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The role of eosinophils in breast cancer

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par

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This work is dedicated to my grandmother Iveta Spisak whose unconditional love and support was taken away from me far too soon. Although she is no longer with us, her spirit and memory remain in my heart.

Abstrakt

Eosinophile sind Granulozyten und gehören zum angeborenen Arm der Immunität. Eosinophile können sich in verschiedenen, basalen oder aktivierten Zuständen befinden, und je nach Art der Aktivierung üben sie unterschiedliche Effektorfunktionen aus. Es hat sich gezeigt, dass diese multifunktionalen Zellen bei verschiedenen Krebsarten eine entscheidende Rolle spielen. Bei Brustkrebspatientinnen haben blut- und tumorassoziierte Eosinophile nachweislich unterschiedliche Auswirkungen auf die Tumorprogression, die von günstig über neutral bis hin zu schädlich reichen. Mehrere präklinische Modelle zeigten sowohl eine pro- als auch eine antitumorale Rolle der Eosinophilen, die entweder direkt oder indirekt durch Veränderungen der Immunumgebung des Tumors zustande kamen.

In meiner Dissertation musste ich zunächst eine zuverlässige Strategie entwickeln, um Eosinophile im Tumorgewebe zu identifizieren und sie von anderen Immunzellen, insbesondere Neutrophilen, zu unterscheiden. Zu diesem Zweck testete ich verschiedene Gating-Strategien mit Hilfe der Durchflusszytometrie und bestätigte meine Ergebnisse durch histologische Analysen. Ich habe verschiedene Methoden ausprobiert, um Eosinophile in Primärtumoren von Mäusen anzulocken und sie in situ zu aktivieren, um ihre Auswirkungen auf das Fortschreiten von Brustkrebs zu analysieren. Eine starke Eosinophilie sowohl im Blut als auch im Tumor wurde durch die Behandlung der Mäuse mit einem Antikörper-IL5 Fusionsprotein erreicht. IL5 ist der wichtigste Differenzierungs- und Überlebensfaktor für Eosinophile und wurde an einen Antikörper gegen Periostin gekoppelt, ein Protein, das in der extrazellulären Matrix von Brusttumoren exprimiert wird, und das resultierende Immunzytokin wurde als AB5-IL5 bezeichnet. Diese Kopplung ermöglichte eine längere Zirkulationszeit sowie eine spezifische Anreicherung im Tumor, was zu einer stärkeren Eosinophilie im Blut und im Tumor führte als bei der Behandlung mit IL5 allein. Ich beobachtete eine konsistente Zunahme der tumorassoziierten Eosinophilie in vier verschiedenen Brustkrebsmodellen, und meine Ergebnisse zeigten, dass die Eosinophilie bei Brustkrebs keinen Einfluss auf das Fortschreiten des Krebses hat.

Ich analysierte auch die Mikroumgebung des Tumors in eosinophilen Tumoren und stellte fest, dass Eosinophile in der Lage sind, die Infiltration von tumorassoziierten Neutrophilen zu beeinflussen, indem sie eine hemmende Wirkung auf diese Zellen haben. Darüber hinaus entdeckte ich in 4T1-Tumoren eine noch unbekannte Art von SiglecF+ Neutrophilen, die stark an Eosinophile erinnern und höchstwahrscheinlich nicht identisch sind mit SiglecF^{+} Neutrophilen, die von anderen in Mausmodellen von Lungenadenokarzinomen beschrieben wurden.

Insgesamt war ich in der Lage, sowohl eine zuverlässige Gating-Strategie zur Identifizierung von Eosinophilen in verschiedenen Brustkrebsmodellen zu etablieren als auch eine konsistente Eosinophilie sowohl in der Zirkulation als auch in den Tumoren zu induzieren. Ich habe festgestellt, dass die Identifizierung von Eosinophilen, insbesondere in Patientendaten, schwieriger ist als ursprünglich angenommen und dass Schlussfolgerungen aus Studien, die die Genexpression zur Identifizierung von

Eosinophilen nutzen, mit Vorsicht zu interpretieren sind. Meine Ergebnisse deuten darauf hin, dass die Eosinophilie keinen Einfluss auf das Wachstum des Primärtumors oder die Metastasierung bei Brustkrebs hat, dass diese Zellen jedoch mit anderen Immunzellen in der Mikroumgebung des Tumors interagieren, vor allem mit Neutrophilen.

Schlüsselwörter

Brustkrebs, Eosinophile, Eosinophilie, tumorassoziierte Eosinophile, Brustkrebs-Mausmodelle, Tumorprogression, Metastasen, Tumormikroumgebung, Lymphozyten, Behandlung, Aktivierung, IL5, IL33, SiglecF, Neutrophile.

Abstract

Eosinophils are granulocytes and belong to the innate arm of immunity. Eosinophils can be in different basal or activation states and depending on which type of activation is applied, they exert different effector functions. These multi-functional cells have been shown to play crucial roles in different types of cancer. In breast cancer patients, blood and tumor associated eosinophils were shown to have different effects on tumor progression, ranging from beneficial through neutral to detrimental. Several pre-clinical models showed both pro- and anti-tumorigenic roles of eosinophils which act either directly or indirectly through modifications of the tumor immune environment.

The goal of my thesis was no analyze the effects of breast cancer associated eosinophilia on cancer progression using orthotopic tumor injections as this type of breast cancer model has been rarely used to analyze how eosinophils affect breast cancer progression. I first established a reliable way to identify eosinophils in tumor tissues and distinguish them from other immune cells, namely neutrophils. I did so by testing different gating strategies using flow cytometry and confirming my results with histological analyses. I tried several methods to attract eosinophils to primary tumors in mice and activate them *in situ* so as to analyze their effects on breast cancer progression. Strong eosinophilia in both the blood and the tumor was accomplished by treating the mice with an engineered version of IL5. IL5 is the main differentiation and survival factor of eosinophils and was coupled to the targeting moiety of an antibody against periostin, a protein expressed in the extracellular matrix in breast tumors and the resulting immunocytokine was termed AB5-IL5. This coupling allowed for increased circulation time as well as specific targeting to the tumor, resulting in stronger blood and tumor eosinophilia as compared to treatment with IL5 alone. I observed consistent increases in tumor associated eosinophilia in four different breast cancer models and my results indicated that eosinophilia in breast cancer has no effect on cancer progression when tumors were injected orthotopically.

I also analyzed the tumor microenvironment in eosinophilic tumors and saw that eosinophils are able to affect the infiltration of tumor associated neutrophils, having an inhibitory effect on these cells. Furthermore, I discovered a yet unknown type of SiglecF^{+} neutrophil in 4T1 tumors which strongly resembled eosinophils and are most likely not the SiglecF^{+} neutrophils described by others in mouse models of lung adenocarcinoma.

Overall, I was able to establish both a reliable gating strategy to identify eosinophils in different breast cancer models as well as consistently induce eosinophilia in both the circulation and breast tumors. I found that identification of eosinophils, especially in patient data, is more difficult than initially assumed and that conclusions drawn in studies using gene expression to identify eosinophils must be interpreted with care. I also discovered that pre-clinical studies about eosinophils in primary breast cancer are virtually non-existent. My results indicate that eosinophilia has no effect on primary tumor growth nor metastatic

seeding in breast cancer but that these cells interact with other immune cells in the tumor microenvironment, mainly with neutrophils.

Keywords:

Breast cancer, eosinophils, eosinophilia, tumor associated eosinophils, breast cancer mouse models, tumor progression, metastases, tumor microenvironment, lymphocytes, treatment, activation, IL5, IL33, SiglecF, neutrophils.

Table of contents

Introduction

Breast Cancer

Cancer is a disease that occurs when tissue cells start proliferating uncontrollably. In the case of breast cancer this leads to the formation of a solid mass in within the breast. Breast cancer can arise either in the milk ducts (ductal carcinoma) or the lobules (lobular carcinoma) of the breast. Ductal carcinoma occurs far more often than lobular carcinoma, approximately 75-90% versus 15-10% and has a decreased patient survival probability[1,2]. The main cause of death is not the primary tumor but rather the metastases formed in distant organs, most commonly the bones, lungs, liver and brain[3]. Breast cancer is usually diagnosed via mammography where the breast is x-rayed using low energy x-rays[4].

Breast cancer statistics

In 2021 the world health organization (WHO) published their most recent findings on breast cancer statistics[5]. They showed that in 2020 7.8 million women were diagnosed with breast cancer between 2015 and 2020. This makes breast cancer the most common type of cancer in women, followed by lung and colorectal cancer[6]. The mortality rates of breast cancer have strongly decreased since the 1980s due to new tools allowing for earlier detection as well as more efficient treatment. In 2021 the mortality rate of breast cancer world-wide was 30%[7]. It should be noted that the mortality rate varies drastically between countries. More developed countries such as Northern America, Europe, Australia and New Zealand have mortality rates between 16%-20% whereas less developed countries such as Africa, parts of Asia and Melanesia have mortality rates above 30%[7]. The high mortality rate in these countries is contributed to the fact that early detection is sparsely, if at all, available for the patients and that appropriate treatment options are too expensive or no available in their geographical area.

The strong decrease in breast cancer mortality rates in developed countries shows that advanced breast cancer treatment is crucial to increase the life span of affected patients, yet a complete cure has not been found.

Molecular subtypes

Breast cancer is not a homogenous disease. There exist many different types of breast cancers and researchers have been using different parameters to classify them. The most common classification was established in the early 2000s based on expression patterns of breast cancer samples from patients. In this classification, breast cancers can be divided into four main clusters according to their molecular profiles: Luminal A, Luminal B, human epidermal growth factor receptor 2 enriched(Her2-enriched) and basal-like or triple negative breast cancer (TNBC)[8–10]. Histologically these subgroups can be defined by analyzing their expression of the hormone receptors (HRs) estrogen receptor (ER) and progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2). Luminal A cancers express high levels of ER and high to

low levels of PR, Luminal B cancers express high levels of ER and high to low levels of PR as well as low to medium levels of Her2, Her2-enriched cancers do not express HR but have a very high expression of Her2 and TNBCs express neither HR nor Her2 (see table1 below). Each subtype is a prognostic factor with Luminal A having the best prognosis, luminal B and Her2-enriched having a medium prognosis and TNBC having the worst prognosis for patient outcome[3,11–13]. In 2007 Herschkowitz et al. discovered an additional subgroup they termed "Claudin Low" due to the lack of adhesion molecule expression in this cluster[14]. Later it was shown that this subgroup has a similar survival as luminal B breast cancer[15].

TABLE 1: THE FOUR DIFFERENT SUBTYPES OF BREAST CANCER AS ESTABLISHED BY THE PAM50 CLASSIFICATION, THEIR HISTOLOGICAL MARKERS, SURVIVAL PROGNOSIS AND STANDARD OF CARE TREATMENT.

Molecular subtype	Histologic markers	Survival	Treatment
Luminal A	ER+PR+	Good	Tamoxifen and aromatase inhibitors
Luminal B	ER+(PR+) Her2+	Medium	Tamoxifen and aromatase inhibitors
Her2-enriched	Her2++	Medium	Anti-Her2 antibodies (trastuzumab, pertuzumab and maretuximab)
Triple negative	HR-Her2-	Worst	Chemotherapy, Radiation, immunotherapy

Approved treatments

The most common treatment in breast cancer, especially TNBC has been chemotherapy, usually in combination with surgery. Several different agents are approved to date for breast cancer chemotherapy. In recent years, early detection methods, targeted therapy as well as immunotherapy vastly improved patient survival in many cases.

Chemotherapy

Doxorubicin (DOX), a family member of the anthracycline family which causes cells death by damaging nuclear DNA and increasing cellular Reactive oxygen species (ROS) production. DOX can have severe side effects, namely damaging the heart muscle and leading to congestive heart failure[16,17], in addition to the fact that many cancer cells remove DOX via membrane transporters. More recently, doxorubicin nanoparticles have been shown to increase both efficacy and safety during treatment[18]. Epirubicin works similar to DOX with the difference that it is generally better tolerated[19].

Paclitaxel, Docetaxel and cabazitaxel belong to the group of taxanes, another group of chemical compounds frequently used in chemotherapy of breast cancer. Taxanes stabilize microtubules by binding to the β-tubulin moiety, thus disrupting cell division[20]. Nab-paclitaxel is an albumin bound version of paclitaxel and has been shown to be more effective in increasing patient survival compared to paclitaxel[21]. 5-Fluorouracil is a fluoropyrimidine. It blocks cancer growth by both incorporating into dividing DNA and blocking thymidylate synthase, an enzyme that synthetizes nucleotides[22]. This leads to disruption of DNA replication and causes rapidly dividing cells, such as breast cancer cells, to go through apoptosis. Cyclophosphamide is a DNA alkylating agent. When metabolized in cells, it binds to guanine in DNA strands, forming covalent bonds, thus locking the DNA into a permanent position, not allowing for DNA replication[23]. Finally, Carboplatin has a similar effect as cyclophosphamide, but the chemistry differs. It also causes stable cross-linkage between DNA strands, thus inhibiting DNA synthesis and cell proliferation[24]. It should be noted that these chemotherapies are often used in combination to avoid resistance, but that in general, combination of chemotherapies does not have the best synergy score in breast cancer[25].

Hormone therapy

Hormone therapy (HR) targets the hormone receptor ER; thus, it is only used in patients with Luminal cancers. The most common used HR is tamoxifen. Tamoxifen and its derivatives 4-hydroxytamoxifen and endoxifen bind to the ER, effectively blocking estrogen from binding. This prevents the translocation of ER into the nucleus, which would lead to increased tumor growth[26]. Both 4-hydroxytamoxifen and endoxifen have a stronger binding ability than tamoxifen to ER, thus catalyzation of tamoxifen into its two derivatives through DYP2D6 is essential for maximum benefit of the therapy[27,28]. Fulvestran is another HR which blocks ER signaling. Similar to tamoxifen it binds to the ER, preventing dimerization which leads to both blockage of ER entry into the nucleus as well as increased degradation of the receptor[29].

Targeted therapy

Targeted therapy is used commonly in Her2-enriched breast cancer. Two anti-Her2 antibodies have been developed in the past: Trastuzumab and Lapatinib. Activation of the Her2 pathway causes increased proliferation of cancer cells, thus blocking its activation in tumors reliant on Her2 generally causes decreased tumor progression[30].

Immunotherapy

Checkpoint inhibitors

Checkpoint inhibitor therapy relies on antibodies which block the inhibition of tumor infiltrating T cells (TILs). Upon T cell activation, programmed cell death receptor 1 (PD-1) is upregulated on their membrane. When PD-1 binds to one of its binding partners, namely PD-L1, PD-L2 or B7-1 (CD80), the T cells become inactive. This T cell inactivation, a process which is crucial to avoid chronic inflammation and tissue damage, is assimilated by tumors to block T cell activity through expression of PD-L1 on their surface[31]. Currently

there are two monoclonal antibodies targeting the PD-1/PD-L1 signaling axis approved by the food and drug administration (FDA) for TNBC in combination with conventional chemotherapy: Atezolizumab[32] and Pembrolizumab[33]. Atezolizumab targets PD-L1 whereas Pembrolizumab targets PD-1[34].

Eosinophils general

Eosinophils

Eosinophils were discovered in 1874 by Paul Ehrlich who gave them their name due to their strong staining with eosin of their basic granules[35]. When analyzed under the microscope, human eosinophils appear with a bilobed nucleus whereas mouse eosinophils have a doughnut shaped nucleus. When stained with eosinophil, Giemsa stain or Sirius red stain, their granules appear bright red-pink.

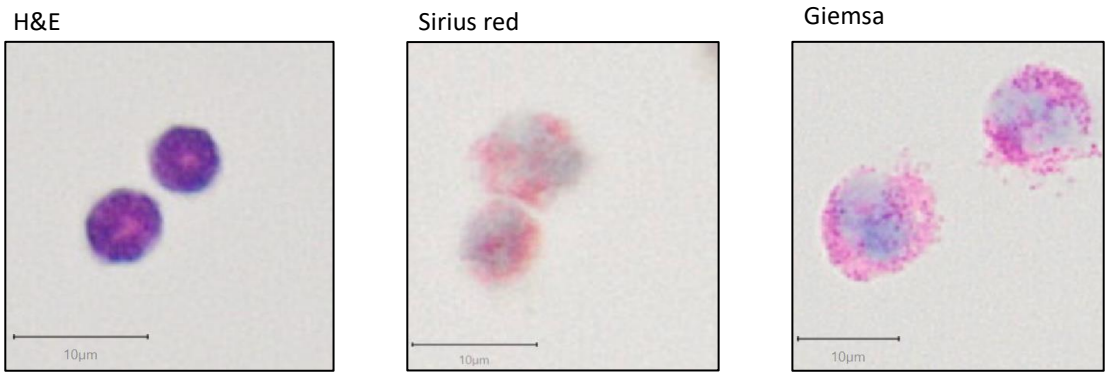


Figure1: Staining of sorted murine splenic eosinophils after cytospins with Giemsa stain (left) and sirius red stain (right). In both stains, the granules appear red-pink.

Eosinophils are part of the innate arm of immunity and are part of the granulocyte family of immune cells. They develop in the bone marrow (see "Eosinophil development") after which they intravasate and rapidly leave the circulation and infiltrate several different organs, such as the intestine, the lungs and adipose tissue.

Normally, eosinophils make up 3%-5% of circulating leukocytes and are present at 350-500 eosinophils/mm^3 in tissues[36]. Any increase of eosinophils compared to those numbers is a phenomenon called eosinophilia. Eosinophilia can occur with and without pathological symptoms, indicating that the activation status of eosinophils determines their destructive capacity. It should also be noted that eosinophilia can be both a temporary condition, due to infection or allergy, or a permanent one, mainly due to genetic variation within the genes responsible for eosinophil development and survival[37].

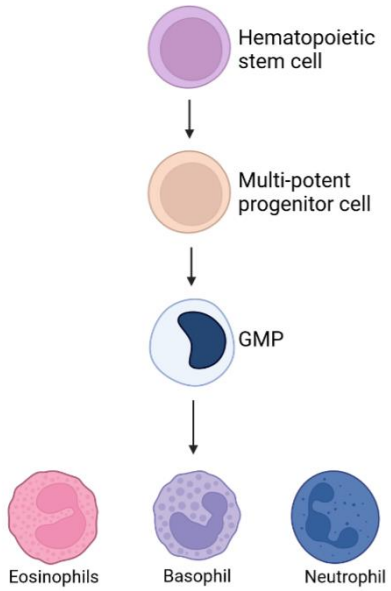


Figure2: Simplified lineage tree of granulocytes. Eosinophils, neutrophils and basophils all differentiate from a common granulocyte-myeloid progenitor (GMP). Created with BioRender.com

For a long time, eosinophils were seen as end-stage effector cells which would swoop into areas of infection, release their granules by bursting and destroy the infectious agent. This view has changed in the last two decades. Not only do we now know that eosinophils have several different and unique methods to release their granules into the environment apart from "exploding", but they have also been shown to be crucial in regulating several homeostatic processes. Because of their vast variety of mediators stored within their granules and surface molecules, eosinophils have been proven to affect many processes and immune cells in health and disease. Importantly, the mediators within eosinophils are pre-formed, allowing them to almost immediately act on the environment in contrast to other immune cells where many cytokines and other effector molecules must first be transcribed and translated.

Similar to other immune cell types, it has become clear that eosinophils consist of a heterogenous population. To date only very few single-cell RNA sequencing (scRNAseq) data exist for eosinophils because the standard preparation process destroys the cells before acquisition, thus most of the information we have is either from bulk sequencing or flow cytometric analyses. One successful method of eosinophil preparation for scRNAseq has been developed by Gurtner et al.[38] and will hopefully allow for further in-depth characterization of this versatile immune cells.

Eosinophil development: from bone marrow to tissue

Transcription Factors

Eosinophil differentiation begins in the bone marrow. GATA-1 and to some extend GATA-2, are the most important transcription factors (TFs) in committing granulocyte-monocyte progenitors (GMPs) towards the

eosinophils lineage[39]. Forced expression of GATA-1 and GATA-2 in human $CD34^+$ hematopoietic progenitor cells (HPCs) *in vitro* caused all the cells to develop into eosinophils, whereas overexpression of dominant negative variants of the transcription factors inhibited eosinophilopoesis[39]. Eosinophils were still present in GATA-1 null mice, most likely due to a compensatory role of GATA-2.

GATA-1 expression is regulated by FOG-1, which suppresses its expression[40,41]. FOG-1 expression is negatively regulated by CCAAT/Enhancer-Binding-Protein (C/EBP). During eosinophil progenitor (EoP) development, an upregulation of C/EBP causes a downregulation of FOG-1 allowing GATA-1 expression which leads to commitment of eosinophil differentiation[40]. The order of GATA-1 and C/EBP expression is crucial in lineage commitment and so is the level of C/EBP expression[42]. If GMPs express GATA-1 before C/EBP they differentiate into neutrophils, whereas C/EBP expressing GMPs become EoP upon GATA-1 induction[43]. C/EBP expression is controlled by Trib1. Bone marrow cells with a Trip1 knockout had decreased levels of C/EBP and were not able to differentiate into eosinophils[44,45]. Another TF regulating GATA-1 and GATA-2 expression and thus eosinophil development, is IRF-8. During early commitment of eosinophils IRF-8 upregulates GATA-1 and GATA-2 expression[46]. XBP-1 is also necessary for GMPs to become EoPs. It does not regulate any of the other mentioned transcription factors but its deletion in GMPs block eosinophil differentiation[47]. Once GMPs have differentiated into EoPs, IL5 signaling causes the precursors to become mature eosinophils. ID1 and ID2 become essential at this stage of eosinophilopoiesis. Downregulation of ID1 and upregulation of ID2 are necessary for eosinophil maturation. In hematopoietic stem cells overexpressing ID1, eosinophil differentiation failed, whereas forced ID2 expression caused all cells to differentiate into eosinophils[48]. Although not necessary for lineage specification, PU.1 is essential for eosinophil maturation, as PU.1 knockout cells are not able to respond to exterior maturation signals, such as IL5[49]. It should be noted that, although eosinophils mainly differentiate within the bone marrow, several studies found EoP within the circulation of asthmatic patients[50,51] and the lung tissue of mice in a model of allergic airway inflammation[52].

The respective up- and downregulation of the different TFs is depicted in Figure3 and their interactions in Figure4.

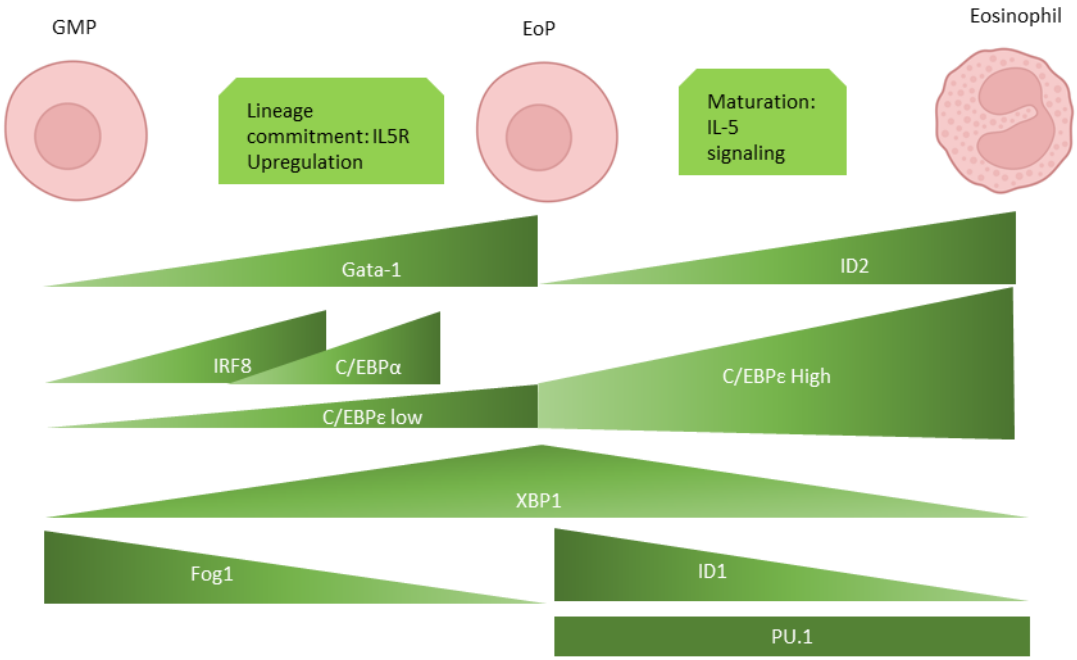


Figure3: Schematic representation of the variation of transcription factors during eosinophil development and maturation.

GMP: Granulocyte-monocyte progenitor. EoP: Eosinophil progenitor. IL5R: IL5 receptor.

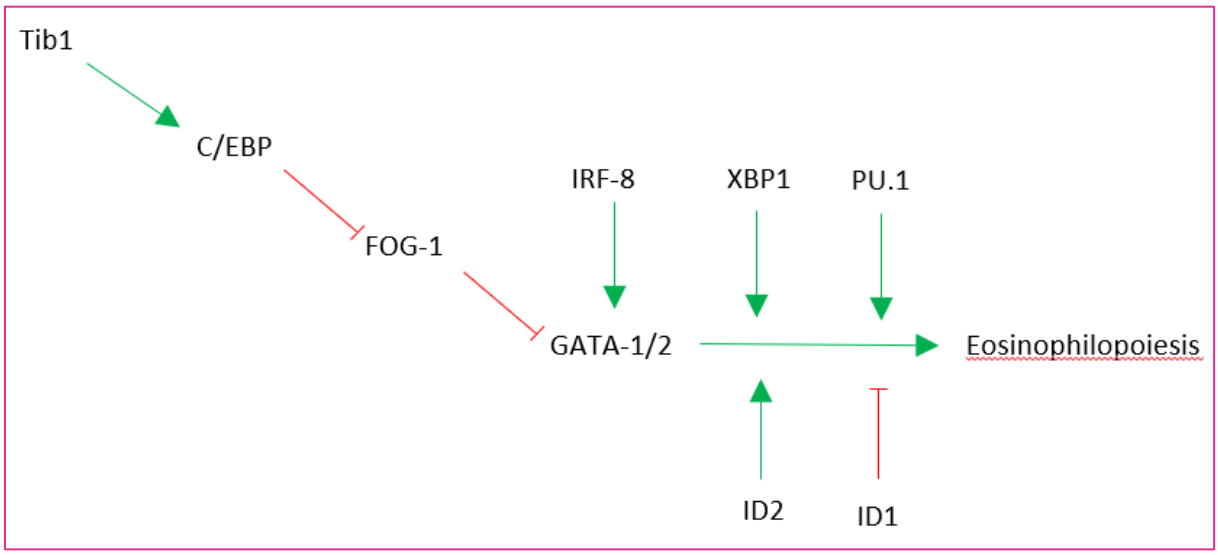


Figure4: Schematic representation of the interplay between the different transcription factors necessary for eosinophil differentiation. Activation is depicted by green arrows and inhibition by red lines.

