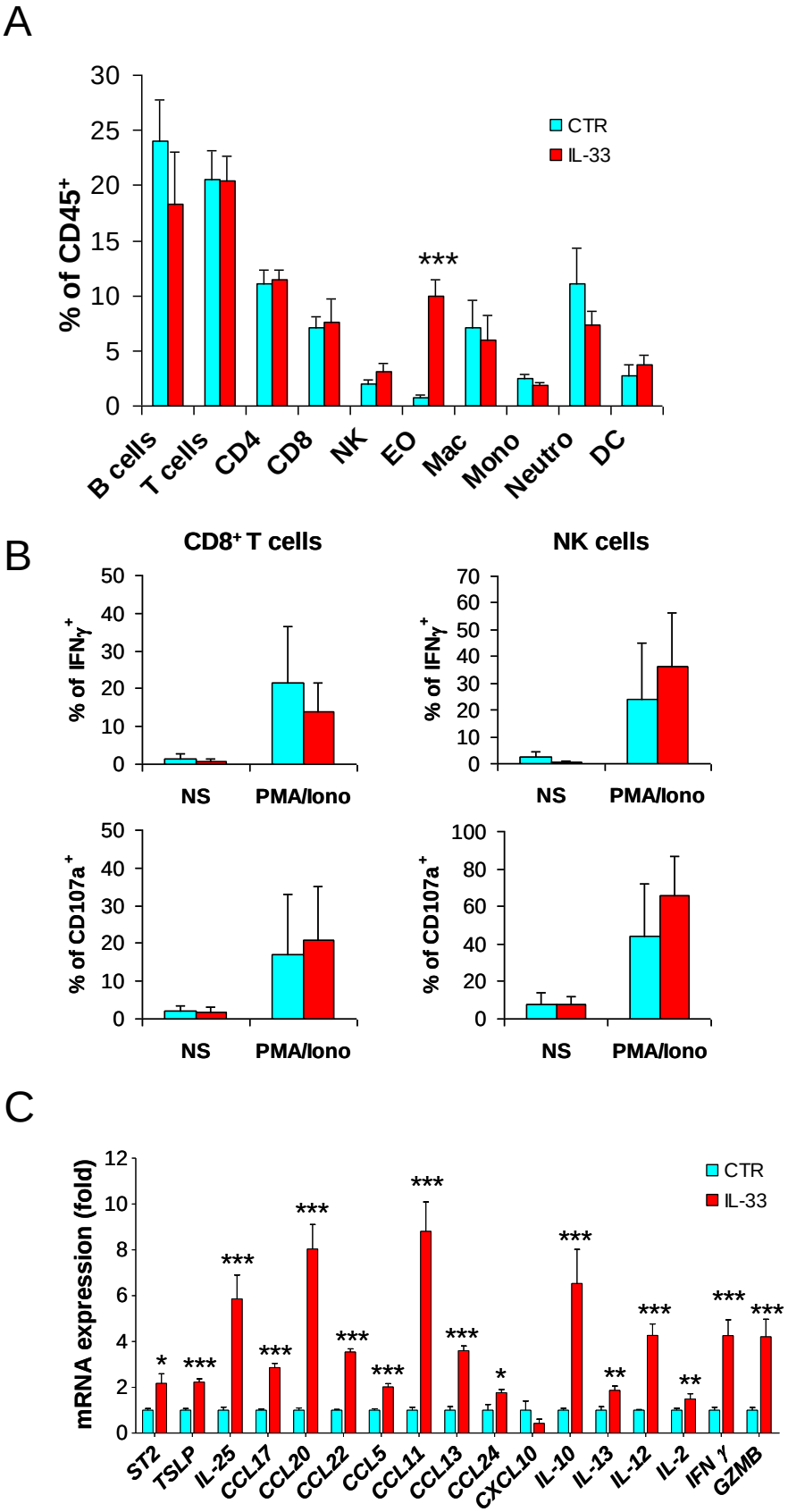


Figure 15: Analysis of pulmonary immune environment following IL-33 treatment. C57BL/6 mice were treated intranasally with IL-33 (2 µg) and then injected intravenously with B16.F10 melanoma cells (0.3 x 10^6). Mice were sacrificed at day 19 post B16 inoculation and

lungs were harvested. (A) Flow cytometry analysis of pulmonary immune-infiltrating cells. Data show the mean frequencies of the indicated $CD45^+$-gated immune cell populations in individual mice ± SD (n=6) from four separate experiments. (B) Flow cytometry analysis of CD107a and IFNγ expression in pulmonary $CD8^+$ T and NK cells following *in vitro* stimulation with PMA/Ionomycin. Data represent the mean percentages from three separate experiments. (C) 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<0.001. CTR: control; NS: not-stimulated.

4.5 IL-33 reduces lung metastasis formation in a ST2-dependent manner

To verify whether the anti-metastatic effect of IL-33 occurred through the activation of IL-33/ST2 pathway, ST2-deficient ($ST2^{-/-}$) mice which don't respond to IL-33 were injected with melanoma cells and treated or not with IL-33 to evaluate lung metastasis formation. Data revealed that $ST2^{-/-}$ mice did not respond to IL-33 and developed macro and micrometastasis to the same extent as untreated $ST2^{-/-}$ mice, indicating that IL-33 inhibits pulmonary metastasis onset in a ST2-dependent manner (Fig. 16A). Of note, $ST2^{-/-}$ mice developed metastasis to a greater extent compared to WT mice (Fig. 16A). 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 20 in comparison to the WT counterparts (Fig. 16B).