Exogenous Signaling molecules

Interleukin-5 (IL5) was found to be the main eosinophil differentiation factor[53–57]. In the bone marrow (BM) the main IL-5 producing cells are $CD34^+$ progenitor cells[58] , stromal cells upon IL-1b stimulation[59] and Cd3+ cells upon sensitization and allergen challenge[58]. IL-5 binds to its cognate receptor IL-5R[60] which is

composed out of two subunits, α and β. The α-chain is specific for IL-5 whereas the β chain is shared between IL5, IL3 and CSF2 (GM-SCF)[61–73]. Binding of IL5 to IL5R on EoPs causes their maturation into eosinophils[74]. Additional roles of IL5 on eosinophil activation and survival will be discussed later. Two alarmins have also been shown to potentially be able to cause eosinophil differentiation as well: Thymic stromal lymphopoietin (TSLP) and IL33. TSLP has been shown to promote eosinophil differentiation *in vitro* from human blood derived $CD34^+$ hematopoietic stem cells. When culturing these cells together with IL3 and TSLP, pluripotent cells became mature eosinophils[75]. IL33 was shown to be able to stimulate circulating human CD34+ cells to produce and release factors typical of eosinophils, such as IL13 and IL6[76]. This does not prove that IL33 induced differentiation, since the authors did not analyze the morphology of the cells and the cells were still expressing CD34, a marker that is lost in mature eosinophils. Another study, using mouse bone marrow derived cells, showed that incubation with IL-33 caused eosinophilopoiesis. When adding anti-IL5 antibodies to the cell culture, eosinophilopoiesis was markedly, although not completely, decreased[77]. This could indicate that IL33 could cause differentiation of progenitors into eosinophils in combination with IL5. Another report supporting these findings showed that *in vivo*, administration of IL33 strongly increased blood eosinophilia which was reduced to base levels when anti-IL5 antibody was administered[78]. The effect of the different exogenous factors and GMPs and EoPs are depicted in Figure5.

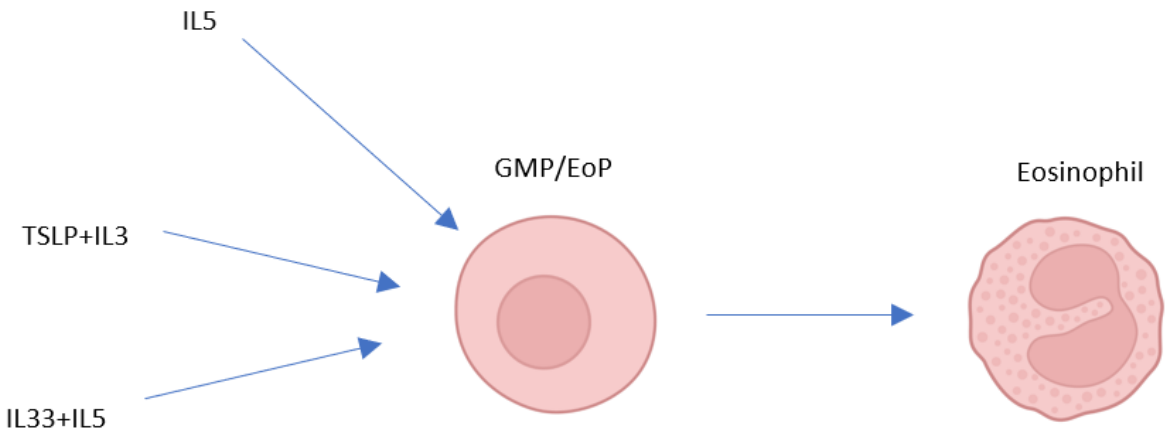


Figure5: Schematic representation of the effects IL5, TSLP + IL3 and IL33 + IL5 on the differentiation of GMPSs or EoP to mature eosinophils.

GMP: Granulocyte-monocyte progenitor. EoP: Eosinophil progenitor.

Modes of secretion and extracellular traps.

Eosinophils have three modes of secretion: Exocytosis, cytolysis and piece-meal degranulation (PMD). Exocytosis is not observed *in vivo* but was shown *in vitro* upon co-culture of eosinophils and heminths[79]. Cytolytic degranulation occurs when the eosinophil undergoes apoptosis and releases its entire content into the environment whereas PMD allows eosinophils to secrete granules containing different cytokines

one by one without dying[80]. These secreted granules remain intact and are capable of releasing their content upon stimulation with environmental cues[81]. Recently the formation of eosinophil extracellular traps (EETs), reminiscent of neutrophil extracellular traps (NETS) and composed of intact granules and mitochondrial and nuclear DNA, were discovered[81–83]. Different stimuli cause the release of granules with different contents, and we still do not have a complete understanding of this selectively. This topic has been summarized in an excellent review by Fettrelet et al.[84]

Eosinophil granule content

As mentioned, eosinophils contain a plethora of cytokines, chemokines, toxic proteins and lipid mediators within their pre-formed granules which they can release upon stimulation. The four main granule proteins contained within in eosinophils are major basic protein (MBP) (not to be confused with myelin basic protein, also abbreviated MBP), eosinophil peroxidase (EPX or EPO), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) and will be discussed in some detail. Table2 summarizes some additional cytokines and chemokines within eosinophil granules and a few examples of their possible effects on immune cells. Note that many factors released by eosinophils can have an autocrine and/or paracrine effect.

MBP, encoded by the PRG2 gene, is an extremely basic protein with an isoelectric point of 11.4. When released, it destabilizes the membrane of cells, single-cell parasites and worms, thus it was thought of for a long time as destroying any cell or organism indiscriminately[85]. In OVA-induced asthma, MBP has been shown not to cause eosinophil infiltration, mucus production or increased airway hyper-responsiveness (AHR)[86], implying that despite its destructive potential, it does not affect eosinophil infiltration and does not always cause tissue damage. MBP has been shown to decrease epithelial barrier integrity in a model of oxazolone induced colitis[87]. During nematode infections of mice with different parasites injected subcutaneously or intravenously into recipients followed by infection of the lungs, MBP was essential to reduce parasite burden[88,89]. In a model of infection where parasites where places in a cell-permeable diffusion chamber and then implanted into mice, MBP also played an important role in larval killing[90]. In addition to direct cell killing, MBP can signal to basophils and mast cells, causing histamine and leukotriene C4 release *in vitro*.

EPX, as its name suggests, is a peroxidase and can produce oxidants which are toxic to the surrounding tissue. In an OVA-induced model of asthma, EPX knockout mice had phenotypes similar to those of their wild type (WT) littermates, namely there was no difference in eosinophils recruitment to the lungs, airway mucus production or AHR. It should be noted that even though not significant, there was a slight trend of reduced airway eosinophilia in $EPX^{-/-}$ mice[91]. These results indicate that EPX alone is not sufficient to cause the lung damage seen during asthma. When mice were challenged intranasally with a fungus which can cause asthma, there was a significant decrease of eosinophils to the respiratory tract in $EPX^{-/-}$ mice compared to WT. Additionally, eosinophils in the spleens and lungs of challenged animals had decreased

levels of TLR4, which interestingly did not affect the ability of these mice to control infection with a gram-negative influenzae bacterium[92]. During nematode infection, the absence of EPX paradoxically increased the number of eosinophils in the thoracic cavity and decreased the number and lengths of the worms. This could be explained by increased IL5 production by other cells in the lung during infection[89] even in the absence of EPX. In dextran sulfate sodium (DSS) induced ulcerative colitis (UC) EPX was causative for disease symptoms such as weight loss, diarrhea and rectal bleeding. In $EPX^{-/-}$ mice, there was a significant decrease in weight loss, diarrhea and rectal bleeding, showing that in this model of UC, the main cause of damage if EPX. Of note, eosinophil number were not changed between challenged WT and $EPX^{-/-}$ colons[93].

Both EDN and ECP belong to the family of RNase A and are also known as RNase 2 and RNase 3 respectively[94–96]. Both EDN and ECP have been used as biomarkers in asthma in order to predict the severity of the disease[97,98]. Additional functions of EDN are clearance of human respiratory syncytial virus (RSV)and human immunodeficiency virus (HIV) *in vitro*[99,100], and repression of plasmacytoid dendritic cell (pDC) IFNα release[101]. Similar to EDN, ECP has anti-viral functions *in vitro* against RSV albeit not as strongly[102]. Interestingly, there seems to be no synergy between EDN and ECP against RSV *in vitro*[102]. There has not been much research into either of these RNases in recent years and their exact roles and functions during homeostatic and pathologic conditions *in vivo* are not yet explored.

TABLE 2: CYTOKINES AND CHEMOKINES WITHIN EOSINOPHILIC GRANULES AND THEIR POTENTIAL FUNCTIONS. ATM: ADIPOSE TISSUE ASSOCIATED MACROPHAGES. NK: NATURAL KILLER CELLS. ILC2: TYPE 2 INNATE LYMPHOCYTES. DC: DENDRITIC CELLS.

Secreted molecule		**Potential functions on the environment**	**Reference**
IL3	Interleukins	Development and activation of basophils and mast cells.	103–106
IL4		Polarization of CD4 T cells, sustaining ATM, mammary gland development.	107–110
IL5		Eosinophil development, survival and intravasation, B cell development and antibody isotype switching.	53–57,111–113
IL12		Polarization of CD4 T cells, activation of NKs.	114
IL13		Eosinophil activation, macrophage polarization, sustaining ATM.	110,115
IL25		Promotion of type 2 immune response.	116,117
IL33		Eosinophil activation, ILC2 and Th2 activation.	118–122

IFNγ		Activation of T cells and M1 polarization.	[123]
TNFα	Pro-inflammatory cytokines	Macrophage activation.	[124]
CSF2 (GM-CSF)		Immune modulation of macrophages, granulocytes and T cells.	[125–127]
CCL11		Eosinophil and basophil attraction and activation.	[128–131]
CCL5 (RANTES)	Chemokines	Activation and attraction of granulocytes and macrophages.	[132–135]
CXCL9/CXCL10		Attraction of T lymphocytes, NKs and DCs.	[136,137]

None-immune roles of eosinophils

Eosinophils have been shown to not only be part of the immune response caused by different infection agents and in pathological conditions such as asthma, but also in the development and maintenance of different tissues. They are important in maintaining a permissive immune microenvironment within the gut, contribute to liver and muscle regeneration, thermoregulation, mammary gland development and adipose tissue homeostasis[80]. Their roles in the two latter processes are discussed in more detail here to illustrate the versatile roles of this immune cell.

Breast development

In 2000, Gouon-Evans et al.[138] made the break-through discovery that eosinophils, together with macrophages, are necessary and sufficient for mammary gland development in mice. First, they observed both cell types next to the terminal end bud (TEB), where macrophages tended to localize at the neck and eosinophils at the end of the TEB. To analyze the functions of macrophages and eosinophils, they used a CFS1 and CCL11 deficient mouse model respectively. In the absence of CSF1, macrophages are severely diminished whereas lack of CCL11 prevented accumulation of eosinophils in the mammary gland. They found that macrophages were crucial for TEB elongation whereas eosinophils were crucial for TEB branching. Of note, no effect on mammary gland associated macrophages was seen upon removal of eosinophils. That eosinophils do not affect TEB elongation was also shown by Sferruzzi-Perri et al.[139] when using mice that overproduced IL5, causing blood and tissue eosinophilia. Branching on the other hand was slightly but significantly decreased at 5 weeks of age but normalized at 7 weeks of age in eosinophilic mice compared to wild type (WT) mice. Estrogen and progesterone play an important role in mammary gland development and were shown to be at least partially responsible for macrophage and eosinophil attraction to the developing mammary gland[140,141].

Adipose tissue homeostasis

It has long been known that anti-inflammatory, alternatively activated type 2 macrophages (M2) are important for adipose tissue (AT) homeostasis[142,143]. But how this M2-like phenotype was sustained in AT was not clear until 2011, when Wu et al.[144] published a paper showing that eosinophils are crucial for this process. In IL4/IL13$^{-/-}$ or eosinophil deficient mice there was an almost complete loss of AT associated M2 macrophages. Thus, the authors concluded that eosinophils maintain M2 macrophages in AT mainly through secretion of IL4 and IL13, both of which have been shown to be potent induces of the M2 phenotype. Innate lymphocyte type 2 cells (ILC2, discussed in more detail below) were shown to produce IL4, IL13 and IL5 upon IL33 stimulation from white adipose tissue-resident multipotent stromal cells (WAT-MSCs). IL5 increased local survival of eosinophils(discussed in more detail below) whereas IL4 and IL13 caused increased production and secretion of CCL11 by WAT-MSCs and increased eosinophil infiltration[145] thus it was proposed that eosinophils are attracted and maintained in AT through a signaling network between ILC2 and AT stromal cells.

Eosinophil activators

Because of their large variety of surface receptors, including adhesion receptors, cytokine and chemokine receptors, receptors for lipid mediators, pattern-recognition receptors (PPRs), complement receptors and Fc receptors, eosinophils can be regulated by many inputs. The activation of eosinophils by colony stimulating factor 2 (CSF2), interferon-γ (IFNγ), lipopolysaccharides (LPS), Polyinosinic:polycytidylic acid (Poly(I:C)) and tumor necrosis factor (TNFα) will be discussed briefly. The effect of IL5, IL33 and CCL11 will be discussed in more detail bellow. Additional activators and their effects are listed in table 3.

CSF2

In a model of colitis, induced by *Helicobacter hepaticus* infections concomitant with IL10R blockage, eosinophils increased significantly in the bone marrow and in the colon of infected wild type mice[146]. These eosinophils produced high levels of the pro-inflammatory cytokines TNF, IL13 and IL6. In mice lacking the CSF2 receptor (CSF2rb$^{-/-}$ mice) on the other hand, there was a significant decrease in overall eosinophils, SiglecF^{hi} activated eosinophils and the colitis score. Depletion of CSF2 using an antibody showed the same results. Additionally, anti-CSF2 treatment *in vivo* decreased eosinophil activation as illustrated by the decrease of EPX activity whereas addition of CSF2 *in vitro* significantly increased the production of TNF and IL13 by eosinophils isolated from healthy colons. Upon stimulation with CSF2 of bone marrow derived eosinophils *in vitro*, eosinophils significantly upregulated a pro-inflammatory signature, including upregulation of the interferon regulatory factor IRF5. Furthermore, CSF2 treatment of splenic eosinophils from IL5-tg (mice with constant eosinophilia, discussed in more detail in "Mouse models to study the function of eosinophils") mice significantly increased their expression of SiglecF and CD11b, both markers used to asses eosinophils activation[147,148]. CSF2 stimulated eosinophils were crucial to reduce tumor burden

in both MC38 and APC$^{min/+}$ mouse models of colon cancer[149]. Interestingly, the addition of CSF2 to eosinophils isolated from IL5-tg mice spleens showed significant increase in expression of MHCII as well as the co-stimulatory molecules CD40, CD80 and CD86[150]. When these activated eosinophils were pulsed with OVA peptide and installed intranasally into OVA sensitized mice, they efficiently activated endogenous T cells as measured by IL4 and IFNγ production.

IFNγ

The effects of IFNγ on eosinophils has not been studied extensively. Yet there are some studies which show that not only do eosinophils express the IFNγ receptor (IFNγR) but that they can be activated through INFγ signaling. Treatment of human peripheral blood eosinophils with IFNγ increased their expression of FcRI (CD64) and FcRIII (CD16). The upregulated CD16 was functionally active as shown by eosinophils depolarization upon binding with an anti-CD16 antibody. It also caused their degranulation and release of pre-stored RANTES[151]. Addition of IFNγ to human eosinophils pre-activated with either CSF2 or IL5 increased their superoxide production and degranulation[152]. It did not however change their expression of CSF2R, IL5R and CD11b.

LPS

Lipopolysaccharides (LPS) are part of the outer membrane in gram-negative bacteria. Our immune system has evolved to recognize LPS through expression of toll-like receptors (TLRs) on our immune cells. The main TLR for LPS is TLR4, expressed on a majority of myeloid cells[153,154]. Stimulation of the eosinophil cell lines dEoL-1 with a combination of LPS and butyrate strongly increased their secretion of a variety of cytokines, such as CXCL12, CCL3, macrophages migration inhibitory factor (MIP), IL8 and CXCL10[155]. *In vivo* treatment of mice with LPS through intrathoracic injections significantly increased CCL11/CCR3 dependent migration of eosinophils into the thoracic cavity[156]. *In vitro* treatment of peripheral blood eosinophils isolated from mildly allergic patients increased both CD69 and CD11b expression upon LPS treatment, indicating increased activation[157]. Furthermore, IL4, IL8 and IL13 production and secretion were also significantly increased. Peripheral blood eosinophils with mild asthma significantly increased eosinophil extracellular trap and reactive oxygen species production upon LPS treatment[158]. When treating blood eosinophils from healthy donors with LPS, both TNFα and eosinophil cationic protein secretion was increased in a concentration dependent manner[159]. Injection of LPS pre-treated eosinophils into the peritoneum caused a significant increase in natural killer cells (NK)[160]. Lastly, LPS increased the capacity of eosinophils to kill hepatocellular carcinoma cells which was partially through TNFα.[161]

Poly(I:C)

Polyinosinic:polycytidylic acid (Poly(I:C)) is used to mimic double stranded RNA which is a hallmark of viral infection[162]. Poly (I:C) is preferentially recognized by RIG-I and MDA-5[163]. Freshly isolated human blood eosinophils expressed high levels of RIG-1 whereas MDA-5 levels were very low[164]. Interestingly, Poly(I:C)

stimulation did not increase eosinophil activation as measured by IL8 and eosinophil derived neurotoxin (EDN) release. In another paper, Poly(I:C) increased the secretion of the cytokines IL8, MIP-1b, NAP2, MCP1 TGF-β2, TIMP1 and IMP2 in eosinophils but had no effect on IL-1β, IL5 and eosinophil cationic protein (ECP)[165]. Finally, no effect on CD11b, superoxide generation and eosinophil survival was shown by Nagase et al[166]. Of note, all these experiments were performed on freshly isolated blood eosinophils, which have a tendency to be less activated than tissue resident eosinophils[167].

TNFα

Not only is TNFα produced by eosinophils, but it can also directly activate them. TNFα significantly increased human blood eosinophil survival, expression of the adhesion molecules ICAM and VCAM, IL8 and CSF2 release as well as ECP secretion[168,169]. IL12 mRNA transcription was also increased upon incubation of human blood eosinophils with TNFα[170] as well as the secretion of CCL22[171]. Importantly, TNFα seemed to synergize with IFNγ or IL4 to increase production of CXCL9 and CXCL10 or CCL17 and CCL22 respectively[171]. It also increased expression of thymic stromal lymphopoietin receptor (TSLPR), which when engaged with thymic stromal lymphopoietin (TSLP) activates eosinophils[172].

TABLE 3: ADDITIONAL ACTIVATORS OF EOSINOPHILS. HBE: HUMAN BLOOD EOSINOPHILS. LTB: LEUKOTRIENE. PGE: PROSTAGLANDIN. TX: THROMBOXANE. EPX: EOSINOPHIL PEROXIDASE. ROS: REACTIVE OXYGEN SPECIES. EDN: EOSINOPHIL DERIVED NEUROTOXIN.

Activator	**Outcome**	**Ref**
PAF	Increased release of superoxide and LTB4 in HBE Increased release of PGE2 and TXB2	173
RANTES	Increased migration and intracellular calcium release in HBE Increased ROS production in HBE	133 174
Periostin	Increased adhesion through αMβ4-integrins in HBE	175
leukotriene	Increased migratory capacity, increased EDN and superoxide release in HBE Increased IL4 release in human umbilical cord derived eosinophils	176 177
prostaglandins	Increased lipid bodies in mouse eosinophils Increased cytosolic calcium and LTB4 release in HBE	178 179
Histamine	Increased cytosolic calcium and LTB4 release in HBE	179
IL4 and IL13	Increased activation of eosinophils	180–184

Complement	C3a and C5a attract HBE, increased polarization and EPX release	185

Interleukin-5 (IL5)

IL5 is the main differentiation, maturation and survival factor for eosinophils. Initially, IL5 was discovered as an important cytokine for B cells differentiation[111] and later its crucial role in eosinophils was shown. Multiple signaling mechanisms have been found for IL-5.[186–194] Binding of IL5 to IL5R causes JAK1 and JAK2[186] activation and signal transduction via STAT1, STAT3 and STAT5[192] which then translocate to the nucleus, leading to cell maturation[53–57], proliferation[186] and suppression of apoptosis[195]. IL5 also signals through PI3K leading to ERK signalling[194]. Overall, IL5 signaling in eosinophils leads to prolonged survival by suppressing apoptosis[195–202], polarization[203] and increased expression of adhesion molecules allowing eosinophils to extravasate[175,204,205] and, depending on the context, degranulate.[206]

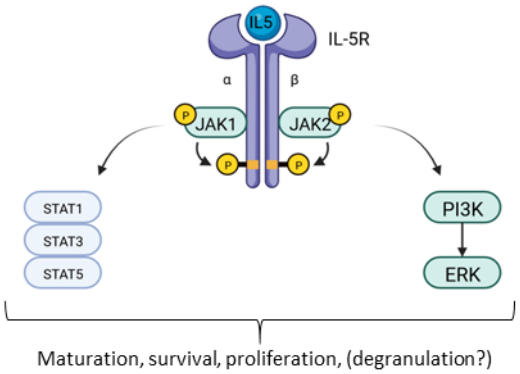


Figure 6: Schematic representation of IL5/IL5R signaling. Activation of IL5 leads to phosphorylation of Jak1/2, causing activation of STAT1/3/5 and PI3K, causing maturation, increasing survival and proliferation and perhaps allowing for degranulation. Created with BioRender.com

Mice overexpressing IL5 have shown that not only is IL5 important for early eosinophil differentiation but also to "pull" mature eosinophils out of the bone marrow into the blood stream and cause blood and tissue eosinophilia.[72,207–213] Indeed, IL5 transgenic mice (IL5-tg) had a 65-265 fold increase in blood eosinophils compared to control littermates. [214] Transgenic mice overexpressing IL5 only in the lung showed dramatic lung pathologies compared to WT mice.[215] IL5 knockout mice as well as mice treated with a specific anti-IL5 antibody, showed decreased basal eosinophil numbers as well as a reduction in eosinophil accumulation during infections and allergic stimulation in the lung[216–229].

IL5 is produced mainly by activated Th2 and innate lymphoid type 2 cells (ILC2)[225,230–237] and in some models by natural killer cells (NK).[238,239] Furthermore, IL5 can also be produced by activated eosinophils, allowing for an autocrine loop to increase eosinophil numbers. [240–245] During eosinophilic asthma, IL5 causes the accumulation of eosinophils within the lung. This is followed by eosinophil degranulation and destruction of pulmonary tissue. Early on, it was discovered that repressing IL5 signaling via a specific antibody against

IL5 could decrease the accumulation of eosinophil in the lungs and thus alleviate asthma related symptoms such as wheezing and destruction of lung tissue[246,247]. In order to attract eosinophils to the lungs, especially in an asthma setting, Th2 cells induce the local production of CCL11 by fibroblasts, epithelial cells and smooth muscle cells via IL4 and IL13 and release IL5 into the circulation in order increase the release of eosinophils from the bone marrow into the circulation allowing intravasation into the lung. This causes a massive infiltration of eosinophils accompanied by their degranulation which in turn causes symptoms such as wheezing, lung tissue fibrosis and airway hyperresponsivness[248–250]. More recently, a new type of innate lymphocyte cell termed ILC2 has been identified and shown to play a major role in IL-5 production. The interaction between these cells and eosinophils will be discussed in more detail below.

Because IL5 has such an important impact on eosinophils, which in turn can cause different eosinophil-related diseases such as asthma or hyperreactivity, a strong interest has been growing to control IL5 in patients. This has resulted in the development of three different antibodies targeting the IL5-IL5R signaling axis, Mepolizumab, Reslizumab and Benralizumab. All three have shown significant reduction in patient eosinophilia, and increased patient well-being.[72,228,251–307]

Interleukin-33 (IL-33)

IL-33 is a relatively newly discovered member of the IL1 cytokine family of alarmins. Its N-terminal domain localizes it to the nucleus whereas the C-terminal domain contains the functional cytokine. Because of the N-terminal domain, IL33 is found in the nucleus of epithelial and endothelial cells under homeostatic conditions and is released upon inflammatory stimulation[308–310], airway inflammation[311], necrosis[312] and necroptosis[313] . Other cells which release IL33 are intestinal epithelial cells upon E.Coli infection[314], keratinocytes during atopic dermatitis induced by house dust mite[315] and activated bronchial smooth muscle cells[316]. Overall, IL33 is preferentially expressed in non-immune cells and released upon cell damage.

IL33 binds to its receptor ST2, encoded by the Il1r1 gene, which causes the recruitment of IL-1 receptor accessory protein (IL-1RAcP)[317] and signal transduction. The co-localization of ST2 and IL1RAcP cause the recruitment of MyD88, IRAK1, IRAK4 and TRAF6 which in turn activate the p38, ERK, JNK pathways as well as NF-κb signaling[316,318–320]. Several mechanisms exist to inhibit IL33 signaling. Soluble ST2 (sST2) can bind to IL33 and act as a sink to prevent signal transductions[321], the cytokine can be retained within the nucleus through its N-terminal domain or caspases can cleave the cytokine domain for inactivation.

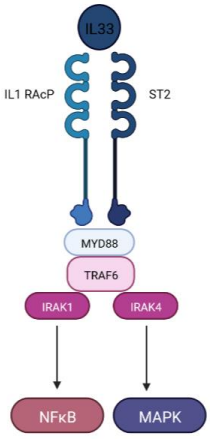


Figure 7: Schematic representation of IL33/ST2 signaling. Binding of IL33 leads to co-localization of ST2 and IL1RAcP which transduce their signal though MyD88, TRAF6 and IRAK1/4. This leads to activation of the MAPK and NFκB signaling pathways. Created with BioRender.com

IL-33 direct effects on eosinophils

To date, the main effect of IL33 on eosinophils is thought to be an indirect one through T cells or innate lymphocytes. There is some *in vitro* and *in vivo* evidence suggesting that IL33 can influence eosinophils directly.

In vitro, IL33 stimulated superoxide and EDN release from human eosinophils, as well as increased survival but has no effect on neutrophils, as the latter were shown not to express ST2[118,318]. It also stimulated the release of CCL2, CXCL8 and IL6[318] and increase expression of adhesion molecules[318,322] in eosinophils. *In vitro* treatment of mouse bone marrow (BM) differentiated eosinophils with IL33 decreased apoptosis and this was dependent on ST2 and MyD88. IL33 treatment increased release of GM-SCF from cultured eosinophils which caused an autocrine loop, necessary for increased eosinophil survival IL-33-Induced Cytokine Secretion and Survival of Mouse Eosinophils Is Promoted by Autocrine GM-CSF[323]. IL-33 caused increased release of IL13 and IL4 from eosinophils via P38 MAPK signaling. *In vitro*, IL33 activated eosinophils mediated an M2 phenotype of co-cultured macrophages.[323] In gonadal adipose tissue (GAT), IL33 blockage decreased the number of eosinophils whereas administration of IL33 via I33 expressing J558L cells increased GAT eosinophils even in the absence of ILC2s, indicating a direct effect of IL33 on eosinophils *in vivo*[324].

IL33 indirect effect on eosinophils through ILC2 and T cells

Recently identified type 2 innate lymphoid cells (ILC2) were shown to be crucial regulators of eosinophils *in vivo*[325]. These ILC2 express ST2 and their regulation of eosinophils strongly depends on IL33 signaling. For instance, during injection with *S. Aureus* into mice, IL33 increased accumulation of eosinophils to the lungs more than 15-fold. But in PLZF knockout mice, which lack ILC2, eosinophils only increased about 2-fold[326]. In an OVA-induced asthma model, depletion of IL33 caused significant decreases in IL5 and IL13, both

cytokines which are important for eosinophil survival and activation[120]. IL33 intranasal administration increased the amount of IL5 and IL13 producing ILCs significantly in the lung which was mainly dependent on mTOR signaling.[119] In mouse GAT, IL33 caused an increase in IL5 producing ILC2s concomitant with an increased number of gonadal adipose tissue (GAT) associated eosinophils. Another study looking at the effect of ILC2 on adipose tissue during ageing showed that administration of IL33 significantly increased ILC2 in visceral adipose tissue, concomitant with a significant increase in eosinophils compared to non-treated mice[327]. Co-culture of eosinophils and IL5^{+}ILC2s showed that the latter caused increased expression of PRG2 mRNA in eosinophils via IL5.[324] Crucially, ILC2 in the bone marrow were shown to be the main source of IL5 for eosinophil differentiation upon intranasal administration of IL33[328].

The other main cell type to regulate eosinophils through IL33 are Th2 cells. A more detailed description of the interactions between eosinophils and T cells can be found bellow. In this paragraph, I will only discuss the effect IL33 has on eosinophils through Th2 cells. ST2 expression on Th2 cells is well known[329] and was in fact found long before ST2 expression in ILC2. *In vitro* polarized Th2 cells were shown to significantly increase the production of IL4, IL5 and IL13 upon IL33 stimulation[119,330]. IL33 has also been shown to stimulate Th2 polarization of naïve CD4 T cells in combination with TCR activation[121]. In a murine model of asthma, activation of Th2 cells with IL33 was shown to be crucial for eosinophils infiltration of the lungs[121].

Effect of IL33 on other immune cells

IL33 has also been shown to affect Tregs, mast cells (MC), basophils and dendritic cells (DC). I briefly summarized the effects of IL33 on these four different immune cell population below:

Specialized Tregs, found mainly in non-lymphoid tissues, express ST2. When IL33 signaling occurs in these cells, they secrete IL-10 and TGF-β in order to dampen the immune response[331]. *In vivo*, in an experimental colitis mouse model, IL33 decreased the severity of the symptoms by promoting tolerogenic DCs which in turn induced immunosuppressive Tregs[332]. In the intestine, IL33 signaling was crucial to maintain homeostasis. Upon ST2 deletion in Tregs, mice developed much more severe symptoms in a model of induced colitis[333]. IL33 signaling was important for *in vitro* proliferation of Tregs from visceral adipose tissue (WAT), lungs and spleens and *in vivo* treatment of mice with IL33 increased Tregs in VAT, lungs and spleens[122].

During colitis, MC upregulate ST2 in the gut[334]. IL33 increases human and mouse MC survival *in vitro* and *in vivo* by maintaining high expression of the anti-apoptotic protein Bclxl[335]. MC are commonly thought of as pro-inflammatory cells, causing damage during a type 2 inflammatory response during gut and airway inflammation. But contrary to this view, MC have been shown to reduce lung eosinophilia upon IL33 treatment through increasing Tregs[336]. Similarly, IL33 signaling in MC during colitis increases mucosal healing and potentiates an immunosuppressive environment[337]. On the other hand, IL33 increased IgE mediated MC degranulation *in vitro*[338] and exacerbated bronchoconstriction in an OVA-induced airway inflammation model[339].

In vitro, IL33 can induce expression of both soluble and membrane bound ST2 in basophils. It can further cause release of the type 2 immunity associated cytokines IL13 and IL4 as well as IL8[340]. During lung development in mice, IL33 signaling in basophils is crucial, as it allows them to cause alveolar macrophage maturation[341]. In a model of allergic rhinitis, IL33 signaling was important for basophil attraction to the nose. IL33 knockout mice showed significant reduction of basophil numbers upon ragweed stimulation[342]. Lastly, human basophils were shown to increase secretion of IL5 upon stimulation with IL33 and IL3[343].

BM derived DC express ST2, mainly intracellularly. *In vitro* treatment of DCs causes them to produce IL6 in a concentration dependent manner. Both MHCII and CD86 were upregulated upon IL33 *in vitro* treatment. Co-culturing IL-33 activated DCs increased the production of IL5, IL13 and CCL11 in CD4 T cells[344]. *In vivo*, IL33 administration caused an increase in lung DCs, as well as the increase of CD40, CD80, OX40L and CCR7 expression in lung DCs. In an OVA-induced airway inflammation model, IL33 pretreated DCs were injected into the lung and caused increased eosinophil peroxidase activity.[345]

It is clear from these results that IL33 is not an eosinophil specific cytokine. Many of its actions seem to be mediated by both the adaptive and innate immune system. Despite its effect on several different cell types, IL33 is a cytokine which can cause eosinophil attraction and activation both directly and indirectly *in vivo*.

IL-33 in breast cancer

IL33 has been implicated in tumor progression in many solid cancers. Depending on the cancer type as well as the study, it can have both pro- and antitumorigenic functions. Here I will briefly present the impact IL33 has on breast cancer, in both humans and mouse models.

The role of IL33 in breast cancer has only recently begun to draw interest from scientist. Only few human studies exist which look at the effect of IL33 on breast cancer. IL33 serum concentration in breast cancer patients was found to be significantly higher than in healthy controls and was increased in more advanced cancers compared to lower stage cancers[346–348]. In breast cancer patients, increased IL33 expression was correlated with a slight but significant benefit for patient survival in all four molecular subtypes of breast cancer and in TNBC[349,350] whereas another study showed that levels of circulating IL33 had no prognostic value[351]. IL33 was also increased in lung metastases of breast cancer patients compared to the adjacent, healthy tissue. Notably, the authors showed that the main source for IL33 were lung fibroblasts and not epithelial cells[352].

Downregulation of IL33 in MCF7 breast cancer cells thought shRNAs decreased the mRNA levels of several stem cell associated genes and decreased *in vivo* tumor growth[348], implying a tumor promoting role of IL33 in this breast cancer model. I must emphasize that because the authors analyzed a human cell line, it is difficult to know if this result is an artifact due to the absence of innate immunity which, as described, is crucial to mediate the effects of IL33. In the 4T1 mouse model of breast cancer, exogenous IL33 treatment increased primary tumor growth, primary tumor cell proliferation, tumor vasculature, lung and liver metastases. It should be noted that in their control treated mice, they saw no lung metastases, a rather

strange finding, considering that after 1 week, the 4T1 cancer cells have already spread in all of our experiments. They also showed that IL33 treatment significantly changed the lung immune environment, increasing overall myeloid cells, Tregs and DCs and decreasing NKs.[353] In a study characterizing the effects of *cordyceps sinensis* extracts, a traditional Chinese medicine, on breast cancer metastases in the 4T1 mouse model, the authors showed that upon treatment, both lung metastases and lung IL33 levels decreased. Although not a proven causative role, there was certainly a correlation between decreased lung metastases and decreased lung IL33 levels[354]. Immunization using viral particles presenting IL33 showed decreased IL33 levels concurrent with decreased primary tumor growth and lung metastases. There also was a decrease in Th2 T cells and Tregs in the lungs and an increase in Th1 T cells[355]. Metastatic seeding of 4T1 and PyMT cells to the lungs caused a significant increase in IL33 expression, mainly by fibroblasts. This increase in IL33 led to increased lung infiltration by immune cells and depletion of IL33 in an orthotopic model of 4T1 metastases decreased both metastatic seeding and metastatic growth in the lungs[352]. Finally, IL33 treated, 4T1 intravenously (i.v.) injected Balb/c mice had significantly reduced lung metastases burden and increased survival compared to PBS treated mice[356]. IL33 treatment significantly increased natural killer cells (NK), ILC2, macrophages, eosinophils and myeloid derived suppressor cells (MDSCs) in the lungs. NKs were shown to be causative for decreased metastases as antibody mediated depletion restored metastases numbers and survival to the same level as PBS treated mice.

Overall, it seems that IL33 does not have an established role in human breast cancer, but it can act in a pro- or anti-tumorigenic role in mice, namely in the 4T1 and PyMT breast cancer models.

CC-Chemokine Ligand 11 (CCL11)

CCL11 belongs to the group of eotaxins, chemokines that attract eosinophils, and is the most specific and potent chemokine for eosinophils[129,357]. In humans, three eotaxins exist, CCL11, CCL24 and CCL26 (eotaxin1, eostaxin2 and eotaxin3 respectively). In mice, only CCL11 and CCL24 are expressed. In my work, I mainly used CCL11, so this eotaxin will be presented and discussed in some detail bellow.

CCL11 is expressed by fibroblasts, smooth muscle cells and epithelial cells[358] in almost all organs in varying amounts. Upon LPS injections into mice, CCL11 RNA expression was significantly increased in the lung, heart, spleen, kidneys and intestine.[359]. In fibroblasts, CCL11 production is dependent on STAT6 signaling as STAT6-negative, STAT6-defective or STAT6 inhibited cells are not able to produce and secrete CCL11[360–362]. Upon IL33 stimulation, lung fibroblasts significantly increased their production and secretion of CCL11[363]. Smooth muscle cells rely on STAT3[364] or MAPK[365] (p38, JNK, ERK1/2) and not STAT6 to produce CCL11. This signaling is activated by IL17A[364] and IL9[366]. Stimulation with IL4[361], IFNγ or interleukin-β1 (IL1β)[367] and TGFβ[368] all increase CCL11 release from smooth muscle cells. When mouse pulmonary epithelial cells were stimulated with TNF-α or IL1β, they increased their mRNA expression of CCL11.[369] Th2 derived IL4 and IL13 increased CCL11 expression and secretion of airway epithelial cells, leading to a rapid accumulation of

eosinophils[370], causing airway inflammatory symptoms[371]. Figure 8 shows a summary of the molecules and cells involved in CCL11 secretion.

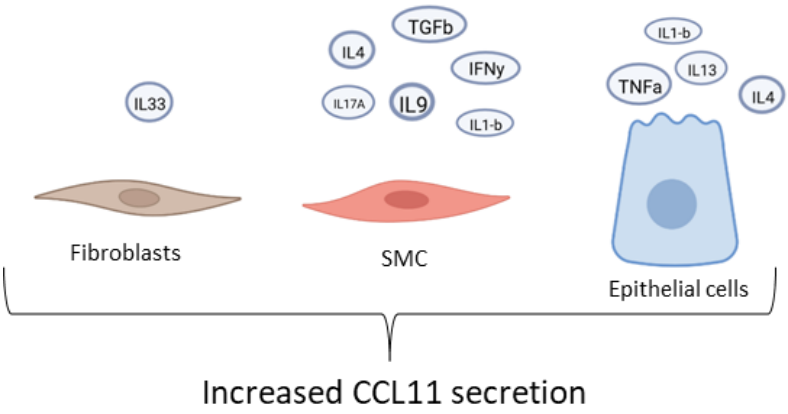


Figure 8: Summary of the different factors and cell types which can secrete CCL11. Upon activation of their respective cytokines, each cell type upregulates CCL11 expression and releases it into the microenvironment, causing accumulation of eosinophil. Created with BioRender.com

CCL11 binds to its cognate receptor CCR3[357], which is a classical G-protein coupled receptor (GPCR)[372] and is also expressed on basophils[373], a subset of CD4 T cells[374] and dendritic cells[375]. In basophils, CCL11 can cause histamine release[373]. The complete signaling pathway of CCL11/CCR3 binding is not yet know. But some studies have implicated several different pathways that are activated upon CCL11 binding. ERK2[131], P38[131], Rho and ROCK2[376] become activated during CCR3 signaling which leads to both chemotaxis and partial granule release *in vitro*[131,376,377]. In the lungs however, CCL11 on its own does not cause degranulation of eosinophils, rather a combination of CCL11 and IL5 is necessary to cause lung eosinophilia and degranulation requires additional stimuli from lung resident CD4 T cells[378]. In atopic patients, subcutaneous injections with CCL11 causes an increase in eosinophils, neutrophils, basophils and macrophages as well as a late-phase allergic reaction which presents as local swelling and redness[379]. Figure 6 shows a schematic representation of the known and not yet fully understood signaling pathways that occur during CCL11 binding to CCR3.

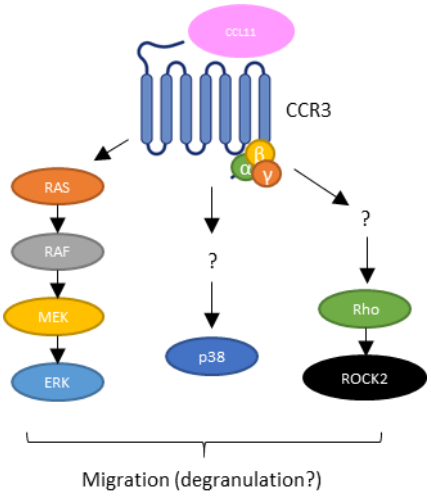


Figure 9: Signaling pathway of CCL11. Upon binding of CCL11 to CCR3 ERK, P38 and ROCK2 become activated, leading to rearrangement of the cytoskeleton, followed by migration. Created with BioRender.com

The effect of CCL11 expression on tumor progression varies between different cancers. High CCL11 expression was shown to have a better prognosis in colorectal cancer compared to low expression[380,381]. *In vitro* treatment with carboplatin and paclitaxel of high-grade serous ovarian cancer cells isolated from 14 different patients induced senescence which was accompanied by increased expression of CCL11[382]. In xenografts of human ovarian cancer cell lines, PARP inhibitors (PARPi), caused an upregulation of CCL11 expression in cancer associated fibroblasts (CAFs)[383]. In head and neck squamous cell carcinoma (H raw p 0.044 high CCL11 expression correlated with slightly increased survival and CCL11 expression was increased in adjacent healthy tissue, compared to tumor tissue[384]. In xenografts of lung cancer, invasive tumors had increased expression of CCL11. The authors speculated that this increase in CCL11 expression came from an increase in tumor associated myeloid derived suppressor cells (T-MDSCs). When tumor-bearing mice were treated with exogenous CCL11, they had increased metastatic burden with a co-occurring increase in PI3K-AKT signaling pathway enrichment. They concluded that CCL11 increases the metastatic capacity of human lung cancer cells[385]. The impact of CCL11 expression in breast cancer is not yet defined. In one study, Thomas et al.[386] raw p 0.014 at CCL11 high and low expressors had a similar overall and relapse-free survivals.

Mouse models to study the function of eosinophils

In 2002, Yu et al.[387] created an eosinophil deficient mouse model by deleting the double GATA-site in the GATA-1 locus, which they termed ΔdblGATA. Even when crossing these mice with IL5 overexpressing mice (discussed below), there were no eosinophils in the blood of hemizygous ΔdblGATA mice. There was no effect on other immune cells, including mast cells. Two years later, in 2004, James Lee et al. created and characterized the PHIL mouse model in which the diphtheria toxin A (DTA) gene was placed under the EPX promoter, restricting its expression to eosinophils[388] and causing congenital eosinophil deficiency. This model allowed them to delineate the exact roles eosinophils play in the symptoms of asthma. They showed

that none of the other immune cell types were affected by the lack of eosinophils under steady-state conditions. During acute allergen challenge, they saw a significant decrease in epithelial hypertrophy, decreased mucus production and increased lung function compared to challenged wild type (WT) littermates. Additionally, there was a significant increase in lung macrophages during allergen challenge in PHIL mice compared to WT mice. Wanting to investigate the role of both major basic protein (MBP) and eosinophil derived peroxidase (EPX) in allergic disease, Doyle et al.[389] accidentally created another eosinophil deficient mouse model. To their surprise, MBP and EPX were shown to be strictly necessary for eosinophil development of eosinophil progenitors (EoP) from granulocyte monocyte progenitors (GMP). There was no change in lymphocytes, monocytes, neutrophils and basophils upon double knockout of MBP and EPX. Leaning on the idea of using DTA to ablate eosinophils specifically, Jacobsen et al.[390] created the iPHIL mouse model. Similar to the PHIL mouse model, DTA was used to ablate eosinophils but instead of expressing the toxin directly in eosinophils under the EPX promoter, they expressed diphtheria toxin receptor (DTR). Upon addition of DTA, all cells expressing DTR were killed thus allowing for conditional ablation of eosinophils. Using their model, they showed that during the sensitization phase, eosinophils are not required to induce a Th2 response in the lungs. On the other hand, loss of eosinophils during allergen challenge caused a massive increase in neutrophils within the asthmatic lung, leading to neutrophilic asthma instead of eosinophilic asthma. Interestingly, macrophages and lymphocytes within bronchoalveolar lavage fluid (BAL) remained the same during allergic stimulation regardless of eosinophil ablation. Another mouse model of genetic eosinophil ablation was created by Bettigole et al. [47]. They ablated the xbp-1 gene in all hematopoietic cells and saw that there was a near complete lack of eosinophils while most other immune populations were not affected. This was due to the fact that XBP-1 is crucial for EoP survival and without it, no EoPs were available for eosinophils maturation. Note, splenic dendritic cells were reduced approximately two-fold.

In contrast to eosinophil depleted mouse models, the IL5-tg mouse model has strong eosinophilia. In 1990, Dent et al.[214] created this mouse model by inserting the il5 gene under human CD2 promoter into T cells. This caused continuous production of IL5 which in turn caused excessive blood and tissue eosinophilia in mice which lasted their entire lives. Interestingly, none of these mice showed any adverse symptoms under steady-state conditions, further supporting the fact that eosinophils need to be activated in order to cause damage. This mouse model is often used to characterize eosinophils as these cells are very rare in wild type mice and thus usually their numbers are not sufficient for analyses[38,391].

Surface markers of eosinophils

Eosinophils express a large variety of different surface markers. Importantly, the expression of surface markers on eosinophils varies with their activation status, the type of activating molecule and their localization and origin. For instance, single-cell RNAseq has allowed the definition of five subsets of eosinophils. The phenotype of these eosinophils was strongly dependent on the organ the cells where isolated from. Furthermore, infection of mice prior to eosinophil sequencing caused a dramatic shift in the

phenotype of eosinophils compared to un-infected mice, especially in the stomach, the colon and the blood[38]. Markers that are used to identify eosinophils and asses their activation state are discussed here.

Surface markers used to identify eosinophils through flow cytometry

The main surface markers used to define eosinophils are SiglecF, CD11b and a high side scatter, indicating their highly granular contents. CCR3, the receptor for CCL11 and IL5R, are used by some in addition but considering that these markers are not necessarily specific to eosinophils (discussed in more detail in "Eosinophils and other immune cells" as well as the result section), they are not ideal. For a long time, eosinophils have been considered to be $Ly6G^-$ but this has also changed (discussed in more detail in "Eosinophils and other immune cells" as well as the result section). F4/80, a marker commonly used to identify macrophages has unequivocally been proven to be expressed on several subset of eosinophils. Ly6C expression is sometimes used to define eosinophils but is not strictly necessary as there are $Ly6C^+$ and $Ly6C^-$ eosinophils. Finally, MHCII has been shown to be expressed on eosinophils in several different circumstances, thus gating strategies which exclude MHCII cells from eosinophils are out of date and should be revised (discussed in more detail in "Eosinophils and other immune cells", "Activation markers on eosinophils" and the result section). The gating strategies most commonly used *in vitro* and *in vivo* for identification of mouse eosinophils and their tissue specificity are listed in table4.

TABLE 4: GATING STRATEGIES USE TO IDENTIFY EOSINOPHILS

Tissue	Gating strategy	Ref
Bone marrow	$CD45^+/SiglecF^+/CCR3^+$	392
	Gated on $SiglecF^+$: $GR1^+$, $GR1^-$ and $Ly6G^+$, $Ly6G^-$	393
	$CD11b^+SiglecF^{med}IL5R^{low}$	391
	$CD11b^+SiglecF^{med\text{-}hi}IL5R^{low}$, IL5-tg mice. Note: CCR3 was expressed at varying degrees from low to high	391
Blood	$CD45^+/SiglecF^+/CCR3^+$	392
	$CD11b^+SiglecF^{med}IL5R^{low}$	391
	$CD11b^+SiglecF^{med\text{-}hi}IL5R^{low}$, IL5-tg mice. Note: CCR3 was expressed at medium levels.	391
	$CD45^+CD11b^+MHCII^-Ly6G^-SiglecF^+$, MC38 tumor bearing mice. Note: CCR3 expression and side scatter intensity were downregulated in tumor associated eosinophils.	149
Spleen	$Cd45^+SiglecF^+SCC^{med}$, IL5-tg mice	394

	$CD45^+CD11b^+SiglecF^+$, melanoma bearing mice	395
	$CD11b^+SiglecF^{med}IL5R^{low}$	391
	$CD11b^+SiglecF^{med\text{-}hi}IL5R^{low}$, IL5-tg mice	391
Lungs	$SSC^{hi}CD11c^{neg\text{-}med}$	396
	$CD45^+/CD11c^-/F4/80^+/SiglecF^+$	397
	$CD11c^-Gr1^{neg\text{-}low}MHCII^-$	398
	$CD45^+CD11b^+SiglecF^+$, B16-F10 i.v. injected mice	395
	$CD45^+CD11b^+SiglecF^+CD11c^-$. Note: Alveolar macrophages had higher SiglecF expression than eosinophils	399
	$CD45^+SSC^{hi}SiglecF^+CCR3^+$, saline or OVA challenged mice. Note: All eosinophils were $CCR3^+$.	400
	$Cd45^+CD11c^-SiglecF^+GR1^-$ and $Cd45^+CD11c^-SiglecF^+GR1^+$, allergen challenged mice	401
	$SSC^{hi}SiglecF^+Ly6G^-CD11c^{low\text{-}hi}$, KP tumor bearing mice	402,403
	$CD45^+SiglecF^+IL5R^+F4/80^+CCR3^{med}$	404
Intestine	$CD45^+/CD11b^+/SiglecF^+/MHCII^-/Ly6g^-/Ly6c^-/SSC^{hi}$, colon and ileum	392
	$CD45^+/SiglecF^+/CD11b^+/SSC^{hi}$, lamina propria	394
	$CD11c^-CD11b^+SiglecF^+SSC^{hi}$, lamina propria. Note: these cells were also $F4/80^+CD40^{low}CD80^{med}$ and some expressed CCR3	405
	$CD45^+CD11b^+MHCII^-Ly6G^-SiglecF^+CD80^+PD\text{-}L1^+$, stomach, colon and SI	38
Bone marrow derived eosinophils	$CCR3^+SiglecF^+$ and $CCR3^-SiglecF^+$	394
	$SiglecF^+SSC^{low\text{-}high}GR1^+$ and $SiglecF^+SSC^{low\text{-}high}GR1^-$	401
	$SiglecF^+SSC^{low\text{-}high}GR1^+Ly6C^+Ly6G^+$ and $SiglecF^+SSC^{low\text{-}high}GR1^+Ly6C^+Ly6G^-$	393
	Gated on $SiglecF^+GR1^+$: $CCR3^+$, $CCR3^-$, $IL5R^+$ and $IL5R^-$	
	Gated on $SiglecF^+GR1^-$: $CCR3^+$, $CCR3^-$, $IL5R^+$, $IL5R^-$	
Breast tumors	$SSC^{hi}CD45^+CD11b^+Gr1^{low}F4/80^+SiglecF^{hi}$, E0771 and MMTV-PyVT	406
Colon tumors	$CD45^+/CD11b^+/SiglecF^+/MHCII^-/Ly6g^-/Ly6c^-/SSC^{hi}$, APC tumors	392
	$CD45^+CD11b^+MHCII^-Ly6G^-SiglecF^+$, MC38 tumors	149

Melanoma	$CD45^+CD11b^+SiglecF^+$, B16-F10	395
Hepatocellular carcinoma	$Cd11b^+Ly6g^{neg\text{-}med}SiglecF^+$, Hepa1-6 tumors	407

Activation markers on eosinophils

There is some consensus about how to identify activated eosinophils. In histology, visual inspection of degranulation can be used but this is very cumbersome and difficult to quantify. *Ex vivo* cultures or *in vitro* cultures can be used to measure the release of granule proteins as well as cytokines and lipid mediators of eosinophils quantitively. Of course, culturing eosinophils or tissue sections *ex vivo* might alter their behavior, possibly introducing artefacts. Thus, researchers have tried to come up with markers that can be analyzed by flow cytometry on freshly isolated eosinophils. Activated eosinophils increase their expression of SiglecF and CD11b which can be measured by their mean fluorescent intensity (MFI). Granularity, as measured by side scatter (SSC) in flow cytometry is also commonly used to characterize the activation status of eosinophils. Primed eosinophils and activated eosinophils are rarely distinguished. Upon priming, a process in which eosinophils are "made ready for battle", eosinophils increased their intracellular granules thus they become more granular. Upon activation followed by degranulation they partially or completely lose their high side scatter. Hence, granularity should be used in concert with additional activation markers to distinguish between non-activated, primed and activated-degranulated eosinophils. Additional activation markers used less frequently are listed in table5.