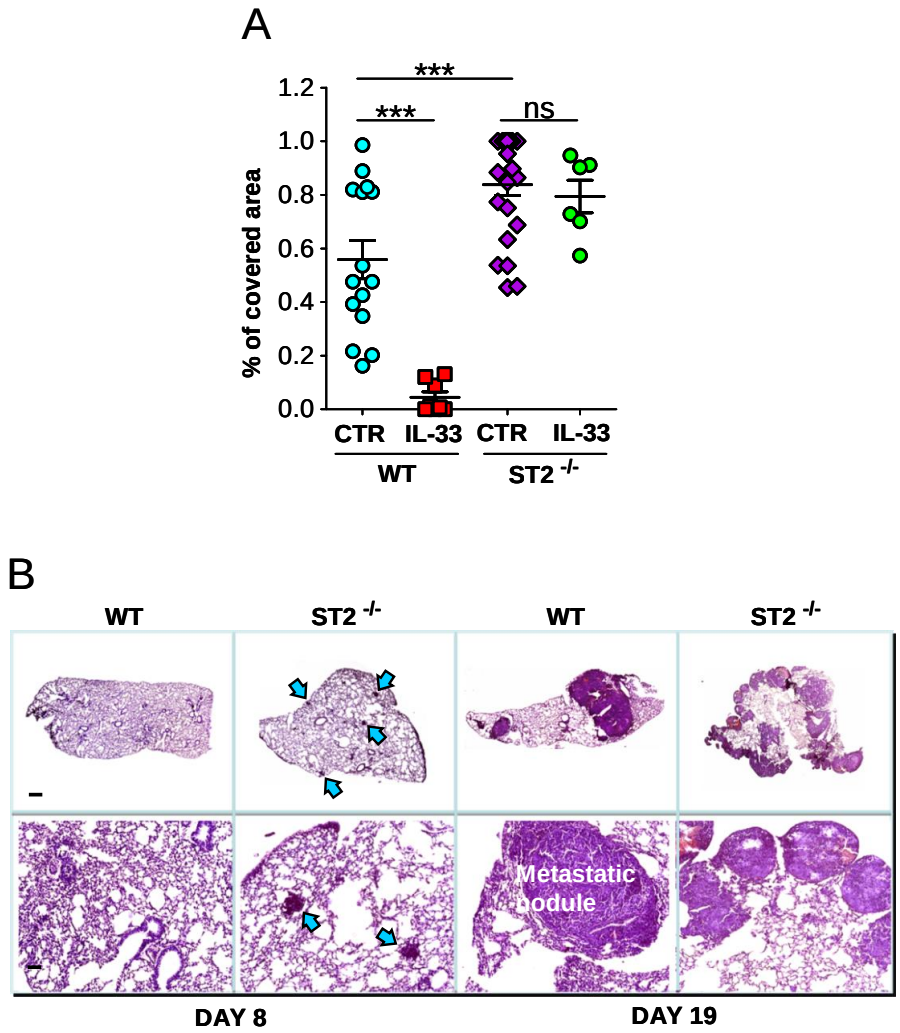


Figure 16: IL-33/ST2 axis controls the formation of melanoma pulmonary metastasis. ST2$^{-/-}$ and WT mice were treated or not with intranasal IL-33 (2 μg) and then injected intravenously with B16 melanoma cells (0.3 x 10^6). (A) Quantitative analysis of lung micrometastasis determined by clonogenic 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 μm (top row) or 40 μm (bottom row). Arrows show metastatic foci inside the lungs.

Flow cytometry analysis of the lung infiltrate evidenced significant reductions in eosinophils and NK cells in ST2$^{-/-}$ mice with respect to WT mice (Fig. 17A-B). However, CD107a/IFNγ 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. 17C). Overall, 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.

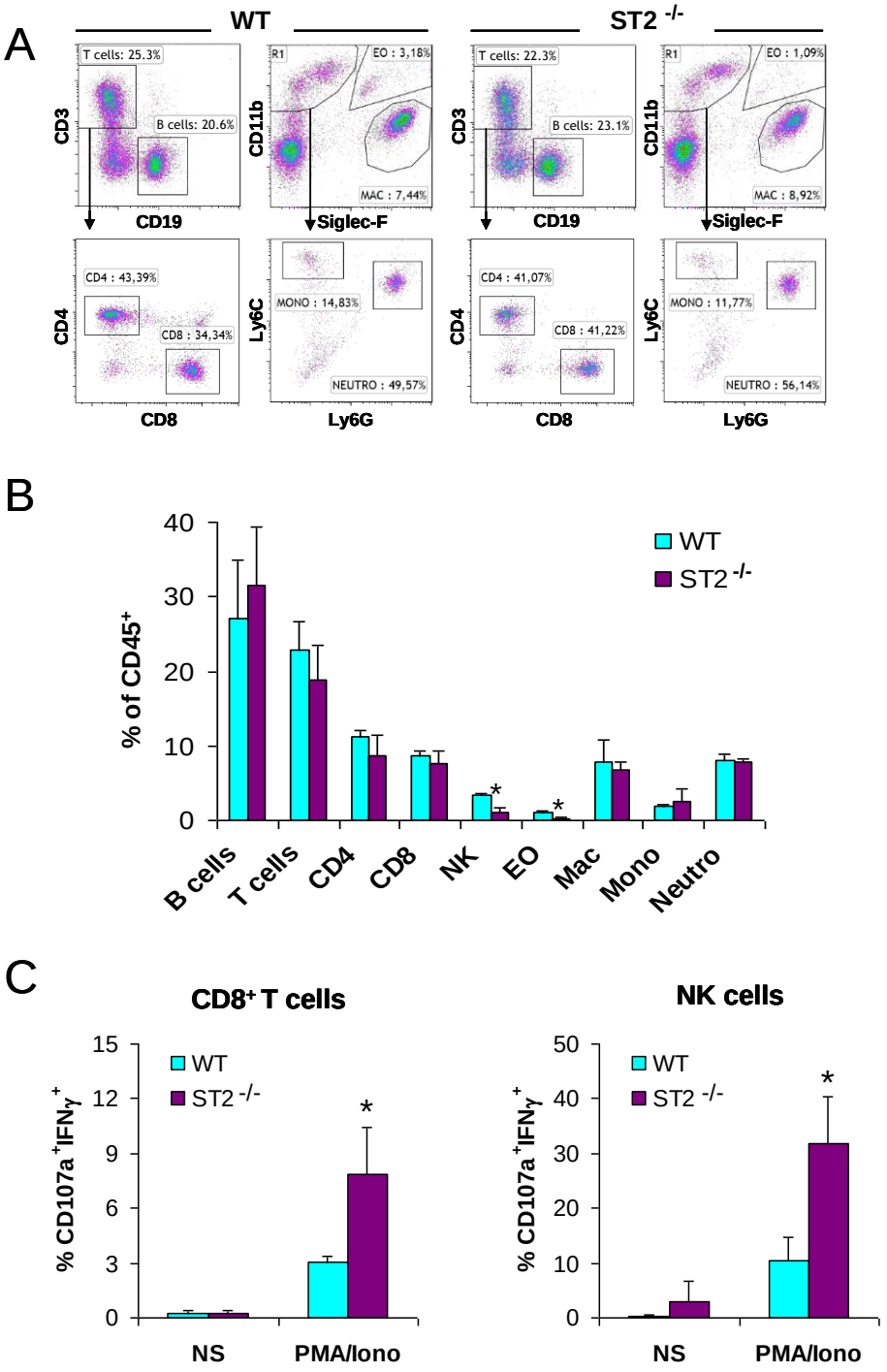


Figure 17: Analysis of pulmonary immune environment in ST2$^{-/-}$ mice following induction of experimental melanoma metastasis. ST2$^{-/-}$ and WT mice were intravenously injected with B16 melanoma cells (0.3 x 10^6) and then sacrificed after 19 days for characterization of lung immune compartment. (A-B) Flow cytometry analysis of pulmonary immune-infiltrating cells.

(A) Gating strategy of immune cell populations analyzed. (B) Mean frequencies of the indicated $CD45^+$-gated immune cell populations in individual mice ± SD (n=6) from three separate experiments. (C) Flow cytometry analysis of $CD8^+$ T lymphocytes and NK cells expressing CD107a/IFNγ following *in vitro* stimulation with PMA/Ionomycin. Data represent the mean percentages from three separate experiments. *P<0.05. NS: not-stimulated

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

We next sought to define the role of eosinophils in IL-33-mediated suppression of lung metastasis. To this aim, 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 sacrificed and lungs assayed for the presence of metastasis (Fig. 18A). Injection of Siglec-F mAb caused efficient eosinophil depletion in the lung (Fig. 18B). Remarkably, restriction of macro and micrometastasis formation in mice receiving IL-33 was completely abolished in the absence of eosinophils (Fig. 18C-D).