In healthy lungs, SiglecF levels on eosinophils tend to be intermediate whereas they significantly increase upon asthma or lung metastases[408]. Similarly, *in vitro* activation with IL33 significantly increases SiglecF MFI in bone marrow derived eosinophils[409]. Splenic eosinophils isolated from IL5-tg mice increased their SiglecF MFI upon *ex vivo* co-culture with CSF2, a known eosinophils activator[149]. Upon infection with the nematode *Nippostrongylus brasiliensis*, whose life cycle includes phases within the lungs and the intestine, lung eosinophil showed increased expression of SiglecF in infected mice compared to WT mice[410]. Single cell RNA sequencing identified active and basal eosinophil subset and SiglecF MFI was decreased in the basal subset compared to the active one[167]. Finally, high SiglecF expression was shown as an activation marker in a mouse model of colitis[146].

CD11b, an integrin crucial for eosinophil adherence to blood vessel walls during extravasation into tissues, is upregulated upon activation During IL33 stimulation *ex vivo* of bone marrow derived eosinophils, CD11b MFI significantly increased[409]. In a mouse model of colitis, Cd11b expression increased on colonic eosinophils and was even further augmented by *in vivo* treatment of CSF2[146]. Tumor associated eosinophils (TAE) showed higher CD11b expression levels compared to blood eosinophils[149]. Blood eosinophils are known to be less active than tissue eosinophils[38]. Furthermore, treatment with CSF2 of IL5-tg splenic eosinophils *ex vivo* also increased CD11b expression[149]. Lastly, eosinophils in the lungs of asthmatic mice showed increased CD11b expression compared to healthy mice[147].

Granularity decreases in TAE in MC38 colon cancers compared to blood eosinophils concomitantly with an increase in CD11b and SiglecF expression indicating that these eosinophils degranulate preferentially upon infiltration into tumors[149]. Activated eosinophils in the intestine during colitis in mice, as determined by high SiglecF expression, showed slightly increased SSC MFI compared to SiglecF^{med} eosinophils. CCL11 treatment of human blood eosinophils significantly increased eosinophil granularity upon *ex vivo* culture[411].

TABLE 5: ADDITIONAL SURFACE MARKERS USED TO IDENTIFY ACTIVATED EOSINOPHIL BY FLOW CYTOMETRY. TAE: TUMOR ASSOCIATED EOSINOPHILS.

Marker	**Eosinophil location and activation condition**	**Ref**
CD63	Human blood eosinophils stimulated with CCL11	412
	Bone marrow derived eosinophils stimulated with IL33	409
	Primed blood eosinophils compared to degranulated TAE	149
CD69	BAL eosinophils upon allergen challenge	147
	Blood eosinophils upon *T. canis* infection	413
	BAL eosinophils from patients with eosinophilic pneumonia	414
	Human blood eosinophils stimulated with CSF2	415,416
MHCII	Blood eosinophils upon *T. canis* infection	413
	House dust mite activated blood eosinophils	417
CD80	Active intestinal eosinophils	167
CD86	SiglecF^{high} TAE from PyMT tumors	408
CD28	Blood eosinophils upon *T. canis* infection	413
CD107	Bone marrow derived eosinophils stimulated with IL33	409

Eosinophils and other immune cells

Due to their huge arsenal of cytokines, chemokines, lipid mediators and receptors, eosinophils can interact with many different types of immune cells. These interactions are determined by the state of the environment, the type of tissue as well as the activation status of eosinophils. Below I will discuss the interactions of eosinophils with T cells and granulocytes. The interactions with type 2 innate lymphoid cells (ILC2) have already been discussed in the context of IL33 activation and will be mentioned again in the context of cancer. It should be noted that there is barely any information about the interplay between eosinophils and natural killer cells (NK).

Eosinophils and T cells

Eosinophils were thought to mainly interact with CD4 T cells, although recent studies have shown that in cancer, eosinophils can interact with CD8 T cells to influence tumor progression[149,408,418–420]. Most studies have focused on the interplay between Type 2 CD4 helper cells (Th2) on eosinophils. This interaction is reciprocal, meaning that Th2 cells influence eosinophils, but eosinophils can also influence Th2 cells.

Eosinophil polarization and attraction of T cells

Naïve CD4 T cells need both TCR engagement as well as co-stimulatory signals, such as IL12 and IFN-γ for Th1 [421–423] and IL4 and IL13 for Th2[424–427] polarization. Eosinophils can secrete large amounts of IL4 under homeostatic and inflammatory conditions[428–432]. During asthma, eosinophils have been shown to infiltrate the draining lymph nodes where they encounter naïve CD4 T cells and could theoretically push them towards Th2 differentiation[433,434]. Recently an important role for IL25 has been suggested for eosinophils-CD4 T cell interactions. During co-culture of human or mouse eosinophils with IL25, house dust mite (HDM) and naïve CD4 T cells, the authors showed that IL25 together with HDMs can induce eosinophils to polarize CD4 cells towards a Th2 phenotype[435]. One study showed that OVA pulsed and intratracheally installed eosinophils were able to cause IL4 release *in vivo* by OVA specific T cells[150], hinting at the possibility that eosinophils have the ability to induce Th2 differentiation *in vivo*, perhaps through antigen presentation, but further research is needed to answer this question. The influence of eosinophils on Th1 differentiation is far less explored. One paper published in the 90's showed that human eosinophils can secrete functional IL12 upon stimulation *ex vivo*[170], but no *in vivo* evidence exists to shows that eosinophils can cause Th1 polarization. In general, eosinophils are associated with a type 2 immune response, although theoretically they have the capacity to induce a type 1 immune response as well.

For a long time, it was believed that during type 2 inflammation, Th2 cells were recruiting eosinophils as effector cells. Yet recent evidence has shown that in many cases of type 2 inflammation, eosinophils arrive in the tissue first and then recruit Th2 cells, expanding on the previous model. During OVA-induced asthma in PHIL mice lacking eosinophils, there was a significant decrease of CD4 and CD8 T cell accumulation in the lung compared to WT mice while T cells in the spleen were unaffected[436]. In the ΔdblGATA mouse model, eosinophils were necessary for CD4 T cell attraction to the lungs during OVA-induced asthma and transfer of eosinophils together with OVA partially rescued this phenotype. It should be noted that treatment with OVA alone or transfer of eosinophils alone did not increase CD4 T cells in asthmatic ΔdblGATA mice, indicating that the activation status of eosinophils is an important factor in their ability to attract CD4 T cells to the asthmatic lung[437]. Lastly, there are many papers which show that eosinophils directly modulate T cells in different types of cancers, which will be discussed in another section[149,408,418,438–440].

Eosinophil activation of T cells

Eosinophils can influence activation of T cells through the secretion of a myriad of different cytokines (discussed in detail in "eosinophil granule content"). Here I will discuss the potential of eosinophils to activate T cells in a yet underappreciated way, namely as antigen presenting cells.

In vitro cultures of eosinophils in the late 80's have shown that these cells are capable to express MHCII on their surface upon CSF2 stimulation. It should be noted that unstimulated eosinophils did not express MHCII, thus they cannot be categorized as professional antigen presenting cells (APCs) but rather as non-professional APCs[441]. This discovery led to further research into the function of eosinophils as APCs[442]. In a case report of a patient with both asthma and chronic eosinophilic pneumonia, eosinophils were isolated from bronchial lavage fluid (BAL) and blood. BAL eosinophils expressed high levels of MHCII and circulating eosinophils upregulated MHCII expression when co-cultured with lung fibroblasts. The addition of CSF2 to the co-culture significantly increased MHCII expression[443]. Blood eosinophils isolated from healthy subjects were able to upregulate MHCII expression upon *ex vivo* stimulation with IL3, the combination of IL3, IL5 and CSF2, the combination of IL3 and IFNγ or IL-1β alone. Contrary to their first report[441], the authors showed that blood eosinophils from healthy subjects do not upregulate MHCII upon CSF2 stimulation *ex vivo*. Interestingly, *ex vivo* cultured eosinophils expressing MHCII were able to increase T cell proliferation in the presence of antigen[444]. Blood eosinophils isolated from donors with idiopathic hyper-eosinophilic syndrome also upregulated MHCII expression upon co-culture with CSF2 or IFNγ. These activated cells were able to cause increased proliferation of T cells after antigen pulsation[445]. When comparing BAL and blood eosinophils from allergen challenged patients, Sedgwick et al. found that MHCII is strongly expressed on BAL eosinophils and not on blood eosinophils[446] and Hansel et al found a similar result but in addition, they found that culturing blood eosinophils with T cell supernatants also increased MHCII expression[447].

The expression of MHCII and additional co-stimulatory molecules on eosinophils was also shown in several studies in mouse models *in vitro* and *in vivo*. When incubating splenic eosinophils from IL5 overexpressing mice (IL5-tg) with CSF2 or *S. stercoralis* antigen (Ag) *ex vivo*, there was a significant increase of the co-stimulatory molecule CD69. Additionally, Ag stimulated eosinophils were able to induce IL5 production by CD4 T cells which was MHCII dependent, as adding anti-MHCII antibody abolished the stimulatory effect of eosinophils on CD4 T cells. Importantly, eosinophils were able to stimulate ILj5 production in naïve T cells in a similar manner as DCs[448]. Eosinophils pulsed with *S. stercoralis* Ag and injected intraperitoneally were able to migrate into the spleen and stimulate T cell expansion as well as increased production of the Th2 cytokines IL4 and IL5. This Th2 cytokine production was MHCII dependent as MHCII deficient eosinophils were not able to induce IL4 and IL5 production[449]. *In vivo* sensitization by OVA injection caused an increased accumulation of $MHCII^+$ eosinophils in the draining thoracic lymph nodes (dTLNs), whereas there were few $MHCII^+$ eosinophils in the blood and even fewer in the lungs. There was also an increase of CD80, CD86 and CD40 expression on dTLN infiltrating eosinophils compared to blood and lung. The expression of these three co-stimulatory molecules in addition to MHCII and the preferred localization to dTLNs suggests that during

lung inflammation, eosinophils are able to present antigen efficiently to CD4 T cells[450]. Further evidence for the capacity of eosinophils to stimulate CD4 T cells was shown when splenic eosinophils were incubated with CSF2 which caused and upregulation of MHCII, CD80, CD86 and CD40. These activated eosinophils, when pulsed with OVA, were able to increase proliferation and IL4 production of OVA specific CD4 T cells. Furthermore, *in situ* analysis of lymph nodes (LNs) showed that there was a physical interaction between OVA pulsed eosinophils and OVA-specific CD4 T cells[150]. A recent paper showed that intestinal eosinophils constitutively express MHCII and CD80 on their surface under homeostatic conditions. The authors speculated that this could be due to the constant expression of CSF2 within the gut. These $MHCII^{+}CD80^{+}$ eosinophils were able to uptake and present OVA antigen when the latter was injected into the intestine[451]. To date there is overwhelming evidence that eosinophils are able to express MHCII as well as co-stimulatory markers in mice *in vivo*. Some human studies show that under inflammatory conditions, lung associated eosinophils also express high levels of MHCII. Thus, an additional role of eosinophils as non-professional APCs has been largely accepted in the field.

Eosinophils and Granulocytes

Mast cells

Mast cells (MC) and eosinophil co-occur in many allergic and autoimmune diseases in patients[452–457]. Because both cells are known to play important roles in type 2 inflammation, the interplay between these two cell types has garnered great interest in science. Mast cells were shown to affect eosinophils and *vice versa*[454,456,458,459]. The main interactions of the two cell types are discussed here.

Effects of mast cells on eosinophils

In vitro stimulated, bone marrow derived mouse MC significantly increased production of IL4, IL5 and IL33 transcription. Notably, addition of IL33 significantly increased expression of IL4 and IL5 in stimulated MC[460]. As discussed above, IL4 and IL5 are important factors for eosinophils activation, survival and differentiation. Eosinophils can be activated by lipid mediators including leukotriene 4 (LT4)[461]. Upon stimulation of bone marrow derived MC with IL3, these cells significantly upregulated their production of LT4[462]. During infection of mice with *Schistosoma mansoni* eggs, MC were crucial for eosinophil IL4 production and infiltration of the peritoneal cavity[463]. Another lipid mediator secreted my MCs which can activate eosinophils is prostaglandin 2 (PGD2)[461,464,465].

Levi-Schaffer et al.[466] looked at the interaction of human blood eosinophils and rat peritoneal mast cell sonicate (PMCS) *in vitro*. They found that addition of PMCS to eosinophils increased survival through autocrine CSF2 signaling in eosinophils. This increased survival was not due to IL4 or IL5. The factor released by MC which increased eosinophil survival was determined to be TNFα. Lastly, activation of eosinophils, as measured by attachment to the culture flask, was increased upon addition of PMCS to the medium. MCs do not only activate eosinophils but can indirectly cause their attraction through release of histamine. *In*

vivo administration of histamine to atopic patients caused an increase in CCL11 producing cells around the injection site and a significant increase of eosinophil infiltration[467]. *In vitro*, endothelial cells incubated with histamine showed a concentration dependent increase of CCL11 expression which in turn caused increased adhesion of eosinophils to endothelial cells. Human blood and mouse bone marrow derived eosinophils (PbEos and BMEos respectively) showed increased migration towards resting human blood derived MC *in vitro* which was increased upon IgE or compound 48/80 stimulation of the latter[468]. Long term co-culture of PbEos or BMEos with resting or activated MC significantly increased EPO activity in eosinophils and TNFα secretion in both cell types. Furthermore, co-culture increased ICAM-1 expression on PbEos but did not affect adhesion molecules on MC. MC derived chymase activated human blood cells *in vitro*. Eosinophil survival, adhesion and secretion of CCL2, CXCL1, CXCL8 and IL6 was increased in a dose dependent manner[469]. IL6 secretion was also increased upon co-incubation with MC tryptase[470].

Effects of eosinophils on mast cells

Eosinophils can cause histamine release from MC through their granule proteins. *In vitro* incubation of purified rat MC with MBP or ECP lead to significant release of histamine in an IgE-independent fashion[471]. Similarly, human heart tissue associated MC released histamine and tryptase upon stimulation with ECP and MBP[472]. Histamine can then act upon MC in an autocrine fashion to increase chemotaxis and activation.[473] Release of tryptase of human blood derived, IgE stimulated MC was significantly increased upon co-culture with human blood eosinophils which was dependent on direct contact between the cell types[467]. Through their production of IL4 and IL5, eosinophils can increase histamine and PGD2 production and release by MC[474]. Importantly, IL4 increased the activation of MC by stem cell factor (SCF) which is known to be important for MC activation and has been found to be expressed in eosinophils[474,475]. There is a strong feedback loop between eosinophil and MC through IL4. IL4 increases the production of IL5 and TNFα, which both in turn can activate eosinophils and cause them to secrete more IL4[476].

Basophils

Basophils act on eosinophils in a very similar manner as mast cells. They secrete eosinophil activating cytokines as well as histamine and lipid mediators which are all capable of activating eosinophils[477,478]. There are not many studies which show evidence for direct interactions between eosinophils and basophils. The most clearly established interplay between these two cell types is that basophils increase attraction of eosinophils under inflammatory conditions. A few examples of this will be discussed here. Because of the large variety of cytokines secreted by both cell types it is likely that they form a secretory communication network, but more data is needed to describe it.

In a model of diphtheria toxin A (DTA) ablation of basophils, eosinophil number in the blood were not changed under homeostatic conditions[479]. But during IgE-mediated chronic allergic dermatitis, there was a significant decrease in eosinophil chemokines CCL11, CCL24 and RANTES as well as the eosinophil activating cytokines IL4, IL5, IL13 and INFγ. Another paper using the same mouse model, showed that basophils were

the main eosinophil attracting cells and the main IL4 producers upon stimulation with IgE[480]. Croton oil induced skin inflammation was characterized by increased infiltration of basophils, eosinophils and neutrophils[481]. Basophils infiltrated the lesions before eosinophils and so the authors speculated that basophils could cause eosinophil attraction. Indeed, when basophils were depleted, there was a significant reduction in the accumulation of eosinophils whereas neutrophils remained unchanged. Bone marrow derived basophils were able to increase activation of eosinophils isolated from the bone marrow of IL5-tg mice as was seen by increased expression of CD69, CD86 and ICAM-1 during co-culture. It must be pointed out that it is not clear in which differentiation state the eosinophils were as the bone marrow contains immature as well as mature eosinophils. Furthermore, bone marrow derived basophils increased the expression of RANTES and CCL11 in mouse embryonic fibroblasts (MEFs) and their own production on IL4 and IL13 upon IL3 stimulation.

Neutrophils

Only few papers have looked at the direct interaction between eosinophils and neutrophils. More research has been done in looking at the co-occurrence of these cells in different pathologies, such as infection with parasites. Both types of interactions will be illustrated here with a few examples.

Direct interactions

Analyses of trans basement membrane migration (TBM) of eosinophils and neutrophils upon stimulation was analyzed in mono- and cocultures[482]. Upon stimulation with IL8, neutrophils did not increase migration when cocultured with eosinophils compared to cultured alone. Eosinophils on the other hand showed increased migration upon IL8 stimulation only in combination with neutrophils. CCL11 and RANTES significantly increased eosinophils migration independent of neutrophils whereas they did not affect neutrophils migration in either of the two conditions. Finally, leukotriene B4 significantly increased eosinophil migration together with neutrophil co-culture but decreased neutrophil migration in the presence of eosinophils. These results indicate that there might be an interplay between eosinophils and neutrophils where one cell type can influence the migration of the other depending on the stimulus present. *In vivo*, eosinophils were shown to be important for neutrophil accumulation in the peritoneal cavity[483]. Wild type (WT) mice treated with CCL11 showed an increased accumulation of eosinophils which was mirrored by an increase in neutrophils. This accumulation of neutrophils was dependent on eosinophils as the same treatment in ΔdblGATA mice which lack eosinophils, showed no increase of neutrophils upon CCL11 administration.

Indirect interactions

When *Strongyloides stercoralis* larvae were transferred into diffusion chambers with varying amounts of eosinophils isolated from the spleens of IL5-tg mice or neutrophils isolated from the peritoneal cavity of thioglycolate treated mice and implanted into naïve WT mice, there was a significant decrease in live larvae

with increased numbers of immune cells. The strongest decrease in larval survival was seen when both eosinophils and neutrophils were present. Depletion of eosinophils through anti-CCR3 antibody strongly increased larval survival in previously immunized mice. Similarly, larvae survival was increased in mice deficient of neutrophils[484]. Major basic protein (MBP) and eosinophil peroxidase (EPX) and myeloid peroxidase (MPO) were necessary for larval killing by eosinophil and neutrophils respectively. Eosinophils from $MBP^{-/-}$ or $EPX^{-/-}$ and neutrophils from $MPO^{-/-}$ mice were not able to decrease larvae numbers[485].

These results could indicate that both eosinophils and neutrophils play a role in the protection against parasitic infections but whether they directly influence each other during infection of play independent roles is not clear.

Distinction between eosinophils and neutrophils is not as straight forward as one would hope

Importantly, the distinction between eosinophils and neutrophils via their surface markers has been put under scrutiny. For example, SiglecF and CCR3, which for a long time have been thought to be exclusive eosinophils markers, have been shown to be expressed on neutrophils[402,403,486,487]. Similarly, a subset of $IL5R^{+}Lin^{+}Ly6G^{+}$ multipotent myeloid cells (MMC) has been recently discovered in the bone marrow of mice. These MMCs showed expression of typical neutrophil markers such as MPO but where able to transdifferentiate into eosinophils upon addition of IL5[488]. On the other hand, Ly6G which has long been thought of as an exclusive neutrophils marker has been shown to be expressed in different subsets of eosinophils in bone marrow, lungs and tumor associated eosinophils[393,401,407]. Considering this apparent fluidity of expression markers between neutrophils and eosinophils, great care has to be taken to ensure that data is properly analyzed. In addition to antibody stains, the granularity should always be considered as well. So far, all studies have shown that eosinophils have a stronger granularity than neutrophils regardless of their origin. Furthermore, histological analyses of these two types of granulocytes allow for easy distinction. Eosinophils stain more strongly with eosinophil and can be identified by Sirius red, a chemical stain that exclusively stains the basic granules contained in eosinophil (and Paneth cells). Major basic protein and eosinophil peroxidase can also be used to conclusively identify eosinophils and myeloid peroxidase can be used for neutrophil identification. Additionally, the morphology of the nucleus varies greatly between eosinophils and neutrophils, being in a doughnut shape or showing hyper-segmentation respectively[489,490]. Unfortunately, histological analysis of tissues is more time intensive and more limited in the number of markers and cells that can be analyzed at any given time compared to flow cytometry. So, it is important to use both of these methods in a complementary way to ensure that eosinophils are properly distinguished from neutrophils.

Eosinophils in asthma and infections

Asthma, allergy and some types of infections are characterized by a type 2 inflammatory response. In many of these diseases, eosinophils play a crucial role. Asthma is a pathology in which the immune system overreacts to a harmless stimulus instead of a pathogen. Infections are a common occurrence and our body

has developed several strategies to fight different infectious agents. During infections with bacteria or viruses, Th1 cells, CD8 T cells, dendritic cells (DCs), macrophages and natural killer cells (NK) play major roles in protection. On the other hand, when a larger organism, such as a fungus or worm enters the body, a type 2 response is activated. Th2 cells, type 2 innate lymphocytes (ILC2), eosinophils, mast cells (MC) and basophils are its main components. Eosinophils have long been known to play important roles in asthma and type 2 immune responses against infections. Here I will briefly discuss the roles of eosinophils in asthma and nematode infections.

Asthma

Asthma is a disease of the lung where the immune system, together with lung fibroblasts and lung epithelial cells, causes uncontrolled inflammation leading to increased mucus production, smooth muscle cell contractions and damage to the epithelium leading to permanent fibrosis. This results in a symptom called airway hyperresponsiveness (AHR) which is defined as the narrowing of the airway and causes asthmatic symptoms such as wheezing[491]. The cause is an overreaction to harmless antigens, such as cat dander, house dust mite (HDM) or pollen as well as harmful fungi. Importantly, fungal infections leading to asthma have become more prevalent in the past few decades[492]. This disease, just like cancer, is very heterogenous and subdivided into different subsets, called endotypes, according to the mechanisms which cause the pathology[493]. Asthma can be subdivided into two main types: Atopic asthma is characterized as dependent on the adaptive immune system, mainly via IgE, whereas non-atopic asthma does not require T cells to occur but is rather caused by irritants such as pollutants, e.g., smoke or asbestos, or microbes. Eosinophil attraction in non-atopic asthma is contributed mainly by ILC2 whereas Th2 cells are thought to be the main recruiters of eosinophils in atopic asthma[494–496].

Mouse models to study asthma

Mice, especially Balb/c mice, are commonly used to model asthma because they have shown to induce a strong Th2 response upon allergy challenge[497,498]. Asthma induction in animals requires two phases: the sensitization phase and the challenge phase. During the sensitization phase, the allergen is administered to mice, usually by intraperitoneal (i.p.) or subcutaneous (s.c.) injections, sometimes in combination with an adjuvant such as aluminum hydroxide (alum). The amount and frequency of allergen administered and the length of the challenge phase varies between publications. After the mice are sensitized, they are challenged introducing the allergen into the airways through different methods such as intranasal instillation (i.n.)[497,498]. Ovalbumin (OVA) is the most commonly used allergen but house dust mite (HDM), ragweed or fungal spores are utilized as well.

Attraction and activation of eosinophils in asthma

Attraction

Upon allergen challenge, there is a significant increase in IL5 production by T cells and ILC2 within the bone marrow and lung which causes increased eosinophil differentiation and release into the blood stream[499,500]. Furthermore, expression of eosinophil chemoattractants such as CCL11 by lung resident smooth muscle cells (SMC), fibroblasts and epithelial cells[501], is significantly increased and causes massive infiltration of blood eosinophils into the lung[502–505]. Of note, some have speculated that lung eosinophilia occurs independently from CCL11 as CCL11 knockout mice showed a similar phenotype during allergen challenge as wild type (WT) mice[506,507]. This apparent contradiction is most likely due to the fact that, even though it is the most potent and specific chemoattractant, CCL11 is not solely responsible the eosinophil recruitment. Regulated and normal T cell secreted (RANTES)[508] and CCL24[509] are also expressed in the lungs of allergen challenged mice and can compensate for CCL11 loss. Mast cells (MC) are also major contributors to eosinophil attraction into the lung during asthma. They increase CCL11 expression in lung resident cells[510] and secrete both IL5 and eosinophil chemokines[511,512] and lung eosinophilia is significantly attenuated in MC deficient mice even during CCL11 administration[513,514].

Activation

Upon entry into the lung, eosinophils become activated by locally produced cytokines. IL4 and IL13 are the main cytokines which activate eosinophils in asthma[512,515,516]. Both of the cytokines are produced by Th2 cells and ILC2 in the lung and their secretion increases upon allergen challenge[515,517–519]. As discussed above, cytokine production in ILC2 is induced mainly through IL33 in asthma[520,521] whereas Th2 cells are stimulated by antigens presented by dendritic cells and local type 2 cytokines[516,522]. Once activated, eosinophils contribute to the symptoms of asthma by increasing mucus production, collagen deposition, TGFβ production leading to fibrosis and smooth muscle contraction and thickening[523]. Furthermore, they directly damage the lung tissue through the release of their granule proteins (discussed in more detail in "Eosinophil granule content") and cause histamine release of by MC[524]. They also act in an autocrine and paracrine manner by releasing CCL11, IL4, IL5 and IL13[80].

Nematode infections

The role initially assigned to eosinophils was to protect against infections with parasites. Their roles in the combat against viruses and bacteria are far less understood and accepted. Here I will discuss some of the possible roles of eosinophils against nematode infection and how several lines of evidence have put this dogma into question. Thanks to the development of eosinophil depleted models (see "Mouse models to study the function of eosinophils"), the role eosinophils play in worm infection has been able to be investigated *in vivo*. Overall, the results indicate that eosinophils both do and do not play a role in controlling worm infection. But a closer look will show that the necessity for eosinophils in combating

parasite infection is dependent on the species, location and whether or not the infection is primary and secondary. In addition to direct effects of eosinophils on worms, they also contribute to increased B cell survival[525,526].

The nematode *Trichinella spiralis* infects the intestine and their larvae migrate into the liver and muscle where they mature and cause significant damage. Upon *T. spiralis* larval infection of the liver, eosinophils were strongly infiltrating liver tissue adjacent to the parasite[527]. Similarly, infection of the intestine with *Trichuris trichiura* caused a massive infiltration of eosinophils into the mesenteric lymph node(LN)[528]. These LN associated eosinophils secreted large amounts of IL4 but where not necessary for worm expulsion as ΔdblGATA mice had the same capacity to eliminate *T. trichiura* as wild type (WT) mice[528]. *Schistosoma mansoni*, another nematode that inhabits the liver upon infection, significantly increased eosinophils within the bone marrow (BM) concomitant with significant increases in eosinophil peroxidase (EPX), major basic protein (MBP) and IL5R transcription[529]. Furthermore, the eosinopoietic cytokines IL4 and IL5 were significantly increased in the circulation. Despite these results, eosinophils were not required to kill the parasites or their eggs[529]. Lastly, *T spiralis* infection on naïve WT or IL5-tg mice showed that there was no difference in the number of larvae recovery[530].