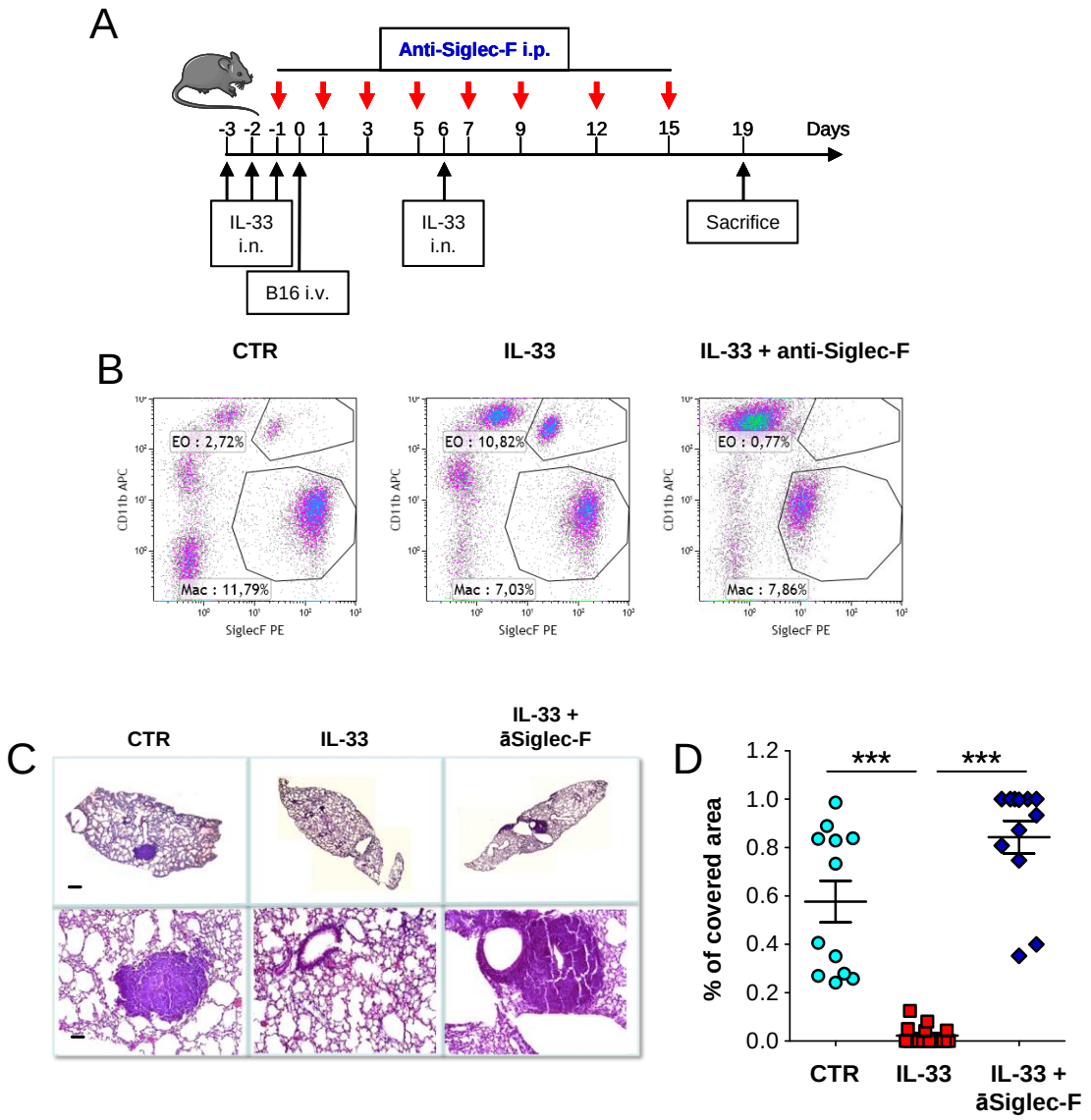


Figure 18: Eosinophils mediate the anti-metastatic effect of IL-33 against melanoma. (A) C57BL/6 mice were treated with intranasal instillations of IL-33 (2 µg) and then intravenously transferred with B16.F10 cells (0.3 x 10^6). For the depletion of eosinophils, a group of mice received repeated intraperitoneal injections of anti-Siglec-F mAb (15 µg per mouse). (B) Selective *in vivo* depletion of eosinophils in lungs from IL-33-treated mice following anti-Siglec-F mAb injections. Eosinophils (EO) and macrophages (Mac) were analyzed by flow cytometry. Percentages inside plots refer to the $CD45^+$-gated population. (C) Hematoxylin/Eosin staining of lung tissues harvested at day 19 post-tumor implant. Scale bar = 200 µm (top row) or 40 µm (bottom row). (D) Clonogenic assay in pulmonary cells. Dots represent individual mice from two separate experiments. ***P<0.001. CTR: control; āSiglec-F: anti-Siglec-F mAb.

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. 19A). Moreover, the percentages of $CD107a^{+}IFN\gamma^{+}$ NK and $CD8^{+}$ T cells in IL-33-treated mice were not modified upon eosinophil depletion (Fig. 19B). Thus, these results indicate that IL-33 inhibits pulmonary melanoma metastasis through recruitment of activated eosinophils 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) or of IL-33 pathway-related molecules (ST2, IL-25, CCL17, CCL20, CCL22; Fig. 19C).

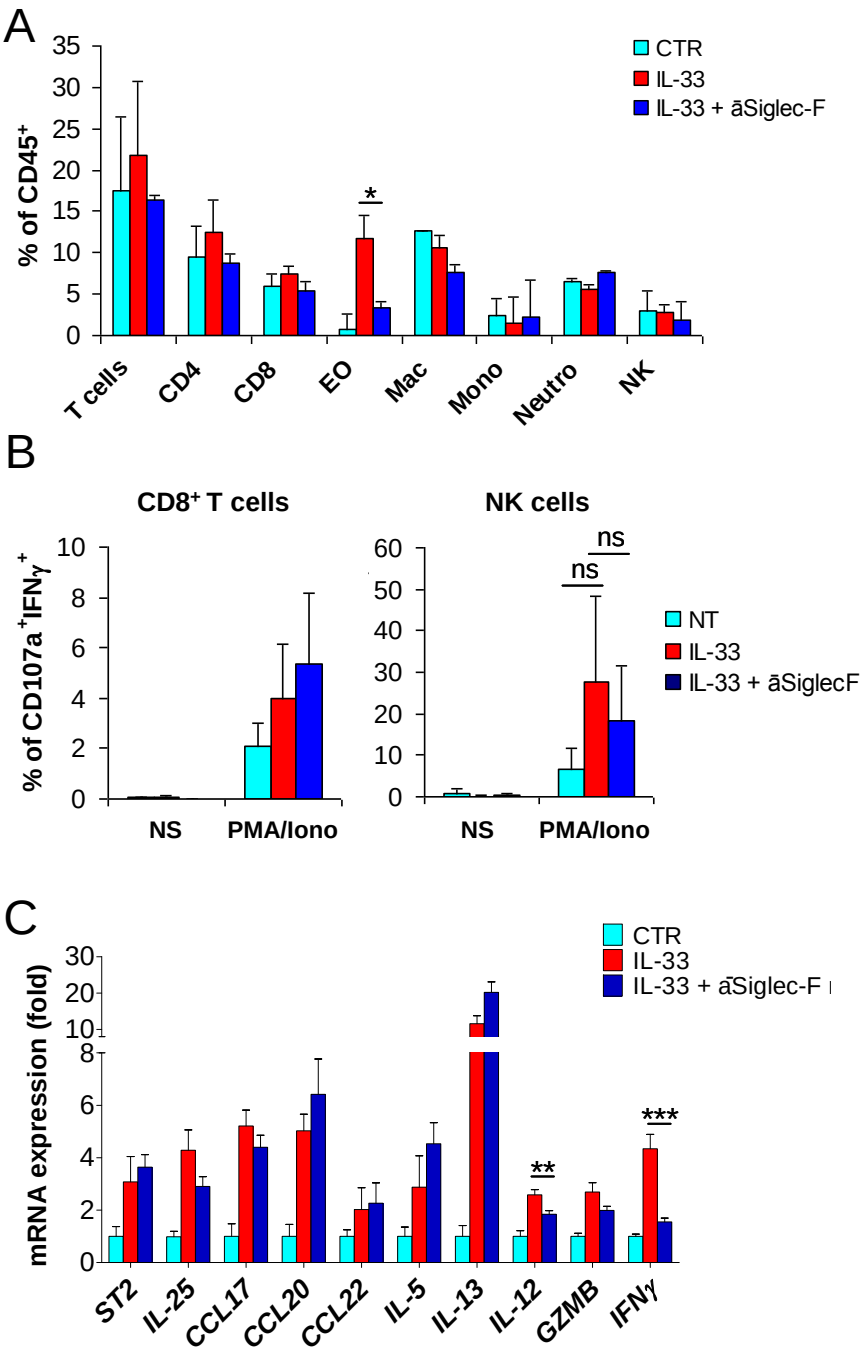


Figure 19: Effect of eosinophils depletion on lung environment. Mice were treated as in Figure 18. (A) Flow cytometry analysis of pulmonary infiltrating leukocytes at day 19. Data show the mean frequencies of the indicated immune cell populations ($CD45^+$-gated) in individual mice (n=6) from two separate experiments ± SD. (B) 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 three separate experiments. (C) Expression of the indicated cytokines and chemokines in lung tissues by qPCR. Data are expressed as fold change of mRNA expression vs control. *P<0.05. **P<0.01; ***P<0.001. CTR: control; āSiglec-F: anti-Siglec-F mAb; NS: not-stimulated.