Surprisingly, several studies have shown that eosinophils not only are not required to combat nematode infection but can in fact increase nematode survival *in vivo*. There was a significant decrease in *T. spiralis* larvae and their growth in ΔdblGATA mice compared to either WT or ΔdblGATA mice after eosinophil transfer[531]. The authors speculated that this might have been due to increased iNOS production from macrophages and neutrophils in eosinophil deficient mice. Eosinophils also augmented the number and growth of muscle associated *T. spiralis* through IL4[532]. Transfer of WT eosinophils into ΔdblGATA mice restored infected area to WT levels whereas IL4 deficient eosinophils were not able to rescue the phenotype. Yet another study using *T.spiralis* larvae muscle infection showed that their survival was decreased in both ΔdblGATA and PHIL mice due to an increased Th1 response in these eosinophil deficient mouse strains compared to WT mice[533].

Other papers showed that eosinophils are able to decrease worm burden *in vivo*, mainly, but not exclusively, during secondary infections. When WT or IL5-tg immunized mice were infected with *T. spiralis* orally, there was a significant decrease in larval burden and replication ability of adult worms in eosinophilic mice compared to WT mice[530]. *Nippostrongylus brasiliensis* infection of WT, IL5-tg and IL5 deficient or ΔdblGATA mice showed that eosinophilic mice had significantly less larvae in the lungs compared to WT and IL5 deficient or ΔdblGATA mice[534]. Both $IL5^{-/-}$ and ΔdblGATA mice showed no change in the number of larvae during primary infection compared to WT animals but interestingly, WT animals had decrease worm burden upon secondary infection which was not seen in either of the eosinophil deficient strains. This could indicate that eosinophils might be important during a secondary infection with *N. brasiliensis* but not during the first. But because larvae were decreased in IL5-tg mice already during the first infection compared to WT mice, further exploration would be needed. Interestingly, larval killing seemed to occur prior to lung

colonization as there were only few eosinophils and other leukocytes found within the tissue. Lastly the idea that eosinophils might play a more important role during secondary rather than primary infection was shown during *T. spiralis* infection. No change of larvae numbers was seen upon primary infection between WT and ΔdblGATA mice whereas eosinophil deficient mice had much more larvae upon secondary infection compared to WT mice[535].

These results show that eosinophils are not required for the protection against nematodes during the first infection, although they consistently accumulate adjacent to the worms, independently of the strain. Yet some evidence exists that eosinophils are important for protection against secondary infections. Importantly, eosinophils were clearly shown to increase the survival and colonization capacity of *T. spiralis*. Whether or not they have a pro-parasitic role for other nematodes need to be investigated further.

Eosinophils in Cancer

In the last three decades, the role of eosinophils in cancers has become a topic of interest amongst researchers. The presence of blood eosinophils or tumor associated eosinophils (TAEs) in cancer patients has led to speculations whether these cells in fact can have a functional role in the development, progression and response of treatment of cancer.[536,537] Human and mouse studies have shown that eosinophils can have neutral, pro- and anti-tumorigenic roles, depending on the cancer type and the study[420,538–547]. Furthermore, eosinophils have been shown to be a prognostic marker for some cancers in patients and are able to influence the outcome of patients undergoing immunotherapy[548–556].

Eosinophils in Breast Cancer

Helen E. Ownby was one of the first researchers to establish a link between eosinophils and survival of breast cancer patients. In the early 80's she and her colleagues showed that breast cancer patients that had a high blood eosinophil count had a better disease-free survival compared to patients with low blood eosinophil counts.[557] Later, these results were confirmed in several independent papers, showing that eosinophils increase the cumulative[558,559] and disease-free[560,561] survival of breast cancer patients respectively. To date, there is no consensus if eosinophils truly play a role in the formation, progression and treatment of breast cancer.

Pre-clinical studies

A handful of papers studying the effects of eosinophils on breast cancer in mouse models have been published over the last few years. The results are summarized in table6.

Hollande et al.[407] used a dipeptidyl-peptidase 4 inhibitor (DPP4i) in an EMT6 breast cancer model in mice. DPP4 has been shown to add posttranslational modifications on several chemokines. The authors showed that DPP4i decreased primary tumor growth and that eosinophil infiltration was causative to this effect, since treating EMT6 tumors with DPP4i and simultaneously depleting eosinophils via anti-SiglecF removed

any benefit of the DPP4i treatment. They also showed that eosinophils can contribute to immunotherapy, enhancing the effect of anti-PD1 and anti-CTLA4 treatment. It should be noted that in their model, the effect of eosinophils on tumor growth was independent from any lymphocytes. Zheng et al.[438] showed that when treating E0771 and PyMT breast cancers with anti-CTLA4 therapy there was a significant increase of tumor associated eosinophils (TAE) which they showed was necessary for blood vessel normalization within the tumor. They also showed that depleting TAE significantly decreased the treatment efficiency, proving that eosinophils can have a beneficial impact on immunotherapies in breast cancer. In their hands, tumor associated lymphocytes (TILs) were essential to attract eosinophils to the tumors, as depletion of the former showed loss of TAE. Grisaru-Tal et al.[408] showed that eosinophils are spontaneously attracted to breast cancer lung metastasis in human samples as well as in breast cancer mouse models. They used both PyMT and 4T1 tumors for metastatic assays and the 4T1 cell line for an orthotopic model as well. In all three cases, eosinophils were increased in metastatic lungs compared to healthy lungs. When eosinophils were transferred to eosinophil depleted ΔdblGATA mice, they preferentially homed to the metastatic lung compared to healthy lungs. Interestingly, this increased migration of eosinophils towards lungs with metastases was CCR3 independent. These lung metastases associated eosinophils decreased metastatic growth by attracting T lymphocytes but had no effect on primary tumor growth. Additionally, they were able to show through RNA-sequencing that metastasis-associated eosinophils displayed a pro-inflammatory signature, namely increased IFNγ and TNFα signaling, able to influence the lung immune-environment such as recruiting T cells. Although they did not show a causative role between T cell recruitment and reduced metastasis burden, one can certainly give credence to this role as T cells have been shown in many instances of cancer to have an anti-tumorigenic role. Cheng et al. [439] showed that upon irradiation of orthotopically injected 4T1 breast cancer there was an increase in TAE which correlated with CD8 T cell infiltration into the tumor. They further showed that irradiation increased a pro-inflammatory T cell signature as well as an activating and attracting eosinophil signature in tumors. Using the 4T1 mouse model of breast cancer, Panagopoulos et al.[562] wanted to analyze the effect of eosinophil peroxidase and breast cancer progression. Eosinophil peroxidase (EPX) is specific for eosinophils and is not expressed in other immune subsets (see eosinophilic granule proteins). They injected EPX intratumorally and observed an increase in primary tumor growth as well as a trend for increased lung metastases. Furthermore, they showed that EPX treatment slightly but significantly decreased primary tumor necrosis and increased deposition of Collagen I and Collagen VI. It should be noted that this is an indirect study of a possible effect of eosinophils, since they did not show any direct evidence of these cells being present in the tumors nor producing and secreting active EPX in the TME. Overall, the pre-clinical data indicates that eosinophils play an anti-tumoral role in breast cancer.

TABLE 6: SUMMARY OF PRE-CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS AND BREAST CANCER

Breast cancer model	**Method**	**Conclusions**	**Additional information**	**Reference**
Orthotopic EMT in Balb/c	Treatment with DPP4i Eosinophils ablation via anti-SiglecF	DPP4i increased tumor eosinophilia which was causatively linked to decreased tumor growth	Eosinophilia combined with anti PD1 and anti-CTLA4 treatment led near complete remission	407
PyMT and 4T1 IV 4T1 orthotopic C57BL/6J and Balb/c respectively	Eosinophilia occurred spontaneously in lung metastases ΔdblGATA mice or anti-SiglecF antibody were used for eosinophil-free conditions	Lung eosinophilia decreased metastatic burden Lung eosinophils causatively increased T cell attraction to the lung Lung metastasis associated eosinophils displayed an increased inflammatory RNA profile compared to naïve eosinophils	Eosinophil recruitment to the lungs was CCR3 independent, no alternative pathway was suggested	408
Orthotopic E0771 and PyMT in C57BL/6J	Anti-CTLA4 immunotherapy Anti-SiglecF depletion of eosinophils	Primary tumor growth was significantly reduced in eosinophilic CTLA-4 treated tumors compared to tumors without eosinophils T cells were required for TAE Eosinophilia caused tumor vessel normalization in	Eosinophilia combined with anti-CTLA-4 therapy increased a pro-inflammatory gene signature in tumors	438

		combination with CTLA-4 therapy		
Orthotopic 4T1 tumors in Balb/c	Irradiation of primary tumor	Irradiation caused increased TAE concomitant with an increase or T cells	No causative link was shown between TAE and TILs in the breast cancer model, their main focus was on melanoma	Cheng et al. [439]
Orthotopic 4T1 tumors in Balb/c	Intratumoral treatment with EPX	EPX treatment increased tumor burden, both higher tumor weights and more lung metastases	The presence of eosinophils in the tumor was not shown	Panagopoulos et al.[562]

Clinical studies

In clinical studies of the effect of eosinophilia on breast cancer one has to distinguish between papers correlating blood eosinophilia with progression and tissue eosinophilia with progression. The results are summarized in table7.

Blood eosinophilia and breast cancer

Ownby[557] and her colleagues showed that a high blood eosinophil counts pre-surgery and pre-treatment correlated positively with increased time to recurrence in a sample group of 592 patients. They did not distinguish between breast cancer subtypes and there was more eosinophilia in patients with low than with high TNM stage. G. Jerusalem and his team published two papers on the relationship between blood eosinophilia and tumor progression[559]. In their first paper they focused on TNBC and Her2+ cancers in 112 patients and showed no significant correlation between high baseline (pre-treatment) relative eosinophil count (REC) and tumor recurrence. They did show a significant correlation between high REC and a pathological complete response (cPR) as well as high REC and patient survival in a neo-adjuvant setting followed by surgery. Interestingly they showed that the product between REC and relative lymphocyte count (RLP), termed eosinophil-lymphocyte product (ELP) was predictive for cPR and survival. This result might indicate that eosinophils could be interacting with B- and/or T cells to decrease tumor progression. In their second study[558] they included all breast cancer subtypes and repeated their analyses in 930 patients. As before, they showed that a high baseline REC correlated with increased breast cancer specific survival (BCSS). As before, the RLP correlated with survival as well, once again indicating that eosinophil's anti-tumoral activity may be influenced by lymphocytes or that eosinophils may enhance lymphocyte activity against the tumor. Finally, they showed that when grouping tumors according to molecular subtype, there

was a non-significant trend for increased survival in Luminal B, Her2+ and TNBC and a significantly increased survival in Luminal A cancer patients. Ghebeh et al[561] looked at the correlation between blood eosinophil count and response to immune checkpoint inhibitor (ICI) therapy in a patient cohort of 14 TNBC patients. They showed that patients who had an increase in blood eosinophilia upon treatment had an increased disease-free survival. It should be noted that this is a very small study and from the 14 treated patients, only 5 responded. Of these five, only 3 showed increased blood eosinophilia. Zenan et al. [563] analyzed 601 breast cancer patients' blood prior to any treatment and looked at different blood parameters and their correlation with patient survival. They separated the breast cancer into luminal A, luminal B, Her2+ and TNBC. Blood eosinophilia had no significant correlation with disease free survival (DFS) or overall survival (OS) even though there was a trend (p=0.089) for increased OS in eosinophilic patients with luminal B breast cancer. A study by Gündüz et al[564] showed that in a small patient set of 62 HER2+ breast cancer patients treated with trastuzumab, a high absolute eosinophil count correlated with a decreased survival time. During the ESMO conference in 2020, Voorwerk et al. [553] presented their findings on 111 TNBC patients enrolled in the phase 2 TONIC trial. The patients were given anti-PD1 immunotherapy and blood eosinophil measurements were taken before and after treatment. The authors showed that anti-PD1 therapy increased circulating blood eosinophils significantly in responders compared to non-responders, suggesting that eosinophilia might be influenced or influence immunotherapy in breast cancer patients.

It should also be noted that so far there are no human studies showing a direct correlation between blood and tumor associated eosinophilia in breast cancer. In fact, Grisaru-Tal et al. [408] analyzed 22 samples of lung metastases from breast cancer patients for intra-tumoral eosinophils as well as blood eosinophilia. There was no clear correlation between tissue eosinophilia in breast cancer lung metastases and blood metastases. Thus, patients with blood eosinophilia cannot automatically be assumed to also have tissue eosinophilia, more studies would be needed to confirm or reject the hypothesis that blood eosinophilia always causes breast cancer tissue eosinophilia.

In conclusion, there is some evidence in patients suggesting that blood eosinophilia is beneficial for breast cancer patients, but variations exist between breast cancer subtypes, treatment and how blood eosinophilia is defined.

TABLE 7: SUMMARY OF CLINICAL STUDIES ON THE ROLE OF BLOOD EOSINOPHILS AND BREAST CANCER

Cancer type and treatment	**Patient sample size**	**Conclusions**	**Additional information**	**Reference**
Mixed	592	Increased DFS, increased time to recurrence, eosinophilia	Patients with low circulating lymphocyte count had shorter DFS	[557]

		correlates with lower clinical stages of tumor		
TNBC and Her2-enriched	112	Baseline blood eosinophilia did not correlate with recurrence but did correlate with increased cPR and patient survival.	A high ELP pre-treatment predicted better patient outcome	[559]
Mixed as well as separated by subtype	930 Luminal A: 133 Luminal B: 131 Her2+: 31 TNBC: 89 Unknown: 9	Increased REC correlated with OS and BCSS survival. Only Luminal A patient survival had a significant correlation between REC and increased DFS.	Increased ELP predicted better patient outcome. BCSS was not increased when patients were separated according to molecular subtype	[558]
TNBC, ICI therapy	14	Patients with eosinophilia upon treatment had an increased probability to respond to treatment	Only a total of 5 patients responded to therapy. Of those, only 3 had eosinophils whereas none of the non-responders had eosinophilia.	[561]
Luminal A, Luminal B, Her2-enriched and TNBC	133, 317, 57 and 94 respectively	No correlation between pre- and post-operative REC with patient survival	Luminal B cancer patients showed a trend for increased survival when REC was present	[563]

Her2-enriched, Trastuzumab	62	Increased REC correlated with shorter DFS	Increased Platelet-to-lymphocyte ratio (PLR) correlated with longer DFS	[564]
TNBC, Anti-PD1 therapy	111	Anti-PD1 therapy increased circulating eosinophils in responders but not in non-responders	Information only from an ESMO conference abstract found	[553]

Tumor associated eosinophilia and breast cancer

A small number of papers have looked at the association of TAE in breast cancer and patient survival. It is important to note that to date, there is almost no single cell RNA seq data available for eosinophils as they require a very specialized process in order to yield a meaningful amount of RNA[38]. The data available for breast cancer samples so far was obtained either by patient-samples analyzed via histology or via bulk sequencing using a computational method called CIBERSORT[565] which allows for identification of up to 22 different immune cell population within a given RNA dataset. The results are summarized in table 8.

A small study by Szalayova et al. looked at the immune infiltration in invasive carcinomas which had undergone prior post-surgical biopsies in 44 patients that had not yet received any form of treatment.[566] They showed that eosinophils were attracted to the area close to the incision but not to the area further away. There was a correlation between increased proliferative cancer cells close to the incision area, but whether there is a causative link between the presence of eosinophils and increased cancer cell proliferation was not established. The first large study including an analysis of the prognostic value of eosinophils in breast cancer was published in 2016 by Ali et al.[567] Using CIBERSORT, they were able to investigate the prognostic effect of eosinophils retrospectively in 10'988 cases of breast cancer of all subtypes. Their results showed that in ER+ breast cancers eosinophils had a significant association with favorable outcome, whereas in ER+-Her2+, ER- Her2- and ER- Her2+ there was only a trend. They further could not find an association between the presence of TAE and the effectiveness of neoadjuvant chemotherapy. It should be noted, that in all the patient samples analyzed, eosinophils made up a very small part of the entire immune microenvironment and there was no distinction between eosinophilic and non-eosinophilic tumors. A second large study by Grisaru-Tal et al. [568] used a similar approach as Ali et al. in order to analyze eosinophil infiltration into several different solid tumors, breast cancer being one of

them, using CIBERSORT. They found that overall, breast cancers contain generally less eosinophils than tissues with mucus producing functions, such as the intestine and the pancreas. They validated these results in tissue tumor arrays of 2'890 individual patient tumors of different origins via EPX staining. 576 of the samples were from breast tumors. Even though there was no association between eosinophils and tumor grade, there was a positive correlation between TAE and tumor stage and primary tumor size, indicating a possible pro-tumorigenic role of eosinophils in breast cancer progression. Finally, they showed a positive correlation between eosinophils and the presence of resting CD4 T cells and activated NK cells and a negative correlation between eosinophils and resting NKs, Tregs, activated and naïve CD4 T cells, CD8 T cells and M2 macrophages, once again indicating that there is an interplay between the immune cells in the tumor microenvironment and eosinophils do not act as single agents, whether they might have a pro-or antitumoral role. A smaller study of datasets analyzed via CIBERSORT by Chouliaras et al.[569] showed that out of 1069 patients, only 40 (3.7%) had tumor associated eosinophilia. Within these eosinophilic tumors there was a significant increase of monocytes, T-follicular helper cells, naïve B cells, resting mast cells and resting CD4 memory T cells compared to the non-eosinophilic tumors. Furthermore, eosinophilic tumors had an increased non-silent mutational load, a decreased TGF-β response and, interestingly, a decreased cytolytic activity score. They also found a significant increase in gene set enrichments of MYC, E2F (proliferation), DNA repair and unfolded protein response. In eosinophilic tumors there was a trend for increase disease-free survival (DFS) compared to non-eosinophilic tumors (p=0.0576) but overall survival did not vary between the groups. Lui et al.[570] also used CIBERSORT to analyze the immune content of 1091 patient samples. They showed that a high eosinophil count in the primary breast tumor correlated with a decreased survival probability and that tumor eosinophils were correlated with the high-risk patient group. Tumor eosinophils had a tendency to negatively correlate with 20 of the other immune populations and positively correlated with monocytes only.

To summarize, human data shows that TAE can have both pro- and anti-tumorigenic functions and their interplay with other immune cell populations may be important to delineate their function. Furthermore, it would benefit patients to establish a treatment regimen that could arm eosinophils to combat cancer.

TABLE 8: SUMMARY OF PRE-CLINICAL STUDIES ON THE ROLE OF TAE AND BREAST CANCER

Cancer type and treatment	**Patient sample size**	**Conclusions**	**Additional information**	**Reference**
Mixed subtypes Treatment naïve patients	44	Correlation between proximity of eosinophils and highly proliferative cancer cells, yet no causative effect proven	Increase in macrophages close to surgical wound	[566]

ER+ Her2+, ER- Her2-, ER+ Her2-, ER+ Her2- Treatment naive or treated with neoadjuvant chemotherapy therapy	10'988	Eosinophils correlate with a better prognosis in cancer patients Eosinophils do not correlate with an increased benefit for the patient in a neoadjuvant setting	Very few eosinophils in all samples No comparison between eosinophilic and non-eosinophilic tumors	[567]
Several solid cancers, mixed breast cancer subtypes Treatment naïve for histology	3088 for RNA analysis, 576 for IHC analysis	Breast cancers contain fewer eosinophils than tumors from mucus producing organs There is a positive correlation between eosinophils and tumor stage and size	Eosinophils occur more often in the tumor rather than the stroma in breast cancer Eosinophils correlate with positively with CD4 T cells and activated NK cells and negatively with resting NKs, Tregs, activated and naïve CD4 T cells, CD8 T cells and M2 macrophages	[568]
Mixed subtypes	1069, 40 eosinophilic (3.7%)	Eosinophilic tumors tended to increase DFS (p=0.0576)	Eosinophilic tumors showed increased monocytes, T follicular helper cells, naïve B cells, resting mast cells, resting CD4 Memory T cells and nonsilent mutations and decreased TGFβ and cytolytic responses Eosinophilia correlated with an increase MYC, E2F, DNA repair and	[569]

			protein unfolding response signature	
Mixed subtypes, no information on treatment status	1091	Tumor eosinophilia correlated with a decreased survival probability Eosinophils were correlated with the high-risk patient group	Eosinophils had a slight tendency to negatively correlate with 20 of the immune subsets analyzed and correlated positively with monocytes	[570]

Eosinophils in other cancers

Colon cancer

Pre-clinical studies

As shown by Reichman et al.[571] eosinophils strongly infiltrated colon cancer in both a DSS-induced mouse model and the $APC^{min/+}$ mouse model upon tumor formation. *In vitro* incubation with colorectal cancer cells line MC38 conditioned medium increased eosinophil survival partially through IL5. Eosinophil deficient ΔdblGATA mice showed increased tumor burden and decreased survival when tumor formation was induced by DSS treatment of crossing of ΔdblGATA mice with $APC^{min/+}$ mice. The decrease in survival was independent of CD8 T cells as ablation through an anti-CD8 antibody did not change the survival of $APC^{min/+}$ or $APC^{min/+}$/ΔdblGATA mice. When comparing TAE with normal colonic eosinophils, they showed that TAEs display an increased IFNγ-signaling pathway signature. To test whether IFNγ can potentiate eosinophils to kill colorectal cancer cells, they incubated eosinophils with MC38 or CT26 colorectal cancer cell lines. There was a significant increase in cancer cell killing of both cell lines upon addition of peritoneal eosinophils isolated from IL5-tg mice which was further increased upon the addition of IFNγ. Kienzl et al.[409] saw that upon treatment of subcutaneously (s.c.) injected CT26 colorectal cancer cells with IL33 there was a decrease in primary tumor growth concomitant with a significant decrease in TAE. The same was seen in a cancer model induced by DSS. Eosinophil migration toward CT26 cancer cells was increased almost significantly ($p=0.0620$) upon IL33 treatment *in vitro*. The decrease in tumor growth via IL33 was dependent on eosinophils as ΔdblGATA treated mice did not show any change in tumor size upon IL33 treatment. Furthermore, transfer of WT eosinophils together with IL33 significantly decreased tumor volume in ΔdblGATA mice. Finally, IL33 treated eosinophils were able to kill CT26 cancer cells *in vitro* but only at very high ratios (Eos: CT26 10:1, 25:1 and 50:1). Mice subcutaneously injected with MC38 colon cancer cells showed that there was a significant increase in TAE within the tumor at day7 compared to later time points[149]. MC38 tumors grew faster in PHIL mice compared to WT mice, implying a role for eosinophils in tumor control. When mice injected with either CT26 or MC38 cancer cells, were treated with anti-IL5 there

was a significant increase in tumor weight whereas IL5-tg mice displayed delayed tumor growth compared to WT littermates. Both CD4 and CD8 T cells increased expression of IFNγ and TNFα expression in MC38 tumors in WT mice compared to PHIL mice upon *ex vivo* stimulation with PMA/Iono. CD8 *ex vivo* stimulation with MC38 specific peptide also increased IFNγ production in CD8 T cells from WT tumors. Inversely, IFNγ and TNFα production were decreased in WT mice compared to IL5-tg mice. The anti-tumorigenic effect of eosinophils was driven by CSF2 as mice which lacked CSF2R specifically in eosinophils were not able to control tumor growth compared to WT mice. Inhibition of CSF2 signaling in eosinophils also decreased IFNγ and TNFα production by CD4 and CD8 T cells, suggesting that CSF2 activation of eosinophils is required for eosinophils to decrease tumor burden in colorectal cancers.

In summary, the role of eosinophils in colorectal cancer is not clear as they can either increase or decrease patient survival. It would certainly be of interest to look at the effect of eosinophils on colorectal cancer in the context of the micro-biota as the latter plays an important role in tumorigenesis and tumor progression in humans.

Overall, it seems that in mouse models of colorectal cancers eosinophils have a beneficial effect. This is in contrast to the human date which clearly showed that eosinophils can have tumor promoting effects. The reasons for this discrepancy can be varied. Because the micro-biota has been shown to have a major impact on colorectal cancer and the microbiota of mice and humans vary drastically, this might be a reason but further investigation would need to be done.

TABLE 9: SUMMARY OF PRE-CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS IN COLON CANCER.

Colon cancer model	**Method**	**Conclusions**	**Additional information**	**Reference**
$APC^{min/+}$ and DSS induced.	Eosinophils spontaneously infiltrated tumors, ΔdblGATA mice were used as controls	TAE significantly decreased tumor burden and increased survival.	The antitumorigenic effect was independent of CD8 T cells.	571
CT26 colorectal cancer cell injected s.c.	Systemic IL33 treatment significantly increased TAE.	IL33 induced TAE significantly decreased primary tumor growth	IL33 treatment significantly activate TAE *in vivo*.	409
MC38 colorectal cancer cells injected s.c. and $APC^{min/+}$ mice.	Eosinophils spontaneously infiltrated tumors, ΔdblGATA and IL5-tg	TAE significantly decreased primary tumor weight	CSF2 signaling was necessary to activate eosinophils.	149

	mice were used as controls			

Clinical studies

Prizment et al.[572] analyzed 441 samples of female colorectal patients via microarray. A pathologist analyzed the samples and classified them according to a pre-established eosinophils score using eosinophils peroxidase staining to identify eosinophils. The more eosinophils infiltrated the tissue the higher the score and *vice versa*. They showed that a high eosinophil score increased patient overall survival and cancer specific survival. Xiong et al.[573] used CIBERSORT to evaluate the impact of eosinophils on colorectal cancer (CRC) patient survival. They combined data from the TCGA and GEO database and ended up with just over 1000 patients. Eosinophils were more frequent in tumor tissue compared to adjacent healthy tissue albeit very rare in both. They found that high eosinophil infiltration in tumors correlates with a worse survival probability for patients although the difference was small. Wu. et al.[574] looked at the prognostic value of different immune population in the circulation of patients with colorectal cancer. They showed that there is no correlation between blood eosinophilia and survival in 153 CRC patients although high blood eosinophilia tended to increase survival compared to low blood eosinophilia. In a study of 144 patients, Jakubowska et al.[575] analyzed colorectal cancer infiltration of neutrophils, macrophages and eosinophils at the invasive front and the tumor center. In about 70% of the cases, eosinophils were present at the invasive front and the center of the main tumor mass respectively. There was a significant correlation between the infiltration of eosinophils at the border and the center of tumors (R^2=0.353) but there was no correlation between tumor associated eosinophils (TAE) and tumor size, stage and malignancy grade nor was there an effect of TAE at the center of the tumor on disease-free survival (DFS). The correlation between DSF and eosinophils at the invasive front was not analyzed. Ramadan et al.[576] showed that in 122 CRC patients, peritumoral eosinophilia was correlated with increase survival. They also looked at the correlation between intratumoral and peritumoral eosinophils and intratumoral or peritumoral budding but saw no significant association. Finally, Mo et al.[577] investigated the role of different immune infiltrates in 316 colorectal cancer patients undergoing chemotherapy. There was a significant upregulation of TAE in tumors with higher tumor grades, compared to lower tumor grades and increased TAE correlated with decreased OS.

TABLE 10: SUMMARY OF CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS IN COLON CANCER.

Treatment	**Patient sample size**	**Conclusions**	**Additional information**	**Reference**
Mixed	441	High TAE increased patient survival.		572
Unknown	<1000	High TAE decreased patient survival.	The difference in survival between	573

			patients with high and low TAE was small	
Mixed	153	High blood eosinophilia increased patient survival.	Tissue eosinophilia was not analyzed	[574]
Mixed	144	There was no correlation between TAE and survival.	High TAE infiltration of tumor border correlated with high infiltration of TAE in the center of the tumors	[575]
Mixed	122	Increased TAE was associated with increased patient survival.	There was no correlation between TAE and tumor budding	[576]
Chemotherapy	316	Increased TAE was associated with decreased survival.	Higher tumor grades were correlated with increased TAE.	[577]

Melanoma

Pre-clinical studies

Ikutani et al.[396] showed that there is a population of IL5 producing innate cells (ILCs) in the lung. IP or intranasal administration of IL33 strongly increased the production of IL-5 by lung ILCs and was accompanied by a massive infiltration of eosinophils into lung tissue of mice with intact IL-5 signaling compared to mice with an IL-5 knockout. IL5 signaling deficient mice injected IV with B16-F10 melanoma cells had increased lung metastases compared to signaling proficient mice. When treating WT mice with rIL-5, there was a strong increase of lung eosinophilia concomitant with a decrease in lung metastases. A study by Zaynagetdinov et al.[397] also analyzed the effect of IL5 knockout on melanoma lung metastases after IV injection. Their results contradict the Ikutani study since in their hands, $IL5^{-/-}$ significantly decreased lung metastases in mice. Carretero et al.[418] showed that depleting Tregs in a melanoma setting significantly reduced tumor growth and strongly increased TAE which was accompanied by a strong reduction of blood eosinophilia. When depleting eosinophils via anti-SiglecF antibody in a Treg deficient setting, tumor growth was significantly increased and overall survival decreased. They did not analyze the effect of eosinophils depletion in the presence of Tregs. The ablation of eosinophils was correlated with a decrease of activated CD8 T cells in the tumor. Tumor associated eosinophils produced large amounts of the pro-inflammatory cytokines IFNγ, TNF, CXCL9 and CXCL10 and their ablation via Anti-SiglecF antibody decreased the production of these pro-inflammatory cytokines within the tumor, leading to a decreased recruitment of CD8 T cells and suggesting a direct impact of TAEs on the inflammatory response in the tumor. Furthermore,

eosinophilia correlated with increased vascular stability in the tumor. Interestingly, IL5 production was increased upon TAE, suggesting that eosinophils themselves could influence local cells to produce IL5 or produce and secrete it themselves. Injection of tumor specific CD8 T cells together with activated eosinophils strongly decreased primary tumor growth of established tumors and mouse survival compared to either cell type injection alone. This was accompanied with an important accumulation of activated eosinophils and tumor specific CD8 T cells in the tumor. It should be noted that co-injection of non-activated eosinophils with CD8 T cells or injection of eosinophils (activated or not) alone did not significantly decrease tumor growth nor affect mouse survival. The authors concluded that eosinophils decrease tumor progression via increased vascular normalization and CD8 T cells attraction in the tumor. One should keep in mind that, except for the cell transfer experiments, all results with TAE were done in mice lacking Tregs and that the melanoma cell line constitutively expressed the OVA peptide. Lastly, Cheng et al.[419] showed that irradiating subcutaneously implanted B16-F10 tumors caused a significant increase in tumor expression of CCL11 and CCR3, concomitant with elevated TAE numbers and a significant increase in CCL5 (RANTES), CXCL9, and CXCL10 production and CD8 tumor infiltration and T cell cytotoxicity. Depletion of eosinophils via anti-SiglecF in irradiated tumors strongly decreased CD8 T cell tumor infiltration, suggesting that eosinophilia is necessary for increased CD8 T cell infiltration of tumors post radiation therapy. Lastly, they wanted to know if eosinophilia could increase the infiltration of tumor specific CD8 T cells. When transferring tumor specific CD8 T cells in eosinophil ablated mice, there was an increase in tumor growth compared to eosinophil proficient mice. There was no significant benefit in primary tumor growth or survival between radiation treatment and radiation and T cell transfer treatment. Without radiation, eosinophils alone were able to control tumor growth, albeit to a lesser extent than together with radiation.

TABLE 11: SUMMARY OF PRE-CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS IN MELANOMA.

Melanoma model	**Method**	**Conclusions**	**Additional information**	**Reference**
B16-F10 i.v. injected cells	Eosinophils spontaneously accumulated in metastatic lungs. IL5 signaling deficient mice and anti-IL5 treated mice were used as controls	IL5 signaling deficient mice could not increase lung eosinophilia which caused increased lung metastases.	IL5 is produced by type 2 innate lymphoid cells upon IL25 or IL33 stimulation	[396]
B16-F10 i.v. injected cells		IL5 knock out mice showed significantly	Eosinophils were not	[397]

		decreased number of lung metastases compared to WT mice	analyzed directly	
B16-F10 MO4 s.c injected cells	Treg depletion through DT-DTR was necessary to cause eosinophilia. Anti-SiglecF was used to ablate eosinophils	Increased TAE significantly decreased primary tumor growth and increased survival.	Eosinophils decreased tumor progression through tumor specific CD8 T cells	[418]
B16-F10 s.c. injected cells	Radiation induced tumor eosinophilia	TAE significantly decreased primary tumor growth	TAE significantly increased CAR T cell efficiency.	[419]

Clinical studies

In a large prospective study with 1412 patients, Wagner et al.[578] showed a strong correlation with increased absolute and relative blood eosinophils counts and patient survival. All patients had newly diagnosed cutaneous melanoma and were treatment naïve at the time of baseline blood eosinophil measurement. Deylon et al. [551] showed that in 73 melanoma patients treated with ipilimumab, eosinophilia correlated with increased overall survival. Gerbhardt et al.[579] looked at the link between eosinophilia in the blood and melanoma progression. In a cohort of 59 anti-CTLA-4 treated patient, all responders had a significant increase in blood eosinophilia compared to the non-responders. This blood eosinophilia occurred after the first treatment and remained stable up to 3-6 weeks after the last injection of antibody. They speculated that increased blood eosinophilia upon treatment could indicate better response to therapy. Similar results were shown by Simon et al[580] and Ohashi et al.[581] Simon et al. reported that in a set of 32 melanoma patients, blood eosinophilia (both the relative count as well as absolute numbers of eosinophils) significantly increased upon immunotherapy in responders but not in non-responders. When analyzing cytokines in the patient sera they found a significant correlation between eosinophilia and IL-16 and CCL2. To my knowledge, this is the first time in melanoma research that the authors showed a strong correlation (r^2=0.6289, p=0.0062) between the number of circulating and tumor infiltrating eosinophils by staining cancer tissue sections with anti-MBP antibody. It should be noted that the staining shown in the paper is at a very low resolution and the MBP stain is not evident. Oshani et al. found an almost two-fold increase in blood eosinophilia in 16 patients responding to anti-PD-1 therapy compared to non-responders, although this difference was not significant. Another study analyzing eosinophilia in the context of melanoma immunotherapy was done by Machiraju et al.[582] In a patient cohort of 113 melanoma patients receiving either anti-CTLA4, anti-PD1 or combined anti-CTLA4/PD1 therapy, they showed that both anti-CTLA4

monotherapy or combined therapy significantly increased blood eosinophilia, whereas anti-PD1 monotherapy did not. This increase in blood eosinophilia correlated with an increase of highly proliferative CD8 T cells. Rosner et al.[583] showed that a higher relative baseline blood eosinophil count correlated with increased overall survival in 209 patients treated with nivolumab and ipilimumab. A higher absolute baseline blood eosinophil count was not predictive for overall patient survival. When investigating the efficiency of addition of high-dose systemic interferon-α2b to DC vaccination for 19 patients with malignant melanoma, Sheng et al.[552] found that increased eosinophil infiltration in the tumors increased progression-free survival. De Coana et al.[584] showed that 43 melanoma patients treated with Ipilimumab had blood eosinophilia after the second round of treatment. They also showed that increased circulating eosinophils were significantly associated with adverse reactions of the patients to the treatment. The correlation between patient survival and eosinophilia was not analyzed. Lastly, in a study investigating the prognostic potential of eosinophils for targeted MAPKi therapy in melanoma patients, Wendlinger et al.[585] showed that responders had an increased absolute and relative amount of blood eosinophils compared to non-responders in a patient group of 216 people. When comparing the surface marker profile of eosinophils from melanoma patients and healthy donors, they did not find any difference between the two groups.

TABLE 12: SUMMARY OF CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS IN MELANOMA.

Treatment	**Patient sample size**	**Conclusions**	**Additional information**	**Reference**
Mixed	1424	Increased blood eosinophilia increased patient survival		[578]
Ipilimumab	73	Increased blood eosinophilia upon treatment significantly correlated with increased survival.		[551]
Anti-CTLA4	59	All responders showed significant blood eosinophilia whereas non-responders did not	Blood eosinophilia remained stable up to 6 weeks.	[579]
Pembrolizumab or nivolumab and ipilimumab	32	All responders showed significant blood eosinophilia whereas non-responders did not	Blood and TAE correlated	[580]

Nivolumab or Pembrolizumab	16	All responders showed significant blood eosinophilia whereas non-responders did not		[581]
Anti-CTL4, anti-PDL1 or anti-CTLA4/PDL1	113	Anti-CTLA4 and anti-CTLA4/PDL1 significantly increased blood eosinophilia.	Increased blood eosinophilia was associated with increased Cd8 T cell proliferation	[582]
nivolumab and ipilimumab	209	There was no correlation between blood eosinophilia and patient survival.	Treatment significantly increased blood eosinophilia.	[583]
DC vaccination combined with high-dose systemic interferon-α2b	16	Increased TAE increased patient survival.		[552]
Ipilimumab	43	Treatment significantly increased blood eosinophils which was associated with increased adverse effect.		
MAPKi therapy		Responders had a significant increase in blood eosinophilia compared to non-responders.	There was no change in eosinophil surface markers between tumor patients and healthy volunteers.	[585]

Lung cancer

Pre-clinical studies

Malignant pleural effusion (MPE) is the accumulation of fluids within the chest cavity. It occurs in different pathologies such as congestive heart failure and lung cancer. Stathopoulos et al.[586] showed that there was an increase in eosinophils within MPE in mice with intrapleural delivery of lewis lung carcinoma (LLC) cancer

cell line in mice. As expected, eosinophilia in MPE was diminished in IL5$^{-/-}$ and anti-IL5 treated mice after intrapleural delivery of LLC compared to WT mice concomitant with a decrease in MPE volume and visceral pleural tumors. Interestingly there was no change in subcutaneously (s.c.) injected LLC cells between wild type (WT) and IL5 deficient mice, indicating that eosinophils increase metastatic spread but do not affect primary tumor growth in lung cancer.

Zaynagetdinov et al.[397] analyzed the effect of IL5 on tumor growth and metastatic spread of LCC lung cancer. They saw that, although primary tumor growth was not affected upon IL5 knockout, there was a significant decrease in the number of lung metastases in s.c. injected mice. There was no change in the size of lung metastases between WT and IL5$^{-/-}$ mice, indicating that eosinophils increase metastatic spread of lung cancer but not their *in situ* growth. Analysis of eosinophils in the lungs of intravenously injected mice with LLC cells showed that there was no change between healthy and metastatic lungs but as expected eosinophils were almost completely absent in IL5 knockout mice. The authors speculated that the effect of IL5 is directed through eosinophils. To confirm this hypothesis, they injected bone marrow derived eosinophils into WT and IL5$^{-/-}$ mice and saw a significant increase of lung metastases in the knockout mice but not in WT mice. Additionally, both lung eosinophils and lung metastases were significantly decreased in mast cell (MC) deficient mice, indicating and interplay between IL5, MC and eosinophils in lung metastatic seeding. To find a possible mechanism of how these players increased metastases, the authors compared the infiltration of Tregs and the expression of several chemokines in tumor bearing lungs in WT and IL5$^{-/-}$ mice. They saw that there was a significant decrease in Treg infiltration upon IL5 knockout in both healthy and tumor bearing mice which was partially caused by decreased expression of CCL22 concomitant with an increase of IFNγ producing natural killer cells (NK). The authors postulated that MC increase eosinophils in the lung through production of IL5 and that these lung-associated eosinophils then increase an immunosuppressive environment, leading to increased metastatic colonization. It should be noted that MC are not the only and most certainly not the most prominent IL5 producing cells in lungs and that likely TH2 cells and ILC2 contribute significantly to eosinophils recruitment.

TABLE 13: SUMMARY OF PRE-CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS IN LUNG CANCER.

Colon cancer model	**Method**	**Conclusions**	**Additional information**	**Reference**
LLC injected into the pleural cavity.	Eosinophils spontaneously infiltrated MPE.	Eosinophils increased MPE volume and pleural cavity tumors.	s.c injected LLC tumors were not affected by eosinophilia	[586]

LLC injected s.c. and i.v.	IL5 knock out mice and transfer of BM derived eosinophils	IL5 increases metastatic spread most likely through eosinophils.	Metastasis associated eosinophils are likely suppress NK cells through CCL22	[397]

Clinical studies

Osawa et al.[587] looked at the time to treatment failure (TFF) in lung cancer patients treated with the immune checkpoint inhibitor (ICI) anti-PD-L1 monotherapy or a combination of ICI and chemotherapy in correlation to increased blood eosinophilia upon treatment. In 180 patients treated with monotherapy and 79 patients treated with combination therapy, there was a significant increase in TFF in patients with blood eosinophilia. These results indicate that blood eosinophilia is correlated with increased treatment efficiency in lung cancer patients. Similar results were shown by Alves et al[555]. 121 patients with advanced non-small cell lung cancer (NSCLC) were treated with anti-PD-L1 and their blood eosinophilia was measured upon treatment and correlated with overall and disease-free survival (OS and DFS, respectively). Almost 30% of patients developed blood eosinophilia upon treatment. Those patients who did show blood eosinophilia had an increase OS and DFS compared to those who did not. Cheng et al.[419] analyzed two different cohorts of patients with NSCLC treated with radiation therapy (RT). Cohort 1 contained 234 patients and cohort 2 contained 123 patients They had previously shown that eosinophilia significantly increased in pre-clinical mouse models of melanoma, breast cancer and colon cancer which in turn increased tumor control through cytotoxic T cells. In both cohorts they saw a significant increase in blood eosinophilia upon RT which correlated with increased patient survival. Finally, a small study of 63 patients with NSCLC by Tatroglu et al.[588] showed that tissue associated eosinophilia occurred preferentially in stage I and stage II patients compared to stage III and stage IV. No correlation with survival and tissue associated eosinophilia was made.

Together these results indicate that eosinophils have a pro-tumorigenic role in mouse models of lung cancer but their role in humans is not clearly delineated. Mouse models suggest that they cause an immunosuppressive microenvironment in the lung thus allowing cancer cells to colonize the organ much easier whereas human data clearly showed that blood eosinophils during treatment was associated with significantly increase OS and DFS. More studies are needed to determine the effect of tumor associated eosinophils in lung cancer.

TABLE 14: SUMMARY OF CLINICAL STUDIES ON THE ROLE OF EOSINOPHILS IN LUNG CANCER.

Treatment	**Patient sample size**	**Conclusions**	**Additional information**	**Reference**
anti-PD-L1 or anti-PD-L1 with chemotherapy	180 and 79 respectively	Increased blood eosinophilia correlated with increased TFF.		[587]
anti-PD-L1	121	Increased blood eosinophilia correlated with increased survival.	30% of patients developed blood eosinophilia upon treatment.	[555]
Radiation therapy	234 patients in cohort1 and 123 patients in cohort2	Increased blood eosinophilia upon treatment significantly correlated with increased patient survival.		[419]
Unknown	63	TAE preferentially occurred in lower stage cancers.		[588]

Materials and methods

Antibodies

Anti-CD90.1-IgE, AB5-IL5, AB5-CSF2, AB5-IFNγ were produced in-house. Commercial antibodies used for flow cytometry were purchased from BioLegend and are CD206 (clone C068C2), F4/80 (clone QA17A29), CD62L (clone MEL-14), Ly6C (clone HK1.4), CD80 (clone 16-10A1), CD11b (clone M1/70), CD86 (clone GL-1), SiglecF (clone S17007L), CD11c (clone M1/70), CD40 (clone FGK45), MHCII (clone M5/114.15.2), Ly6G (clone 1A8), CD45 (clone 30-F11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD3 (clone 17A2), FoxP3 (clone 206D), CD44 (clone IM7), CD69 (clone H1.2F3), PD1 (clone MH5A).

Commercial antibodies used for histological stains were purchased form Mayo Clinic and were EPX (clone MM25.82.2.1) and MBP (clone MT2-14.7.3).

Cytokines

Commercial cytokines were purchased from Peprotech. IL5 (Ref : 215-15), IL33(Ref : 210-33), SCF (Ref : 250-03), FLT-3L (Ref : 250-31L), CCL11 (Ref : 250-01) IFNγ (Ref : 315-05), TNFα (Ref : 315-01A), IL2 (Ref : 212-12).

Plasmids

CCL11 and CCL24 were expressed in a PiggyBac vector under a CAG promoter with blasticidin resistance. CD90.1 was expressed a lentiviral plasmid under a human PGK promoter. TNFα and IL33 were expressed in a SleepingBeauty vector under a CAG promoter with neomycin resistance. Cloning of plasmids was done by our technician Pierre Dessen.

Cancer cell culture

4T1, CD90.1^{+}4T1, E0771, the PyMT cell line, MC38 and B16-F10 cells were cultured in normal cell culture dishes (PS) in DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Invitrogen), 10% FBS and 1% P/S (Invitrogen) and kept at 37°C and 7% CO2. Cells were kept at 80% confluence.

Primary MMTV-PyMT cancer cells were isolated by mincing primary MMTV-PyMT tumors and digestion in DMEM-F12, TM and TH liberases (1:166, Roche) and DNase (1:400, Roche) at 37°C for 30 minutes. They were plated on collagen coated plates and cultured o/n in DMEM-F12 (Invitrogen), 2% FBS and 1% P/S (Invitrogen).

Collagen-coating solution was composed of HBSS, 100 µg ml BSA, 20 mM Hepes pH6.5, 1% Vitrogen 100 collagen (CellTrix) and filtered at 0,2 µM. Solution was added to the plates and incubated 30 minutes at 37C. The solution was aspirated and the plates were dried under a hood.

Cancer cells were prepared for injection by washing 2x with 1X PBS followed by trypsinization with pre-heated (37°C) 0.25% trypsin (Invitrogen) for 1-5 minutes at 37°C. Trypsin was neutralized by adding

medium. Cell was spun at 400g for 4 minutes at room temperature and supernatant was aspirated. Cells were washed in 1X PBS at 400g for 4 minutes at room temperature and supernatant was aspirated and resuspended in 1X PBS. Cell numbers were determined using the Countess3 automatic cell counter.

Cell transfections

Cells were transfected using electroporation. 1 million cells were resuspended in 1ml OptiMEM (Invitrogen) and mixed with 8.8ug construct plasmid and 1.3ug transposase plasmid per condition. Voltages between 100V and 300V were tested with pulse length between 5ms to 1ms. Transfected cells were plated in 6-well plates. Antibiotic resistant selection was done by adding 10ug/ml blasticin or 0.1mg/ml neomycin until all control transfected cells were dead, usually 3 days. All surviving cells were pooled and gene expression was verified by qPCR.

Ex vivo TIL stimulation

TILs were isolated from tumors, cultured with IL2 and stimulated *ex vivo* with PMA/Ionomycin and Golgi-Plug. PMA/Iono stimulation activated T cells in a non-specific was and causes translation of cytokine transcripts whereas the Golgi-Plug prevents secretion of these cytokines into the medium, allowing for intracellular quantification of cytokine production.

Eosinophil *in vitro* differentiation

An established protocol was used for eosinophil differentiation from bone marrow cells. Mice were euthanized with CO2 and femurs were collected. Both ends of the bone were cut off and the bone marrow was flushed out with a syringe using PBS. Red blood cells were lysed using ACK (0.15M NH4Cl, 0.01M KHCO3, 0.0001M EDTA in PBS) buffer for 5 minutes at room temperature and lysis was stopped by addition of PBS. Cells were spun at 400g for 4 minutes at room temperature and supernatant was aspirated. Cell were plated on low attachment flasks with RPMI (Invitrogen), 10%FBS, 1%P/S, 100ng/ml SCF and 100ng/ml FLT-3L (Peprotech) for 4 days at 37°C and 7% CO2. On day 5 medium was replaced with RPMI (Invitrogen), 10%FBS, 1%P/S and 10ng/ml IL5 (Peprotech) and not changed for 4 days. Starting at day8, medium was changed every two days and cells were kept at 1mio/ml.

Activation assays Eos

In vitro differentiated eosinophils were cultured with 100ng/ml IFNγ or 200ng/ml TNFα or 10ng/ml LPS or 10ug/ml Poly(I:C) o/n for activation. The cells were then washed by spinning down the supernatant, adding PBS, spinning and resuspending in culture medium. Cell were then analyzed by FACS, incubated with T cells or cancer cell lines in RPMI medium, 10% FBS and 1%P/S.

Activation assays T cells

T cells were isolated by crushing the spleen through a 70um cell strainer into a 6-well. Red blood cells were lysed using ACK buffer (0.15M NH4Cl, 0.01M KHCO3, 0.0001M EDTA in PBS) for 5 minutes at room temperature. Lysis was stopped by addition of PBS and cells were washed. The T cell isolation kit (Miltenyi) was used to isolate T cells from other splenocytes, and T cells were cultured with RPMI, 10% FBS and 1% P/S and 10ng/ml IL2. T cells were activated by incubation with 10ug/ml SL8-peptide (OVA257-264 SIINFEKL) for activation assays or anti-CD3/CD28-beads at one bead per cell for proliferation assays. Activation was measured by CD69 expression and proliferation was measured with 10uM CFSE (LifeTech).

Eosinophil T cell co-culture

Eosinophils and T cells were cultured in RPMI, 10% FBS, 1% P/S at a 1:1 ratio. Prior to co-culture, T cells were isolated and stained with 10uM CFSE (LifeTech) and eosinophils with Celltrace violet (LifeTech). In brief, T cells were resuspended in 1ml per 10^7 cells in PBS and 10uM CFSE. Staining was done for 15 minutes at 37°C under agitation. 2x the volume of FBS was added to stop the reaction. Cells were washed two times and plated. Eosinophils were resuspended at 2.5 million in 1ml with CellTrace violet (10uM) and incubated for 15 minutes under agitation at room temperature. 5x the volume of FBS was added to stop the reaction. Cells were washed two times and plated.

Killing assays

Cancer cells were resuspended at 2.5 million in 1ml with CellTrace violet (10uM) and incubated for 15 minutes under agitation at room temperature. 5x the volume of FBS was added to stop the reaction. Cells were washed two times and plated. Cancer cells were plated at 10’000 cells per well in flatbottom 96-well plates and left to attach overnight. Eosinophils were added at different ratios and incubated overnight. Killing was measured by PI and Annexin-FITC stain by flow cytometry.

qPCR

RNA was prepared using a standard trizol protocol. In brief, tumor pieces were homogenized in 1 ml Trizol and 200ul chloroform was added followed by 2-minute incubation at room temperature. The clear phase was separated through centrifugation at 15’000 rpm at 4°C and RNA was precipitated by addition of propanol and spun down at 15’000 rpm at 4°C. The pellet was washed with 70% ethanol and resuspended in H2O. Complementary DNA were generated using the Maxima H Minus Reverse Transcriptase kit according to manufacturer’s instructions (Ref: EP0741) and quantitative PCR was performed in triplicates in a StepOnePlusthermocycler (Applied Biosystems) using the Power SYBR green PCR Master Mix (AppliedBiosystems). For each sample, 200nM of each primer, 200nM DNA and 5ul of SYBR green PCR Master Mix was used and completed to a total volume of 10ul per well. Selected housekeeping gene is ribosomal protein S19.

Amplification protocol

The qPCR amplification was done in three stages for all genes analyzed.

1. Holding stage: 95°C, 10 minutes

2. Cycling stage: 95°C 15s, 58°C for 15s and 72°C 15s for 40 cycles

3. Melting curve stage: 95°C 15s, 72°C 15s, 92°C 15s.

Primers:

dT-primers: TTTTTTTTTTTTTTTTTTTT

RPL19 forward: CTGATCAAGGATGGGCTGAT

RPL19 reverse: GGCAGTACCCTTCCTCTTCC

CCL11 forward: CTC ACC CAG GCT CCA TCC

CCL11 reverse: CAA CCT GGT CTT GAA GAC TAT GG

CCL24 forward: TGC ATC TTC CCC ATA GAT TCT G

CCL24 reverse: TTG GTG ATG AAG ATG ACC CC

IL33 forward: CAT CCA AGG AAC TTC ACT TTT AAC

IL33 reverse : AGT AGC ACC TGG TCT TGC

TNFa forward: CCA AAG GGA TGA GAA GTT CCC

TNFa reverse: GGT GGT TTG CTA CGA CGT G

FACS

Preparation of samples for flow cytometry analysis was done by isolating cells from tumor pieces through digestions as described for PyMT cancers or *in vitro* cells. Cells were washed with PBS 1% FBS and spun at 400g for 4 minutes at 4°C twice. For primary tumor derived cells or blood cells, red blood cell lysis was done using ACK buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 0.0001M EDTA in PBS) at room temperature and lysis was stopped by adding PBS. Cells were washed and resuspended in medium.1 mio cells were plated into one well on 96-well U-bottom plates. Washes were done by spinning the plates at 400g and the supernatant was removed by inverting the plate. Antibody staining was done in parallel with FC block and LiveDead stain for 20 minutes at 4°C. Samples were washed and fixed by 4% PFA and kept as 4°C or on ice in the dark until acquisition. Samples were acquired using the plate reader on the LSR SORP.