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

To evaluate the phenotype of IL-33-recruited lung eosinophils, we analysed 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 to resting controls (Fig. 20A). In contrast, they expressed significant levels of IFNγ and Granzyme B (Fig. 20A-B), suggesting that these cells display an activated phenotype without expressing T cell-attracting chemokines.

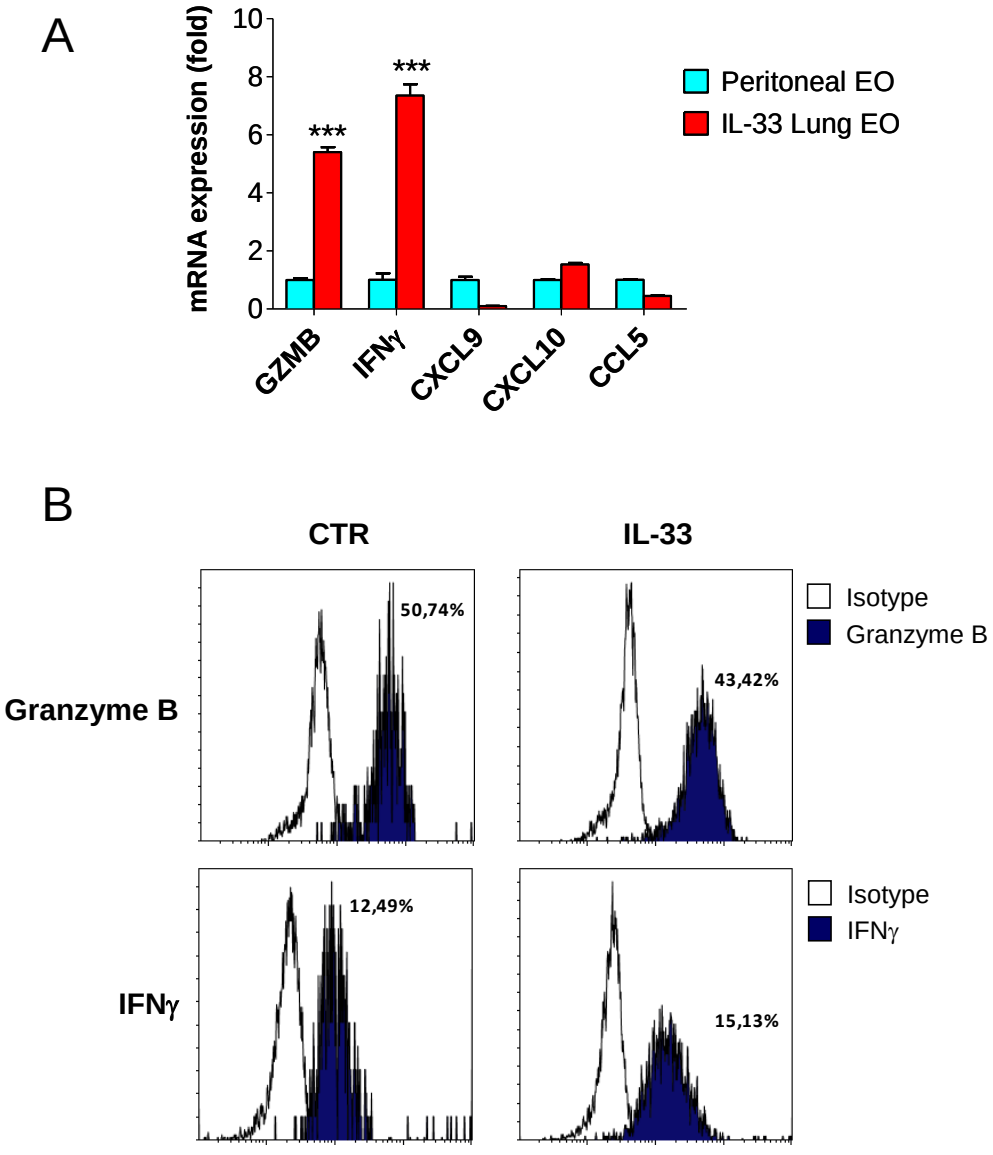


Figure 20: Analysis of pulmonary-infiltrated eosinophils. (A) qPCR analysis of indicated cytokines and chemokines in pulmonary-infiltrating eosinophils from IL-33-treated mice *vs* control peritoneal eosinophils. Data are expressed as fold change of mRNA expression *vs* peritoneal EO. (B) Flow cytometry analysis of intracellular levels of Granzyme B and IFNγ in gated eosinophils ($CD45^+CD11b^+Siglec\text{-}F^+CD3^-Ly6G^-CD11c^-$). One representative experiment out of two is shown. ***P<0.001.

Since eosinophils are known to exert direct cytotoxic effects against various targets, including cancer cells [110], 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 employed 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 days of culture followed by IL-33 for the last 6 days of culture. 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 up-regulation of CD69 and CD11b expression (Figs. 21A-B). Furthermore, IL-33 EO expressed increased levels of Granzyme B and IFNγ, with respect to IL-5 EO, in resemblance with IL-33-recruited lung eosinophils (Fig. 21C). 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 to IL-5 EO (Fig. 21C).

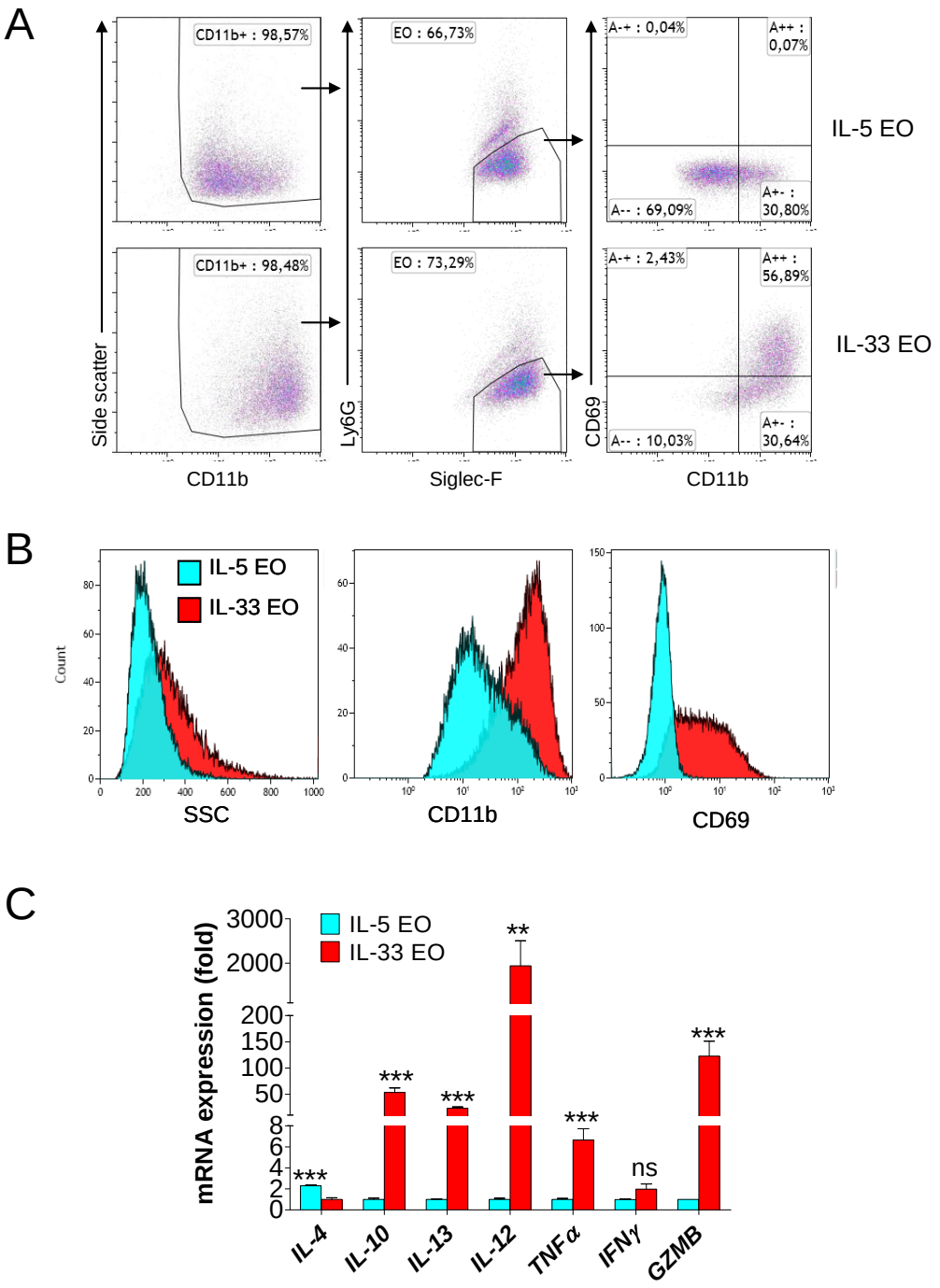


Figure 21: BM-derived eosinophils terminally differentiated with IL-33 are highly activated. BM-derived eosinophils were differentiated in presence of IL-5 (IL-5 EO) or IL-5 followed by IL-33 (IL-33 EO) from BM of naïve mice as shown in figure 4. **(A)** Flow cytometry analysis of IL-5 EO and IL-33 EO phenotype. (B) Flow cytometry analysis of CD69, CD11b and side scatter. (C) qPCR analysis of indicated cytokines in 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.

Next, we assessed the cytotoxic activity of these eosinophils against B16 melanoma cells. To this end, B16 cells were labeled with the red-fluorescent dye PKH26 and 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 in $PHH26^+$ B16 cells. 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 to 9.5% and 13% apoptosis observed with the respective amounts of IL-5 EO (Fig. 22). 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 anti-tumoral function of IL-33.

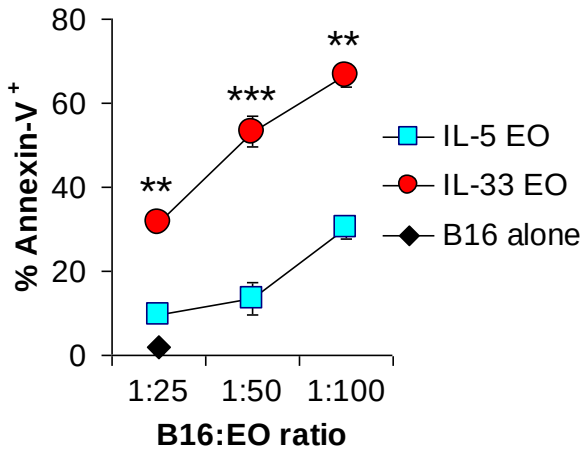


Figure 22: IL-33-EO induce apoptotic cell death on B16 melanoma cells. Flow cytometry analysis of B16.F10 melanoma cell death following co-culture for 5 hours with IL-33 EO *vs* IL-5 EO eosinophils at the indicated ratios. Bars correspond to mean Annexin-V^+ PKH-labeled melanoma cells from several samples (n=5-8 replicates per condition). **P<0.001; ***P<0.001.

4.8 Eosinophils preferentially migrate towards IL-33-conditioned B16 cells

To further investigate the crosstalk between immune cells and melanoma cells in relation to IL-33/ST2 axis we used the organ-on-chip technology, a microfluidic-based approach recently developed in our laboratories as a reliable tool to measure tumor-immune cells interactions [99, 117]. An *ad hoc* fabricated device, composed by three main fluidic chambers and two narrow gel-containing chambers, interconnected by two

arrays of microchannels, was used to co-culture immune cells with B16.F10 melanoma cells. Thus, melanoma cells labeled with the PKH67 green-fluorescent dye were resuspended in Matrigel in presence or absence of IL-33, and loaded into each narrow chamber, in order to embed the tumor cells in a gel matrix. PKH26 red-labeled immune cells (splenocytes from naïve syngeneic mice) were loaded into the central fluidic chamber. To verify if IL-33 has a key role in the recruitment of immune cells, as observed *in vivo*, we carried out fluorescence analysis of immune cells migration (red) into the two lateral chambers containing melanoma cells (green), treated with IL-33 (right side) or untreated (left side). After 24 h of culture, splenocytes visibly migrated towards the right-side microchannels and at 48 h infiltrated the gel matrix containing B16.F10 melanoma cells plus IL-33 (Fig 23). In contrast, no migration or infiltration was observable towards the left microchannel array connecting to untreated melanoma cells (Fig 23). This result evidences that IL-33 treatment of melanoma cells selectively induces potent attractive signals that recruit immune cells.