Permeabilization for intracellular stain was done using the FoxP3 CellPerm kit from Invitrogen according to manufacturer's instructions.

Cytospins

100'000-200'000 cells were fixed with 4% PFA at room temperature for 10 minutes under agitation and collected in 150ul of PBS and spun onto SuperFrost glass slides. Cytospins were left to dry o/n at room temperature then stored at 4°C until staining.

Histological staining

For histological examinations, fixed or frozen tissues were cut into 5um sections.

Sirius red staining was done by deparaffinizing tissue sections through sequential xylene and ethanol steps if necessary. Nuclei were stained with Meyers Hematoxylin for 10 minutes at room temperature. Blueing of nuclei was done by washing slides for 10 minutes under running water. Slides were equilibrated by ethanol for 1 minute. Granules were stained by Sirius red solution during 2h at room temperature. Remaining Sirius red was washed off and slides were dried at room temperature and then put into xylene for dehydration followed by mounting using a xylene based mounting medium.

Deparaffinization, H&E staining and coverslip mounting were done by the histology core facility.

Fluorescent staining was done by fixing fresh tissue sections in acetone at -20°C for 20 minutes followed by drying under a chemical hood. Slides were washed in 1X PBS under agitation for 30s. Unspecific antibody binding was blocked by incubation of slides with a 1% BSA solution in PBS for 1h at room temperature under agitation in a humidification chamber. Slides were put into a humidification chamber and primary antibodies were added diluted in the blocking solution and incubated at 4°C overnight. Sections were washed 5 times in 1XPBS under agitation for 30s and secondary antibodies were added in the blocking solution and incubated for 1h at room temperature with DAPI. Coverslips were mounted using a water-based mounting medium and sections were acquired using the Olympus slide scanner at 20X or 40X.

Mice

These studies were conducted under federal guidelines for the use and care of laboratory animals.

WT Balb/c mice, WT FVB mice and WT C57BL/6J mice were bred in-house or purchased from Charles River. Mice were kept under controlled conditions in the SPF facility before experiments and transferred to the conventional facility for experiments. They had water and food pellets *ad libitum*. All operations were done in a ventilated hood.

In vivo cancer cell injections

For orthotopic breast cancer cell injections, I used 50'000 4T1 cells injected in Balb/c mice, 500'000 E0771 cells and 1'000'000 PyMT cell line injected in C57BL/6J mice. Mice were put to sleep with isoflurane on a heating pad and morphine was applied for analgesia subcutaneously (Temgesic). The area of operation was shaved and a small incision was made in the skin above the fourth mammary fat pad Cells were injected in

30ul of PBS into the fat pad bellow the lymph node. The wound was closed using surgical clips. Mice were left to recover on the heating pad after the operation and food pellets were put directly into the cage for three days post-surgery. 400mg/500ml dafalgan was administered for one day prior to surgery until two days post-surgery. Wound clips were removed on day 11 post-surgery by putting the mice asleep with isoflurane on a heating pad.

For metastatic assays, mice were put under a heating lamp for 2 minutes and then restrained. 50'000 4T1 cells were injected in a total volume of 100ul PBS into the tail vein.

In vivo systemic treatment

For systemic administration of treatment, AB5-IL5, AB5-CSF2, AB5-IFNy and aCD90.1-IgE were injected i.p. into mice at varying amounts and frequencies. Mice were restrained and the treatment was injected on the contralateral side of tumors.

Statistical analysis

Data were analyzed using GraphPad Software (Prism version 9.4.1). To test normality, the Shapiro-Wilk test was used, in which the null-hypothesis is that the samples are normally distributed (i.e. $p > 0.05$) and the alternative hypothesis is that the samples are not normally distributed (i.e. $p \leq 0.05$). If the number of samples was too small to use a normality test, the distribution of the samples was assessed visually on the graph in addition to careful analysis of the QQ plot. If the data followed a normal distribution, a parametric test was applied, otherwise a non-parametric test was used.

Comparisons between two groups with equal standard deviations (SD) were done by a two-tailed unpaired t-test. If the SD between the groups varied significantly, as determined by the F-test, a Welch correction was used.

Comparisons between more than two groups with equal SD were done by one-way analysis of variance (ANOVA) with multiple comparisons. If the SD between the groups varied significantly, as determined by a Brown-Forsythe test, Welch ANOVA with multiple comparisons was used.

Comparisons of more than one variable between more than two groups as well as tumor growth were done by two-way analysis of variance (ANOVA) with multiple comparisons.

Correlations were calculated using a simple linear regression. The R2 value describes the percentage the change is variable y is explained by the changes in variable x.

P values < 0.05 were considered as significant. Non-significant changes are only shown if a strong tendency is present and in these cases the p-values are given.

Scope of thesis

The goal of my thesis was to analyze the effect of eosinophils on breast cancer. I chose this topic for three main reasons: Human data indicates that blood eosinophilia in breast cancer patients leads to increased patient survival in most cases, human data which analyzes the effects of tissue associated eosinophilia (TAE) in breast cancer is not conclusive and eosinophilia in the context of breast cancer in pre-clinical studies, especially in orthotopic mouse models, is an understudied area.

Initially I wanted to find a reliable gating strategy to define eosinophils via flow cytometry as to date there is no set marker profile for these cells yet (see introduction and discussion). Because eosinophils are a rare immune cell population in breast cancer, I next needed to establish an experimental workflow which would reliably induce breast cancer eosinophilia in order to study the effect of this cell on tumor progression. Finally, I wanted to know if and how eosinophilia can affect other immune populations within the tumor microenvironment.

Results

High CCL11 expression is associated with patient outcome in some datasets

Few publications exist that analyze the effects of tumor associated eosinophils (TAE) on breast cancer progression and most of those define eosinophils via gene expression rather than histology or flow cytometry, which can be problematic (see discussion). I decided to use a different approach. Instead of inferring the presence of eosinophils through expression of typical eosinophil genes, such as PRG2, EPX and SiglecF, I assumed that breast tumors with high CCL11 expression would be more likely to contain eosinophils compared to breast tumors with low CCL11 expression. The division into the two groups was done using the "scan" mode which results in the largest possible separation between groups. The minimal group size was defined as eight patients. I first analyzed the effect of CCL11 expression on the overall dataset and then subdivided the datasets into the four different subtypes of breast cancer (only available for the Bergh, Bertucci and Booser datasets).

My analysis, using the dataset from Chin[589], Zhang[590], Bergh[591], Bertucci[592] and Booser[593], showed that CCL11 expression was associated with different outcomes in different datasets (Fig10 A). Increased CCL11 expression in breast tumors was associated with increased survival in the Zhang and the Bertucci datasets and decreased survival in the Chin and Booser datasets whereas there was no correlation in the Bergh dataset. When the patients were divided into the breast cancer subtypes (Fig10 B, C and D) I observed that high CCL11 expression correlated with increased survival of patients with basal breast cancer in the Bertucci and Boser dataset (Fig10 C and D). The other subtypes showed varying results, depending on the dataset.

I concluded that the effect of CCL11 expression and thus the assumed eosinophilia varies between different types of breast cancer and chose to work with breast cancer models that most closely resembled the basal subtype.

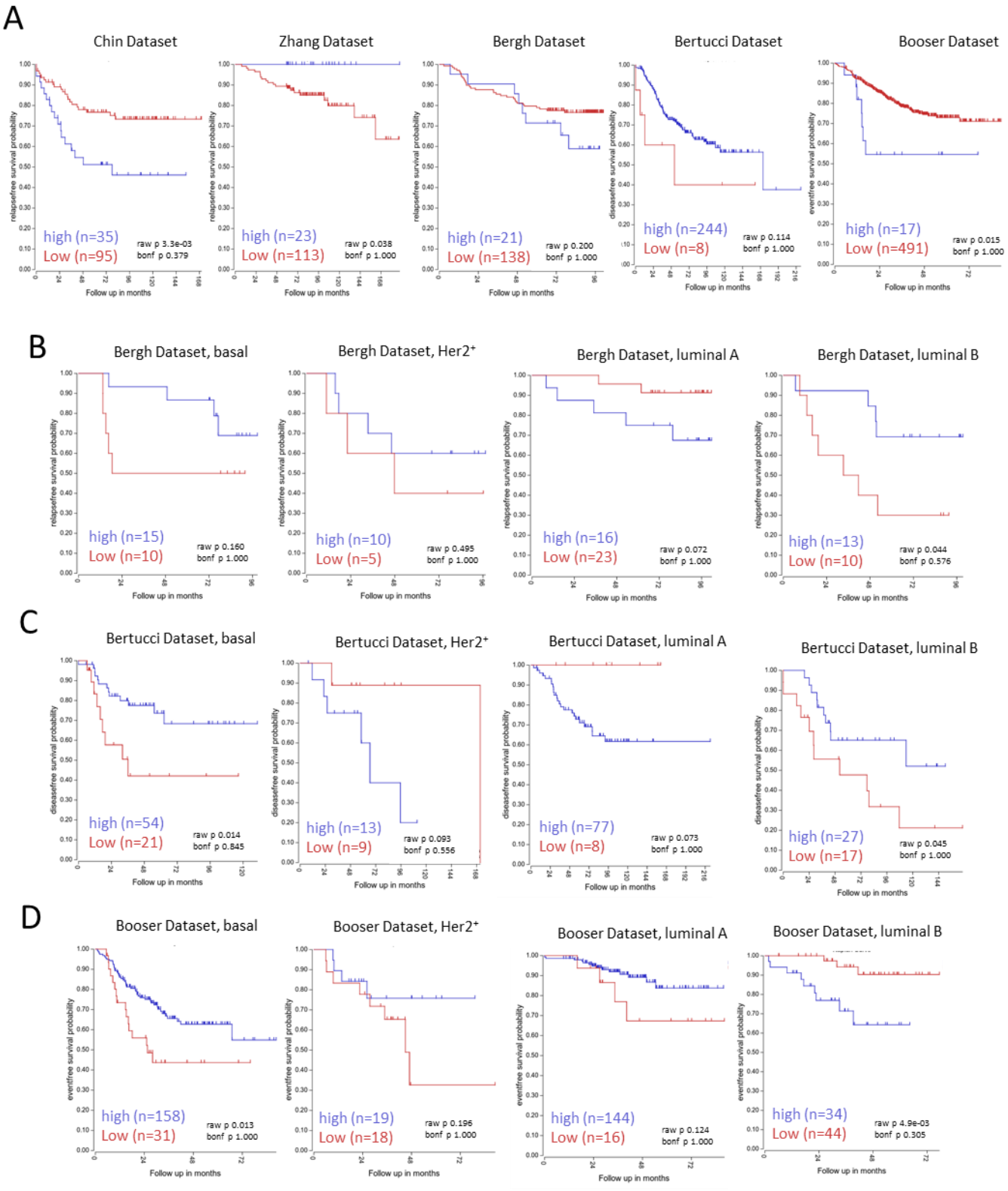

A
Chin Dataset
high (n=35)
Low (n=95)
raw p 3.3e-03
bonf p 0.379
Zhang Dataset
high (n=23)
Low (n=113)
raw p 0.038
bonf p 1.000
Bergh Dataset
high (n=21)
Low (n=138)
raw p 0.200
bonf p 1.000
Bertucci Dataset
high (n=244)
Low (n=8)
raw p 0.114
bonf p 1.000
Booser Dataset
high (n=17)
Low (n=491)
raw p 0.015
bonf p 1.000
B
Bergh Dataset, basal
high (n=15)
Low (n=10)
raw p 0.160
bonf p 1.000
Bergh Dataset, Her2+
high (n=10)
Low (n=5)
raw p 0.495
bonf p 1.000
Bergh Dataset, luminal A
high (n=16)
Low (n=23)
raw p 0.072
bonf p 1.000
Bergh Dataset, luminal B
high (n=13)
Low (n=10)
raw p 0.044
bonf p 0.576
C
Bertucci Dataset, basal
high (n=54)
Low (n=21)
raw p 0.014
bonf p 0.845
Bertucci Dataset, Her2+
high (n=13)
Low (n=9)
raw p 0.093
bonf p 0.556
Bertucci Dataset, luminal A
high (n=77)
Low (n=8)
raw p 0.073
bonf p 1.000
Bertucci Dataset, luminal B
high (n=27)
Low (n=17)
raw p 0.045
bonf p 1.000
D
Booser Dataset, basal
high (n=158)
Low (n=31)
raw p 0.013
bonf p 1.000
Booser Dataset, Her2+
high (n=19)
Low (n=18)
raw p 0.196
bonf p 1.000
Booser Dataset, luminal A
high (n=144)
Low (n=16)
raw p 0.124
bonf p 1.000
Booser Dataset, luminal B
high (n=34)
Low (n=44)
raw p 4.9e-03
bonf p 0.305
Follow up in months

Figure 10: High CCL11 expression is associated with increased survival in patients with basal breast cancer.

Kaplan-Meier survival plots of different breast cancer dataset.

A: Each dataset was subdivided into CCL11 high and CCL11 low expressing tumors and disease-free survival was calculated.

B, C and D: The Bergh (top), Bertucci (middle) and Booser (bottom) datasets were subdivided according to the molecular subtypes of breast cancer into basal, Her2-enriched, luminal A and luminal B. Blue lines indicate high expression and red lines indicate low expression. P values are in the bottom right corner.

Establishing an eosinophilic breast cancer model

Blood and tissue eosinophilia is induced by IL-5 and CCL11

To establish an eosinophilic breast cancer model, blood eosinophilia induction was first tested. To increase circulation time and cause a local IL-5 accumulation within tumors, a periostin specific ScFv (termed AB5) was coupled to Interleukin-5 (IL5), from now on referred to as AB5-IL5 (Fig11 A). Periostin, a protein expressed in the extracellular matrix (ECM) can be found within the stromal compartment of several breast cancer models (data not shown) but also in the bone marrow where eosinophil production occurs. In addition to targeting IL5 to the tumor ECM, the larger size of the interleukin-antibody fusion is supposed to increase time in circulation and thereby effective dose.

Wild type (WT) mice were treated with PBS, 50ng of IL5 or 50ng of AB5-IL5 intravenously (i.v.) or intraperitoneally (i.p.) and blood was collected 30 minutes after treatment to analyze the number of blood eosinophils (Fig11 B). I.v. treatment with AB5-IL5 caused significant blood eosinophilia in FVB mice compared to PBS treatment whereas IL5 treatment increased blood eosinophilia non-significantly (Fig11 C, top panel). In Balb/c mice, only i.v. treatment with IL5 caused a significant increase in circulating eosinophils compared to PBS or IL5 i.p. treatment (Fig11 C, bottom panel). Representative flow cytometry plots for quantification of blood eosinophils in FVB mice treated with AB5-IL5 are shown (Fig11 C, right).

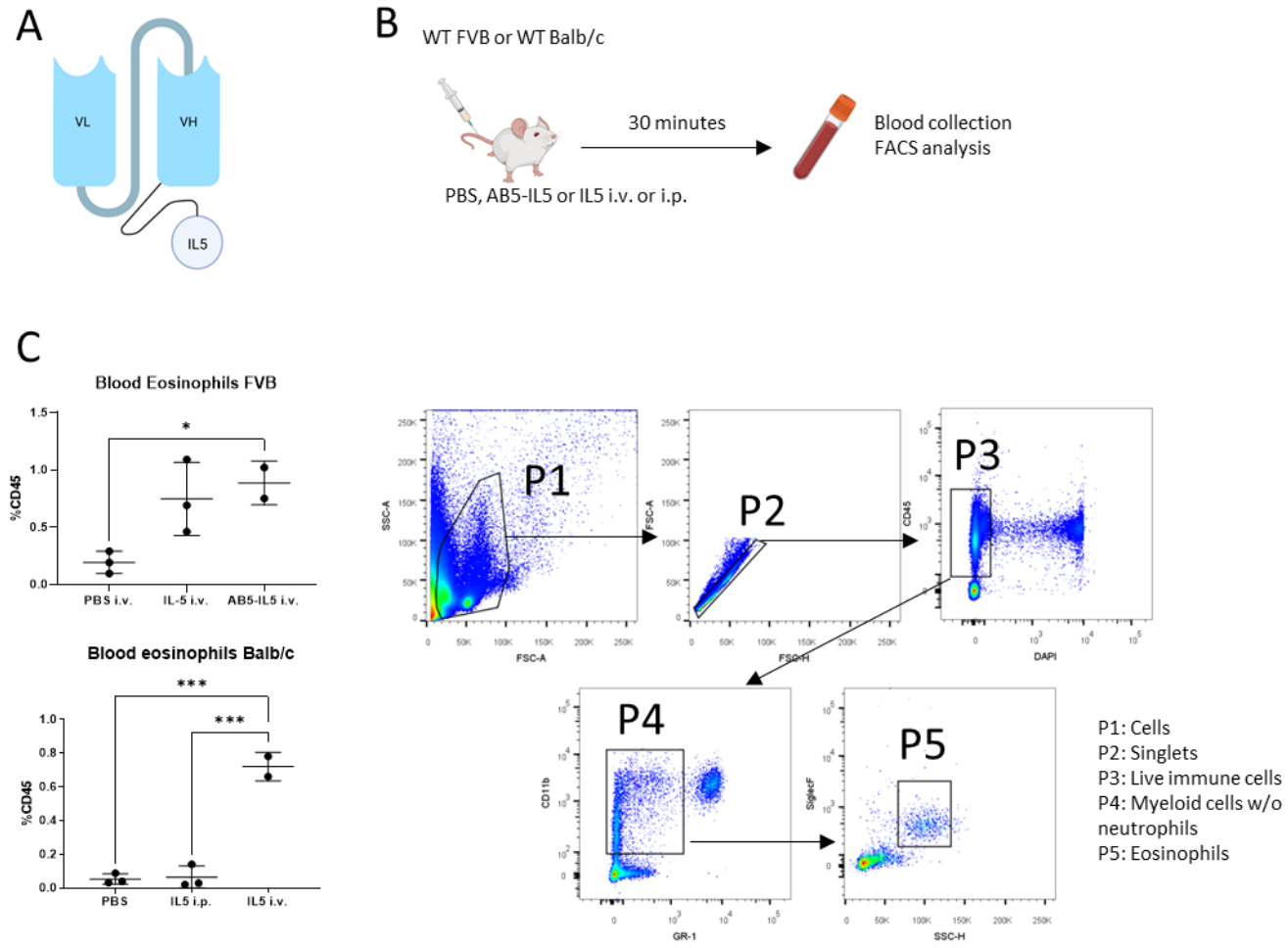


Figure11: Blood eosinophilia is induced by IL5 and AB5-IL5 treatment.

Healthy WT FVB and WT Balb/c mice were administered 50ng of IL5, 50ng of AB5-IL5 or PBS i.v. or i.p. 30 minutes after the injection, whole blood was collected and analyzed via flow cytometry for eosinophils.

A: Schematic representation of AB5-IL5. The variable region (ScFv) of an anti-periostin antibody was coupled with IL5. Created with BioRender.com.

B: Experimental workflow.

C: Eosinophils were defined as $CD45^{+}CD11b^{+}GR\text{-}1^{neg\text{-}med}SiglecF^{+}SSC^{high}$ (one-way ANOVA). Representative flow cytometry plots are shown for one AB5-IL5 treated FVB mouse.

i.v.: Intravenous. i.p.: Intraperitoneal. WT: Wild type.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

Because a single injection did not cause prolonged blood eosinophilia (data not shown), I decided to test a longer treatment period. Prolonged treatment of mice with AB5-IL5 (Fig12 A) increased blood and spleen eosinophilia more than a single injection in WT Balb/c mice. One week of daily i.v. injections with AB5-IL5 caused an 8-fold and a 12-fold increase in eosinophilia within the blood and the spleens respectively (Fig12 B, left panel). As expected, neutrophils were more frequent within the circulation than in the spleens in

both PBS and AB5-IL5 treated mice. AB5-IL5 tended to increase neutrophils within the spleens but not within the blood (Fig12 B, right panel). Representative flow cytometry plots for quantification of blood and splenic eosinophils and neutrophils in AB5-IL5 treated mice is shown (Fig12 C).

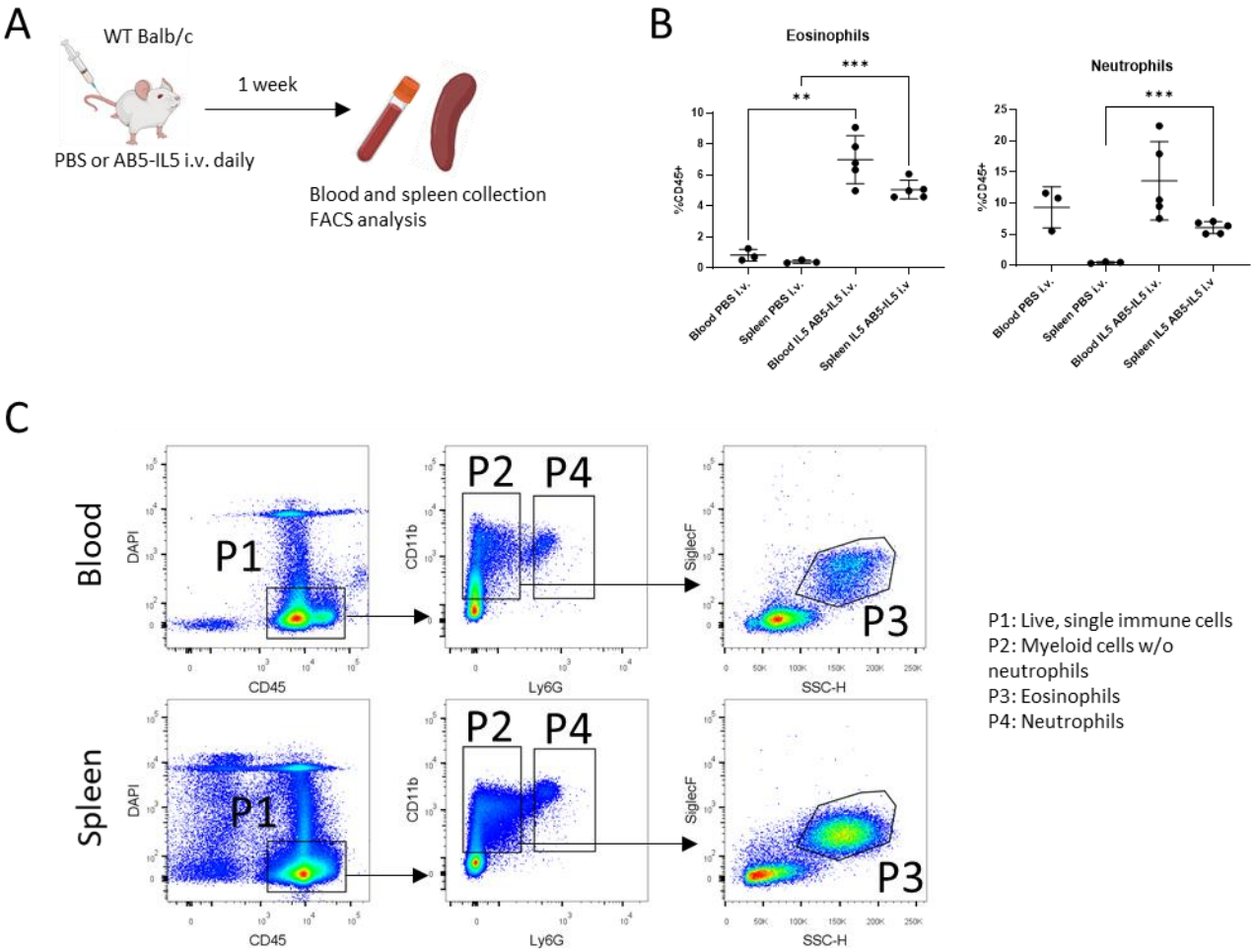


Figure12: AB5-IL5 treatment of Balb/c mice causes eosinophilia in blood and spleen.

WT Balb/c mice were treated with PBS or 100ug AB5-IL5 i.v. daily for one week. Blood and spleens were analyzed by flow cytometry.

A: Experimental workflow.

B: Quantification of immune cells in blood and spleen (Brown-Forsythe one-way Welch ANOVA). Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}SSC^{high}$, neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$.

C: Representative plots used to identify the different immune populations form an AB5-IL5 treated mouse blood (top) and spleen (bottom).

i.v.: Intravenous. WT: Wild type.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

Tumor eosinophilia is induced by AB5-IL5 treatment and CCL11 tumor expression

The goal of these experiments was to establish an eosinophilic breast cancer model, using induction of blood eosinophilia via AB5-IL5, accumulation of eosinophils within tumors by local CCL11 expression and prolonged eosinophil survival via AB5-IL5 localization to primary tumors. To prove that eosinophils preferentially homed to CCL11 expressing tumors, mice were injected with either control or CCL11 expressing 4T1 tumor cells and tumors were left to grow for two weeks without treatment. Starting at D14, tumor bearing mice were treated every second day for two weeks with 50ug AB5-IL5 or PBS i.p. and tumors were analyzed via Sirius red (Fig13 A), a chemical stain which colors eosinophilic granules in pink-red and cell nuclei blue. There was a gradual increase of tumor associated eosinophils (TAEs) from control tumors treated with PBS, control tumors treated with AB5-IL5, CCL11 expressing tumors treated with PBS and CCL11 expressing tumors treated with AB5-IL5 (hereto forth referred to as control PBS tumors, control AB5-IL5 tumors, CCL11 PBS tumors and CCL11 AB5-IL5 tumors respectively), as expected (Fig13 B, left). To quantify this increase, 10 fields of vision were chosen in tumors from each group and eosinophils were counted manually. The numbers of eosinophils per field of vision were normalized to the number of total cells within each field of vision (Fig13 B, right).