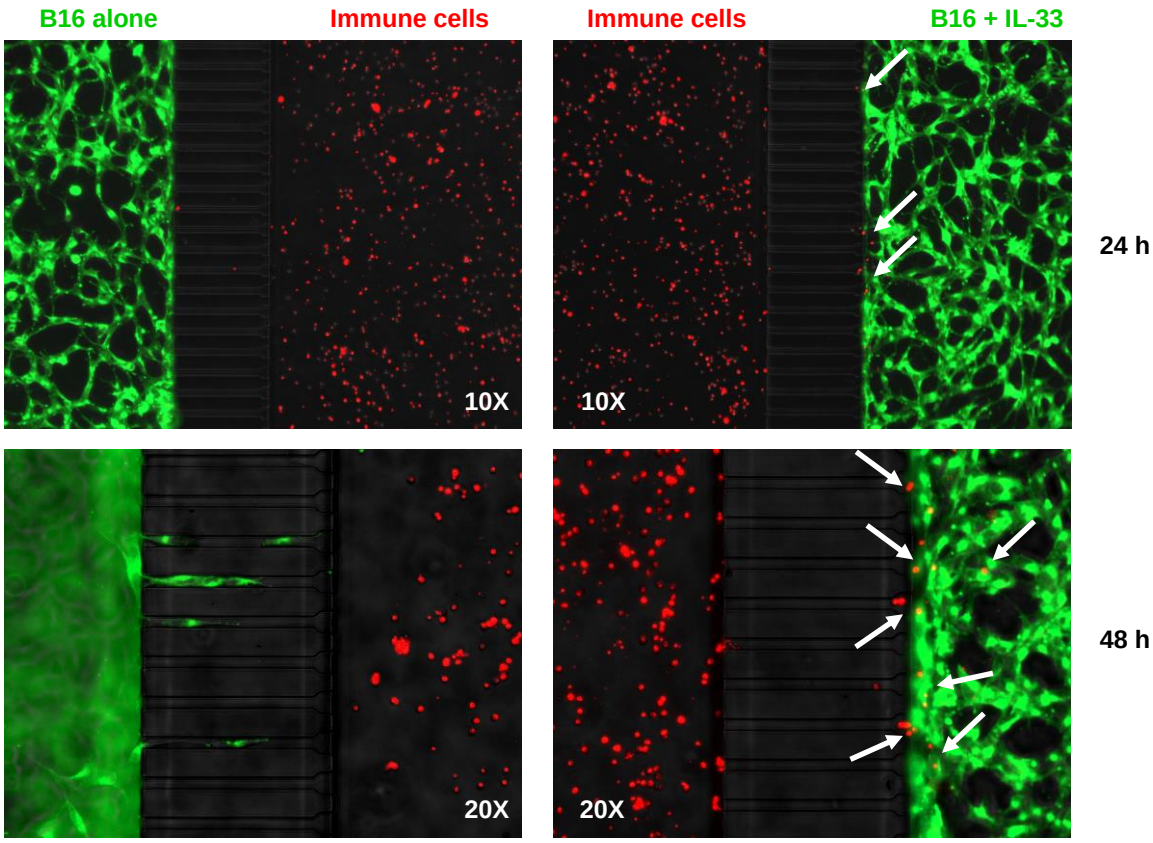


Figure 23: Splenocytes preferentially migrate towards IL-33-treated melanoma cells in a microfluidic device. PKH26-labeled (red) splenocytes (1 x 10^6) were loaded in the central chamber of microfluidic devices. PKH67-labeled (green) B16.F10 (2 x 10^4) resuspended in

Matrigel were placed in lateral chambers in absence or presence of IL-33 (100ng/ml). Fluorescence images were obtained after 24 and 48 h of culture.

In order to confirm the role of eosinophils emerged from *in vivo* experiments, we evaluated the ability of IL-33 to recruit them at tumor site in a microfluidic device. Thus, PKH26-labeled BM-derived eosinophils were loaded in the central chamber of the device and measured for their ability to migrate in response to IL-33 treated melanoma cells. Notably, eosinophils visibly migrated (Fig. 24) and infiltrated (Fig. 25) the right-side microchannels, whereas no migration was observable towards untreated B16.F10 melanoma cells (left chamber). Indeed, quantitative analysis of red fluorescence intensity, carried out using ImageJ software, underscored a strong preferential migration of eosinophils toward the chamber containing melanoma cells with IL-33 treatment (slope >0; Fig. 24 and 25B). Notably, such preferential migration was not observed when Matrigel plus IL-33 alone, without B16.F10 melanoma cells, was loaded into the chamber (Fig 26). These data indicates that IL-33 does not act as a direct chemoattractant for eosinophils, but rather acts stimulating melanoma cells to secrete factors that recruit eosinophils at tumor site.

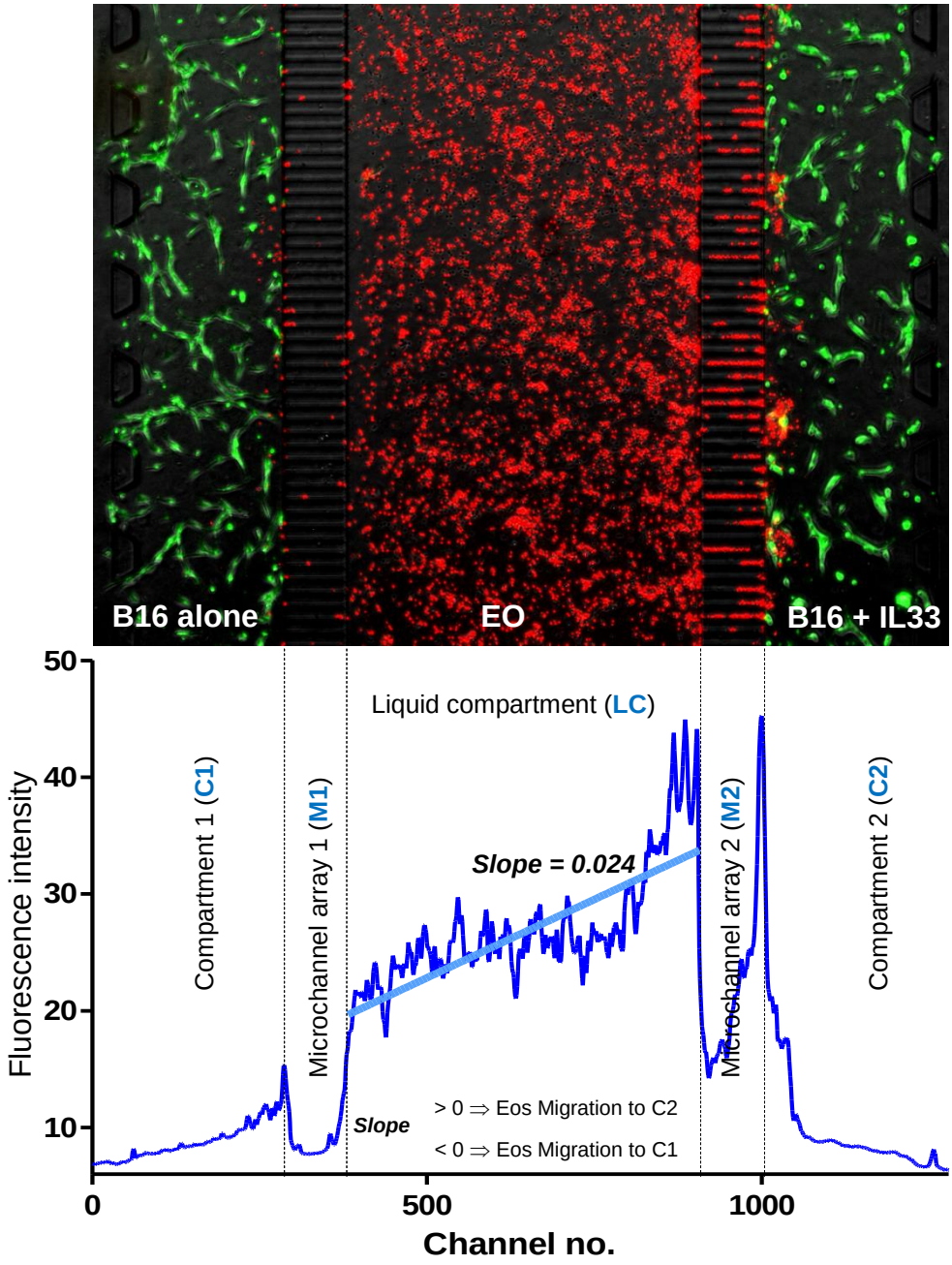


Figure 24: Eosinophils migrate towards IL-33-treated B16 melanoma cells in a microfluidic device. BM-derived eosinophils (1 x 10^6) were stained with PKH-26 (red) and loaded in the central chamber. B16.F10 melanoma cells (2 x 10^4) were stained with PKH-67 (green), resuspended in Matrigel and loaded in the lateral chamber in presence or not of IL-33 (100ng/ml). The profile of eosinophils migration were analyzed with ImageJ software after 48 h of co-culture. Slope> 0 indicates eosinophils migration toward compartment 2 (B16.F10 plus IL-33), on the contrary for slope<0 eosinophils migrate toward compartment 1 (B16.F10 untreated).

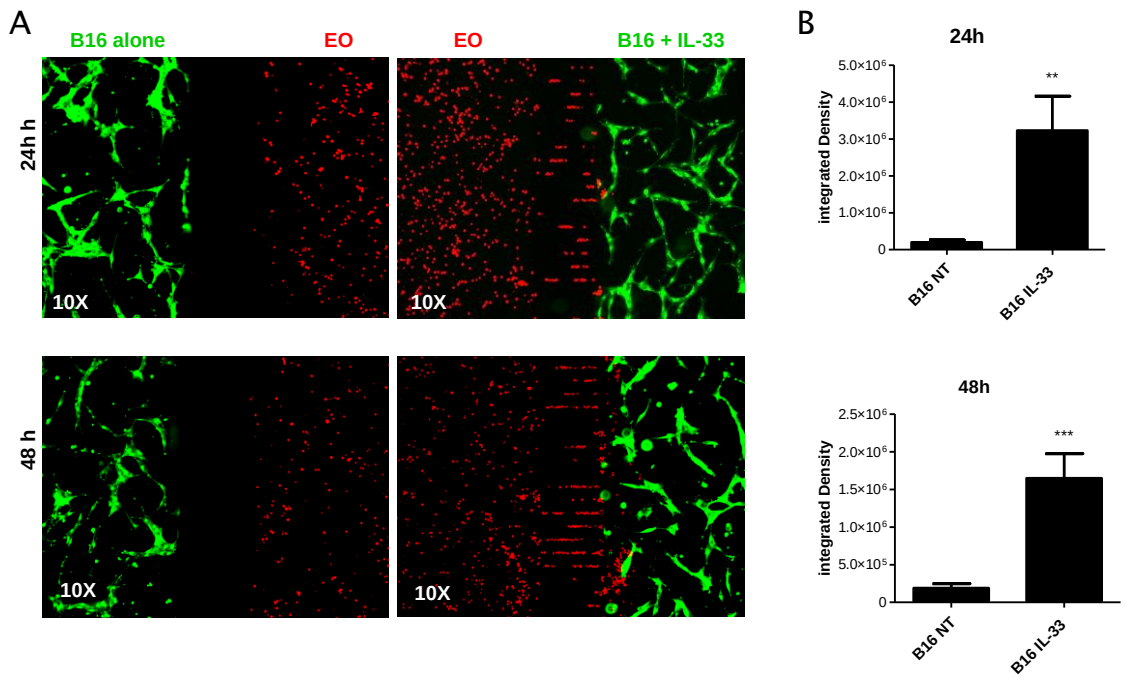


Figure 25: Eosinophils infiltrate preferentially B16 melanoma cells treated with IL-33. PKH26-labeled (red) eosinophils were loaded in the central chamber of microfluidic devices. PKH67-labeled (green) B16.F10 melanoma cells treated or not with IL-33 were placed in lateral chambers. (A) Fluorescence images obtained after 24 and 48h of culture. (B) Quantitative analysis with ImageJ software of eosinophils infiltration in IL-33 or untreated (NT) melanoma channels calculated as red fluorescence values in right/left channels at the indicated times. **P<0.01; ***P<0.001.

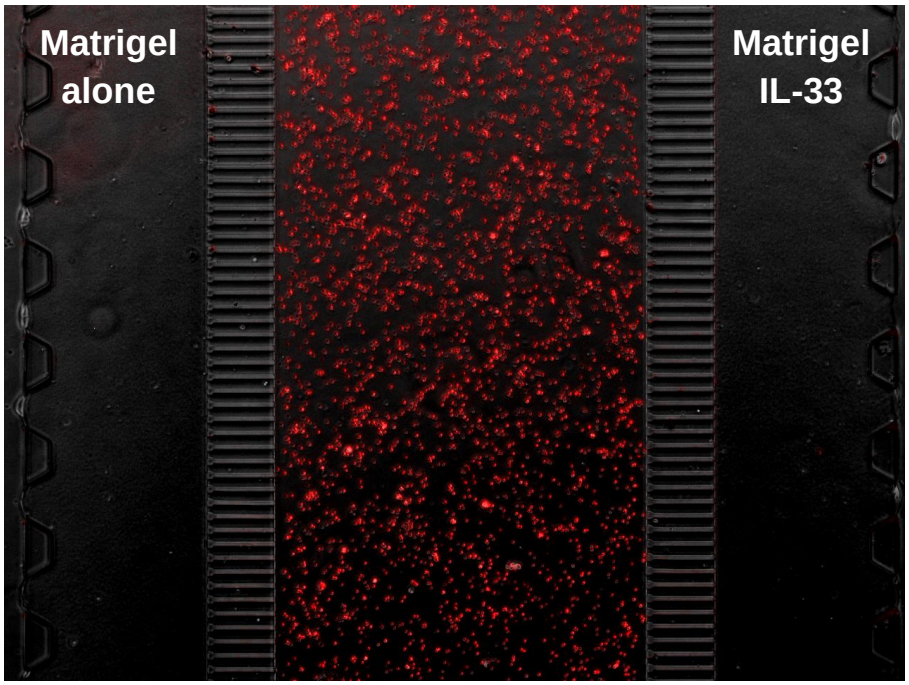


Figure 26: IL33 alone does not elicit the recruitment of eosinophils. BM-derived eosinophils (1 x 10^6) were stained with PKH-26 (red) and loaded into central chamber. Matrigel alone or Matrigel plus IL-33 (100ng/ml) was loaded into lateral chambers. Eosinophils migration is evaluated after 48 h of culture.

5. Discussion

Protective antitumor immunity is classically thought to require a Th1-type of immune response. However, some clinical evidences in melanoma patients indicate that eosinophilia and other parameters associated to Th2-type of immunity may also correlate with response to therapy and favorable outcome [105]. In this regard, the peculiar features of IL-33 in boosting both Th1 and Th2 responses implicates this cytokine as a possible novel candidate for harnessing the melanoma immune environment [39, 79]. However, the current literature indicates a controversial role of IL-33/ST2 axis in tumorigenesis, showing both immunoprotective and tumor-promoting effects, depending on the tumor histotype and the site of expression. In mouse models of breast or colorectal cancers IL-33 was shown to promote cancer progression and metastasis [71, 120]. In addition, high levels of IL-33 intratumoral expression were found to negatively correlate with tumor grade in clinical breast [121] and colorectal [78] cancer. In melanoma, limited *in vivo* studies have been produced, most of which employed transgenic overexpression of IL-33 in tumor cells or in the host immune system. In these reports, IL-33 was reported to exert an anti-tumoral effect through a mechanism depending on induction of $CD8^+$ T cell and NK cell-mediated immunity [81, 82]. In a study employing 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 [83], although the anti-tumor role of these cells was not directly assessed.

In the present study we report that IL-33 exerts an important anti-tumor function against melanoma, restricting both primary tumor growth and metastasis formation through active recruitment of eosinophils, though our data suggest that different anti-tumor mechanisms may be operated by eosinophils at the two sites.

First, we demonstrated that systemic injections of IL-33 are able to hinder melanoma growth, in relation to the starting tumor size, with greater therapeutic efficacy when melanoma is smaller, indicating a correlation with tumor progression. Within the primary tumor microenvironment, the anti-tumor effect of IL-33 was associated with accumulation of both $CD8^+$ T cells and eosinophils, accompanied by up-regulation of chemokines that drive the recruitment of these cells. We demonstrated that tumor-infiltrating eosinophils expressed high levels of $CD8^+$ T cell-attracting

chemokines following IL-33 exposure. The selective depletion of eosinophils, resulting in abrogation of IL-33 anti-tumor efficacy, inhibited the homing of $CD8^+$T cells at tumor site and also their activation. These results indicate 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 study [109]. 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 [79, 81, 122]. 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 [123].

At the tumor site, IL-33 induced the expression of cytokines and chemokines correlated with the IL-33/ST2 axis (IL-25, TSLP, CCL17, CCL20, CCL22) as well as cytokines related to the so-called atypical Th2 response (IL-5, IL-10, IL-13, but not IL-4). Of note, tumors from mice receiving IL-33 also expressed increased levels of Th1 cytokines (IL-12 and Granzyme B), thus confirming the pleiotropic function of IL-33 and the possible action in anti-tumor immune response [102, 105, 124]. 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 anti-tumor 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[69, 72] and colorectal cancer [66, 125]. On the other hand, another study showed that administration of IL-33 inhibits the *in vitro* differentiation and *in vivo* accumulation of MDSCs in the tumor and spleen of B16F1 melanoma-bearing mice [84]. It has been recently reported that IL-33 promotes anti-tumor responses through the accumulation and the activation of tumor-infiltrating mDC increasing Ag cross-presentation to $CD8^+$ T cells [122]. 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) employed in our study with respect to the report of Dominguez and colleagues.

IL-33/ST2 signalling is known to control the homing of immune cell populations at the pulmonary level [37]. Indeed, we demonstrated that local administration of IL-33 via ST2 conditions the pulmonary microenvironment to protect mice from the onset of experimental pulmonary melanoma metastasis. Of note, this effect correlated with selective recruitment in the lung of eosinophils, but not of $CD8^+$ T cells, in contrast to what observed at the primary tumor site. Accordingly, while eosinophils-recruiting chemokines were up-regulated 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. Moreover, in the lung of mice receiving IL-33 we observed increased expression levels of both Th1 (CCL5, CXCL9, CX3CL1, IL12, GZMB and IFNγ) and Th2 (CCL17, CCL20, CCL22) cytokines and chemokines. We found that 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* [114, 126]. 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 [127]. 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 metastasis 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 [100, 128]. However, it remains to be determined whether and how basal levels of eosinophils contribute to endogenous IL-33-mediated protective effect. Of note, depletion of eosinophils in IL-33-treated

mice suppressed the up-regulation of pulmonary Th1, but not Th2, cytokine transcripts. This observation suggests that IL-33-recruited eosinophils play a crucial role for the Th1 arm of immune response induced by IL-33.

The induction of eosinophils recruitment after IL-33 treatment on melanoma cells could also be shown by means of an organ-on-chip approach, a microfluidics-based *in vivo*-like system for investigating the crosstalk between immune cells and cancerous cells [97, 99]. By confronting IL-33 treated melanoma cells and untreated melanoma cells, we could observe that eosinophils preferentially migrate toward the IL-33 treated melanoma cells with respect to control cells. Our analysis revealed an active migration of eosinophils within the gel matrix embedding the melanoma cells, thus closely mimicking the tumor microenvironment.

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 towards Th1 or Th2, respectively, type of immunity [119]. Our BM-derived eosinophils terminally differentiated with IL-33 (IL-33-EO) expressed higher levels of Th1 cytokines (TNFα, IFNγ, 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 by IL-33-recruited pulmonary eosinophils (particularly IFNγ) may serve for boosting $CD4^+$ T cell responses. This hypothesis is worth considering, since $CD4^+$ T cells were shown to reject CTL-resistant MHC class-II negative melanoma tumors [100, 129]. Alternatively, through secretion of Granzyme B eosinophils may contribute to amplify pro-inflammatory responses, as also described for other immune cell types [130].

Of note, terminal differentiation of BM-EO with IL-33 also resulted in phenotypic activation of eosinophils, as denoted by marked up-regulation of CD69 and CD11b and by high side scatter, indicative of presence of cytotoxic granules, confirming that IL-33 can directly activate eosinophils [131, 132]. 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) [106, 133, 134].

The anti-tumor 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 as accessory cells attracting tumor-reactive $CD8^+$ T cells. At the pulmonary site, our data strongly suggest that IL-33-recruited lung eosinophils may prevalently play a direct anti-tumor role via tumor cell killing (Fig. 27). The underlying molecular basis 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 [135]. 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-associated eosinophilia has been correlated with tumor regression and increased survival in several clinical and experimental cancers [136]. Overall, our studies identify a previously unrecognized eosinophil-dependent anti-tumor mechanism triggered by IL-33/ST2 axis that restricts melanoma growth and metastasis, which may open to novel therapeutic options against melanoma.

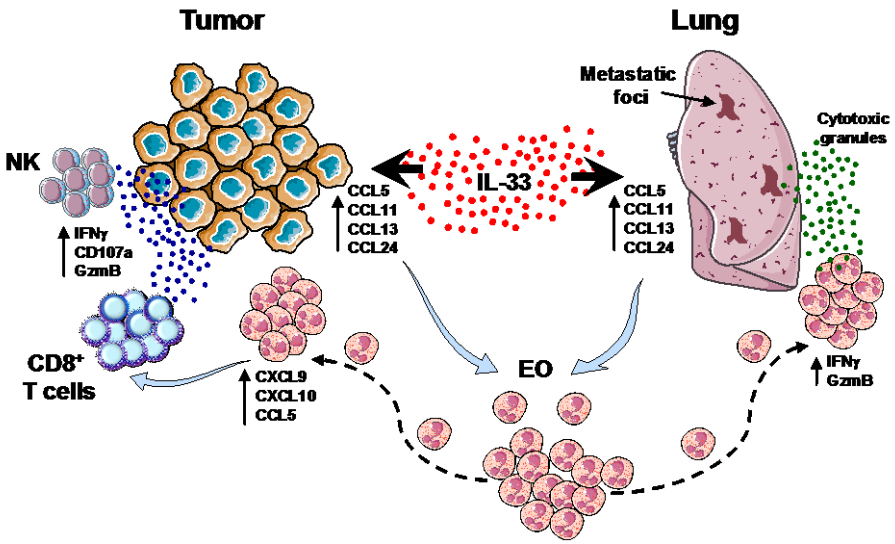


Figure 27: Possible model of IL-33 antitumor activity. IL-33 promotes the upregulation of CCL5, CCL11, CCL13, CCL24 that attract eosinophils at tumor site. Eosinophils are able to induce the recruitment of $CD8^+$T cells at primary tumor level via CXCL9, CXCL10, CCL5 expression and the activation of tumor-reactive CD8 T and NK cell responses. At pulmonary level eosinophils have a direct role in tumor cell killing through the upregulation of Granzyme B and IFNγ.

6. Concluding remarks

The IL-33/ST2 axis has gained much interest in the past few years and its role in host immune response is emerging far beyond Th2-related immunopathologies, to include anti-tumor immunity. In melanoma, IL-33 exerts a clear antitumoral effect, reducing tumor growth and pulmonary metastasis formation with a critical involvement of eosinophils and possibly other immune cell types. A better understanding of the mode of action, regulation and function of IL-33 in human melanoma would facilitate the development of novel therapeutic strategies. In this context, the use of combined therapies for the treatment of melanoma aimed at improving the efficacy of IL-33 antitumoral action could have a relevant translational impact. In this regard, immunotherapies targeting immune checkpoint inhibitors directed against CTLA-4 and/or PD-1 have already demonstrated a high degree of success in advanced-stage melanoma. Based on these premises, it would be of interest to test if IL-33/ST2 targeting would enhance the therapeutic response to immune checkpoint blockade, thus opening perspective for novel therapeutic options in clinical oncology. Finally, given the association of IL-33-mediated eosinophilia with allergic diseases, our studies hold epidemiologic implications on tumor and pulmonary metastasis incidence in allergic subjects, such as asthmatics.

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