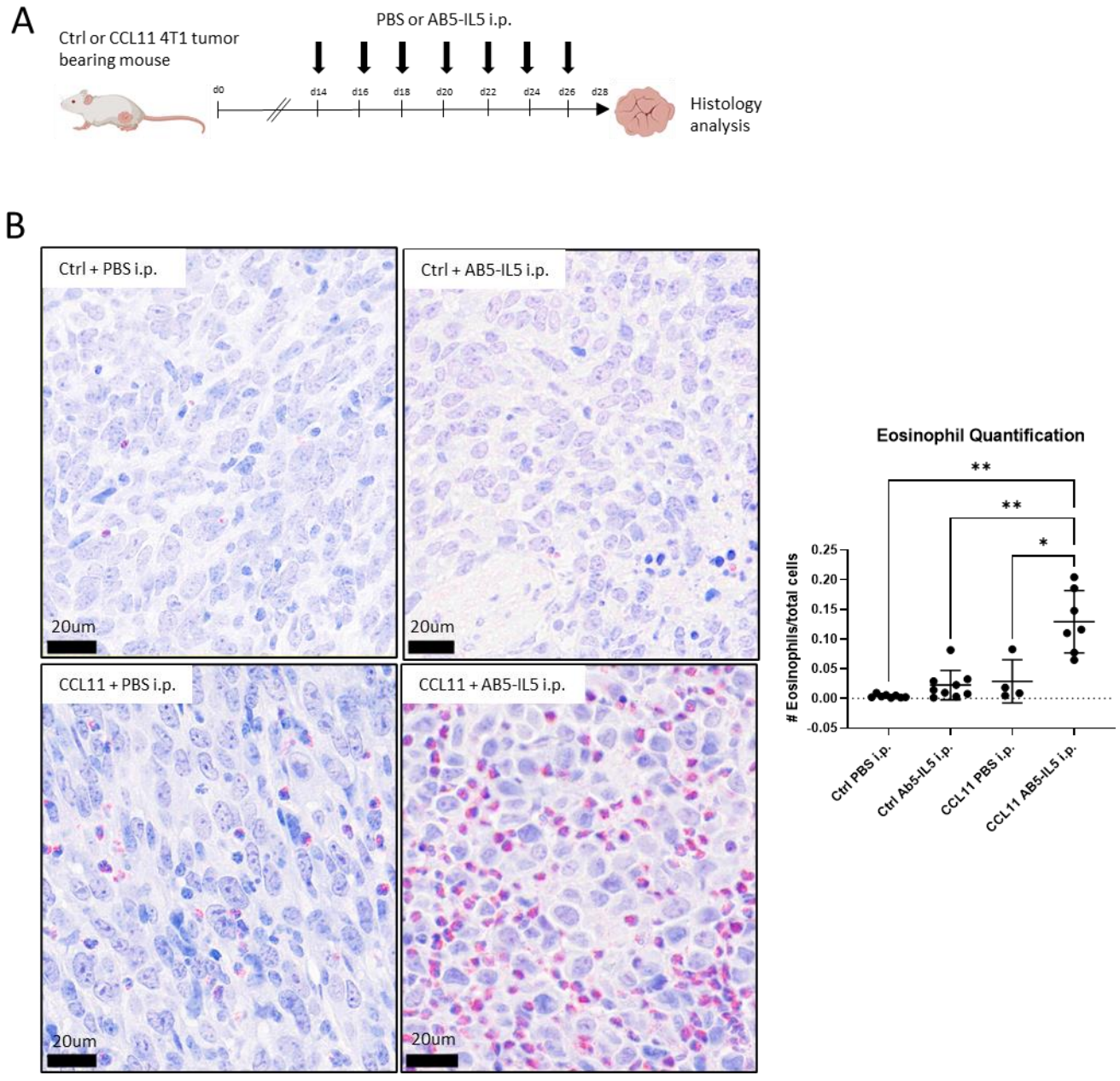


Figure13: Tumor eosinophilia is induced by AB5-IL-5 and CCL11.

WT Balb/c mice were injected with control or CCL11 4T1 cancer cells and treated with PBS or 50ug AB5-IL5 every second day starting at day 14 post tumor injection. Tumor eosinophilia was analyzed by histology. Tumors were fixed in 4% PFA and analyzed via Sirius Red for eosinophils.

A: Experimental workflow.

B: Eosinophils are stained pink-red and nuclei are stained blue. Scale bar 20um. The quantification is shown on the right where the number of eosinophils were normalized to the number of total cells in each field of vision (Brown-Forsythe one-way Welch ANOVA).

WT: Wild type, i.p.: Intraperitoneal, i.v.: Intravenous.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

My results showed that both blood and tissue eosinophilia can be induced via a combination of systemic AB5-IL5 treatment and tumor localized CCL11 expression. This model was used for future experiments to study the effects of eosinophils on tumor progression *in vivo*.

Defining a gating strategy for eosinophils using flow cytometry

In the literature, there is some controversy about which surface markers should be used to identify eosinophils through flow cytometry. Most papers agree that SiglecF is a marker mainly expressed on eosinophils and that eosinophils express CD11b and have a very high granularity, which translates to a very high side scatter (SSC) in flow cytometry. Recently it has been shown that SiglecF can also be highly expressed by neutrophils[402,594–596] and that eosinophils can express Ly6G under certain circumstances[597–599], thus it was important that I established a gating strategy which could reliably distinguish eosinophils from neutrophils.

SiglecF

To ensure that SiglecF was an eosinophil marker, CCL11 AB5-IL5 tumors were frozen and prepared for immunofluorescent (IF) staining with SiglecF, major basic protein (MBP) and eosinophil peroxidase (EPX). SiglecF co-localized with both MBP and EPX, showing it to be a *bona fide* eosinophil marker (Fig 14).

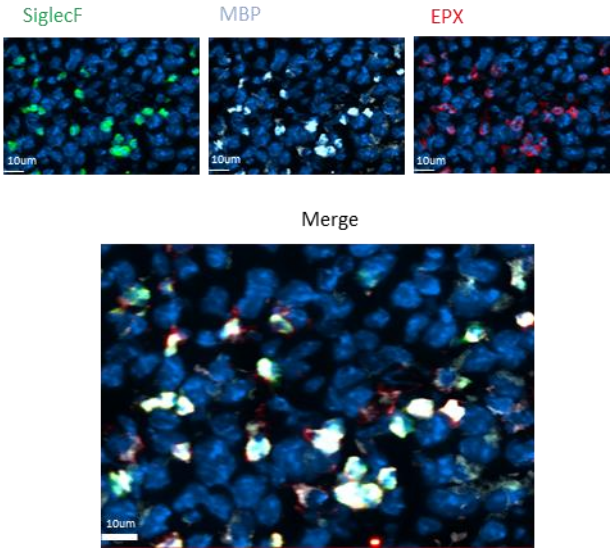


Figure14: SiglecF is expressed in tumor associated eosinophils.

WT Balb/c mice were injected with CCL11 4T1 cancer cells and treated with 50ug AB5-IL5 every second day starting at day 14 post tumor injection. Tumors were frozen for IF staining. Eosinophils were stained with SiglecF (green), MBP (cyan), EPX (red) and DAPI (blue).

i.p.: Intraperitoneal. IF: Immunofluorescence. MBP: Major basic protein. EPX: Eosinophil peroxidase.

Ly6G

Although many publications define eosinophils as Ly6G negative in their text, their results demonstrate that these cells do express Ly6G but at a lower level than neutrophils. To test if this was the case in my model, I decided to make use of fluorescence activated cell sorting (FACS) combined with histological analysis of tumor associated eosinophils (TAEs). Mice were injected with CCL11 expressing 4T1 cancer cells and treated with AB5-IL5 for two weeks. Following tumor resection and homogenization, I stained the cells with CD45, CD11b, Ly6G and SiglecF. Cd11b^{+}Ly6G$^{neg\text{-}med}$, CD11b^{+}Ly6G$^{neg\text{-}med}$SiglecF^{+} and CD11b^{+}Ly6G^{high} cells were sorted (Fig15 A) and then spun onto glass slides. It should be noted that I was not able to recover enough CD11b^{+}Ly6G^{high}SiglecF^{+} cells for analysis. These cytospins were stained with Sirius red to assess the number of eosinophils present in each group. Representative images of cytospins from each gate are shown (Fig15 B) Using this stain, I found that CD11b^{+}Ly6g$^{neg\text{-}med}$ cells contained 24% of eosinophils, CD11b^{+}Ly6g$^{neg\text{-}med}$SiglecF^{+} cells had a population enriched in eosinophils (88 %) and CD11b^{+}Ly6G^{high} cells contained about 1% of eosinophils, highlighted by a red arrowhead (Fig15 B). Importantly, I did not see any neutrophil contamination in the CD11b^{+}Ly6g$^{neg\text{-}med}$SiglecF^{+} population (Fig15 B, bottom left panel). The contaminating cells (highlighted with blue arrowheads) made up 12% of the population and were most likely monocytes, although their exact identity could not be determined. These contaminating cells did not stem (solely) from the inclusion of Ly6G^{med} cells, as the latter made up 50% of the SiglecF^{+} eosinophil gate. It should be noted that F4/80 cannot be used to distinguish eosinophils from macrophages, as the former have been shown to express this marker in some cases[438], a phenotype I observed myself (data not shown).

Interestingly, there was no overlap between EPX and Ly6G staining in the same tumors (Fig15 C), despite the fact that my gating strategy clearly proved Ly6G expression in eosinophils. This could be due to IF being less sensitive and that low levels of expression cannot be assessed.

Overall, I was able to establish a robust gating strategy that allowed me to specifically gate on eosinophils without any neutrophil contamination and showed that in my system, eosinophils express none to moderate levels of Ly6G.

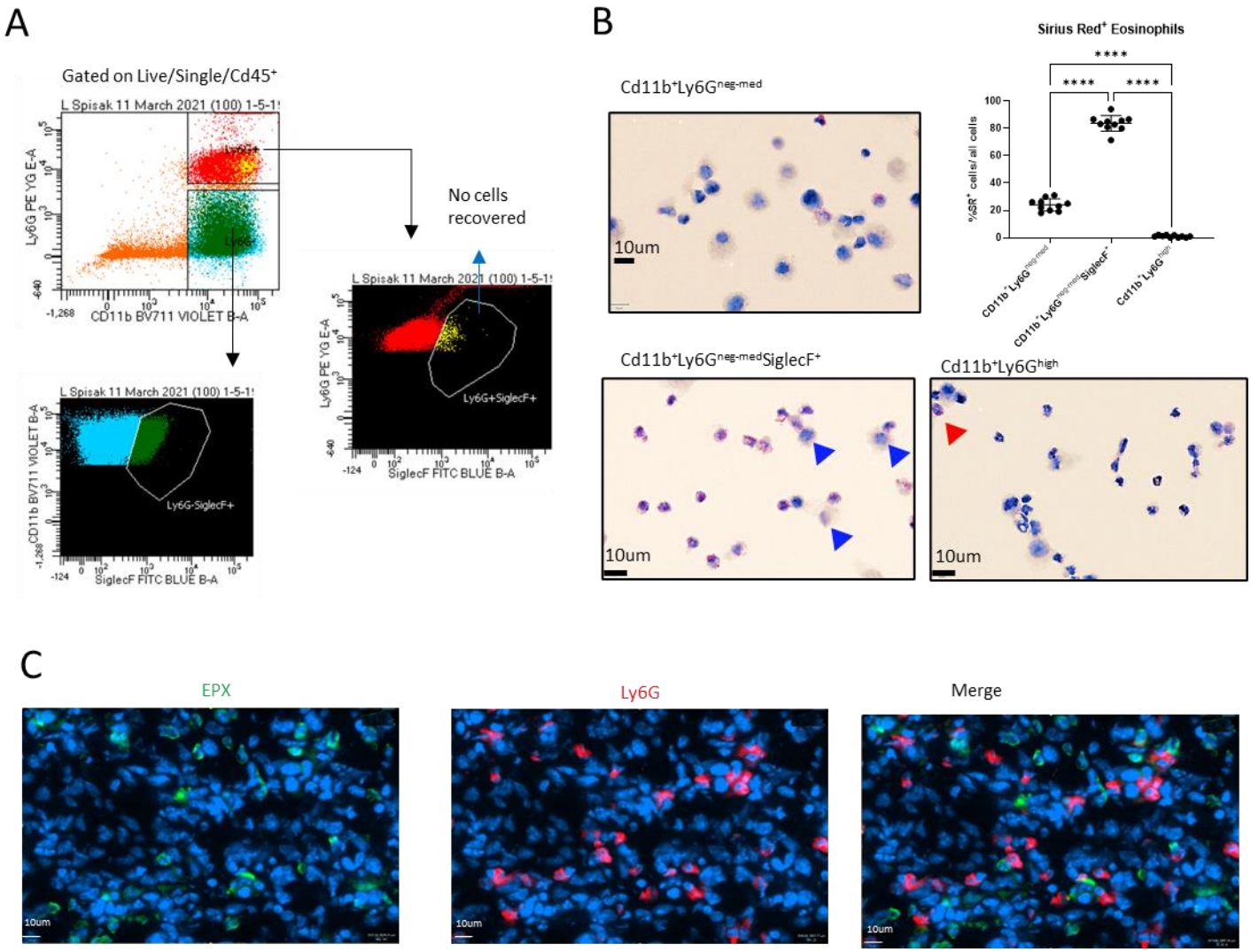


Figure15: Ly6G is expressed in tumor associated eosinophils.

CCL11 tumor bearing mice were treated with 50ug AB5-IL5 i.p. every other day for 30 days starting at day 14 post tumor injection. Tumors were homogenized and prepared for fluorescence activated cell sorting (FACS) and subsequent cytospin analysis. The sorted cells were fixed in 4% PFA and spun unto glass slides and then stained with Sirius red.

A and B: Representative scatter plots (left) and cytospins (right) are shown for the three sorted populations: $CD11b^{+}Ly6G^{neg\text{-}med}$, $Cd11b^{+}Ly6G^{high}$ and $CD11b^{+}Ly6g^{neg\text{-}med}$ $SiglecF^{+}$. The red arrowhead shows a Sirius red positive cell in the $Cd11b^{+}Ly6G^{high}$ population and the blue arrowheads show non-eosinophils in the $CD11b^{+}Ly6g^{neg\text{-}med}$ $SiglecF^{+}$ population. Scale bar 10um. Quantification of sorted samples are shown in the upper right corner (One-way ANOVA).

C: IF staining of tumors. Neutrophils were stained with Ly6G (red) and DAPI (blue) and eosinophils were stained with EPX (green). Scale bar 10um.

IF: Immunofluorescence. EPX: Eosinophil peroxidase.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

IL5R and CCR3

Both IL5R (also known as CD125) and CCR3 (the CCL11 receptor) are known to be expressed by eosinophils and are used by some authors to identify these cells[523,600,601]. CCR3 expression has been almost exclusively ascribed to eosinophils, although basophils have also been shown to express it[602] and IL5R can be expressed on both neutrophils and alveolar macrophages[487,601] in addition to eosinophils. I wanted to test if I could use these markers in my tumor model to both identify eosinophils and distinguish them from neutrophils. I analyzed the expression of IL5R on eosinophils in control PBS and CCL11 AB5-IL5 tumors via flow cytometry and observed that it was barely present (Fig 16 A). I used the same antibody to stain sections of the same tumors and did not observe a membrane stain but rather a staining pattern reminiscent of the extracellular matrix (Fig 16 B). CCR3 was expressed in a majority of tumor associated eosinophils but was also expressed in almost all tumor associated neutrophils (Fig 16 C). I confirmed this finding through histological staining with CCR3 and Ly6G (Fig16 D and E).

I concluded that although it might be a good eosinophil marker, I could not use the IL5R antibody and testing another one was not possible at the time. Furthermore, CCR3 is not a good marker for TAEs in my model because it was expressed in almost all tumor associated neutrophils. It should be noted that I did not validate either of these markers by qPCR.

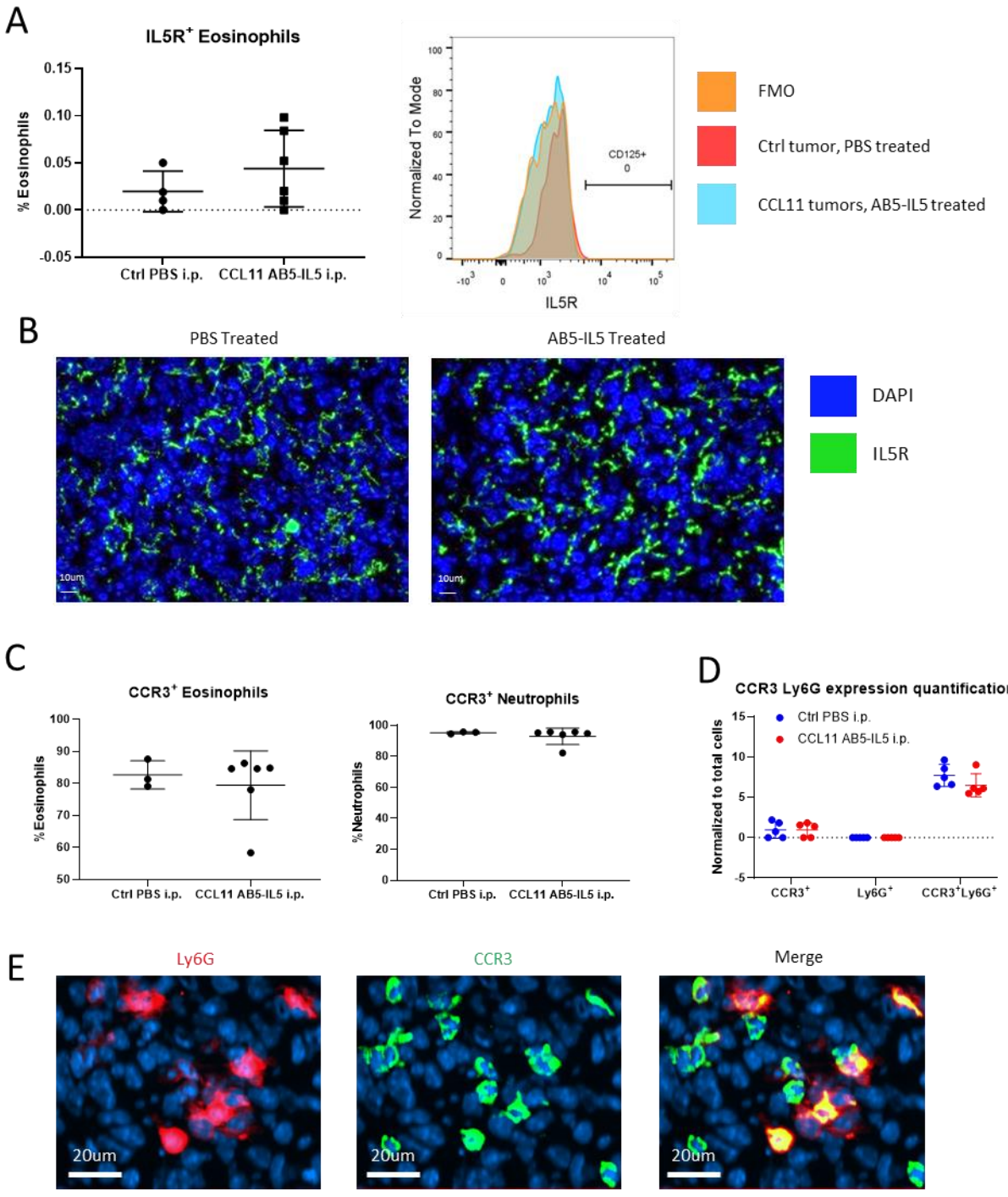

A
IL5R+ Eosinophils
% Eosinophils
0.15
0.10
0.05
0.00
-0.05
Ctrl PBS i.p.
CCL11 AB5-IL5 i.p.
Normalized To Mode
CD125+
IL5R
FMO
Ctrl tumor, PBS treated
CCL11 tumors, AB5-IL5 treated
B
PBS Treated
AB5-IL5 Treated
10um
DAPI
IL5R
C
CCR3+ Eosinophils
% Eosinophils
CCR3+ Neutrophils
% Neutrophils
Ctrl PBS i.p.
CCL11 AB5-IL5 i.p.
D
CCR3 Ly6G expression quantification
Normalized to total cells
Ctrl PBS i.p.
CCL11 AB5-IL5 i.p.
CCR3+
Ly6G+
CCR3+Ly6G+
E
Ly6G
CCR3
Merge
20um

Fig16: IL5R and CCR3 cannot be used to identify tumor associated eosinophils.

WT Balb/c mice were injected with Ctrl or CCL11 expressing 4T1 cells and PBS or 50ug AB5-IL5 was administered systemically every second day. IL5R and CCR3 expression were analyzed by flow cytometry and IF.

A: Analysis of IL5R expression on TAEs in control tumors treated with PBS and CCL11 tumors treated with AB5-IL5 via flow cytometry (left, unpaired two-tailed t-test). Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6G^{neg-med}SiglecF^{+}SSC^{high}$.The expression of IL5R from one representative sample of each group is shown in comparison to the control stain as a histogram (right).

B: Representative images of IL5R IF staining in each group. Tumor sections were stained with IL5R (green) and DAPI (blue). Scale bar 10um.

C: Analysis via flow cytometry of CCR3 expression on TAEs (left) and TANs (right) in control PBS tumors and CCL11 AB5-IL5 (unpaired two-tailed t-test). Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6G^{neg-med}SiglecF^{+}SSC^{high}$ and neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$.

D: Quantification of $CCR3^{+}$, $Ly6G^{+}$ and $CCR3^{+}Ly6G^{+}$ cells in both groups as determined by IF staining (two-way ANOVA).

E: Representative images of Ly6G and CCR3 staining in an AB5-IL5 treated CCL11 tumor. Tumor sections were stained with Ly6G (red), CCR3 (green) and DAPI (blue). Scale bar 20um.

Ctrl: Control. IL5R: IL5 receptor. IF: Immunofluorescence.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

My final gating strategy to identify tumor associated eosinophils

I saw that I could use SiglecF to reliable identify eosinophils, although it was necessary to include a high side scatter to try and exclude monocytic contamination. I also found that I could not exclude all Ly6G positive cells, as tumor associated eosinophils expressed no to moderate levels of Ly6G. Finally, I observed a $SiglecF^{+}$ tumor associated neutrophil populations which displayed the same high granularity as eosinophils (Fig17). Overall, I showed that it is not as straightforward as some might think to reliably identify eosinophils and distinguish them from other immune cells, especially neutrophils.

My final gating strategy markers consisted of CD45, CD11b, Ly6G, SiglecF and side scatter and I used it throughout my experiments. Representative plots from a CCL11 AB5-IL5 tumor are shown in figure 17.

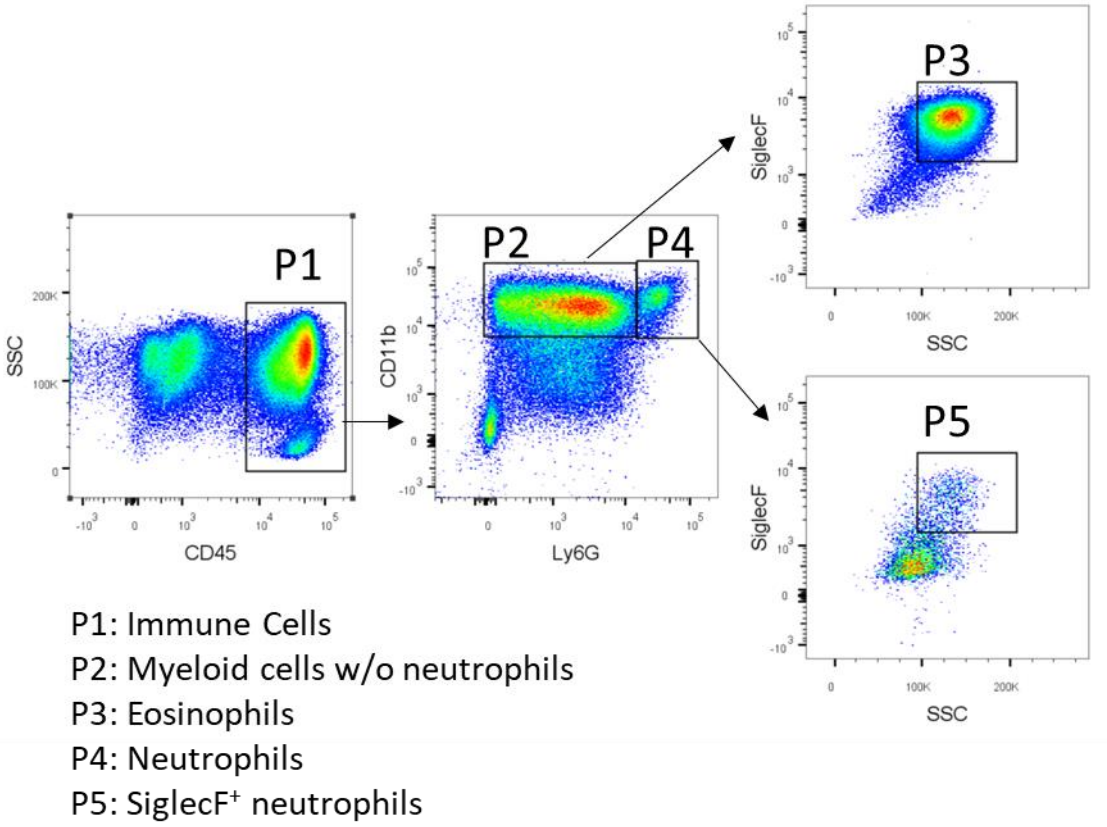


Figure17: Final gating strategy to identify tumor associated eosinophils.

Representative plots are shown from an AB5-IL5 treated CCL11 tumor. Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}SSC^{high}$ and neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$.

Analyzing the effects of eosinophilia on breast cancer

Late onset eosinophilia does not affect primary tumor size or lung metastases

To see if tumor eosinophilia had any effects on tumor progression *in vivo*, I injected control or CCL11 4T1 tumor cells orthotopically into wild type (WT) Balb/c mice. After 2 weeks I began systemic PBS or AB5-IL5 treatment and mice were euthanized at day 30. Immune infiltration of tumor associated eosinophils (TAEs) as well as tumor associated neutrophils (TANs), CCL11 *ex vivo* tumor expression, tumor weight, lung metastases and primary tumor necrosis were analyzed. The results shown are from two independent experiments. Each experiment is color coded (Fig18 A).

In a manner similar to results shown in figure13, TAE numbers gradually increased upon addition of AB5-IL5 treatment and tumor CCL11 expression (Fig18 B, left). Notably, highest eosinophil tumor infiltration was achieved upon combination of CCL11 and AB5-IL5. TANs were significantly decreased in CCL11 AB5-IL5 tumors compared to the other groups (Fig18 B, right). CCL11 expressing 4T1 cells formed CCL11 expressing tumors and the expression remained high throughout the experiment compared to the control transfected cells (Fig18 C).

Tumor weight and lung metastases did not change between groups (Fig18 D left and middle). Because eosinophils are known to induce tissue damage during degranulation, I wanted to know if primary tumor necrosis changed upon treatment. A significant increase in necrosis was seen between control AB5-IL5 tumors and CCL11 AB5-IL5 tumors (Fig18 D, right).

My results showed that AB5-IL5 treatment and CCL11 tumor expression increased tumor infiltrating eosinophils which increased tumor necrosis but did not affect primary tumor size nor lung metastases. I saw a significant decrease in TANs in highly eosinophilic tumors and finally, *in vivo* CCL11 expression remained elevated.

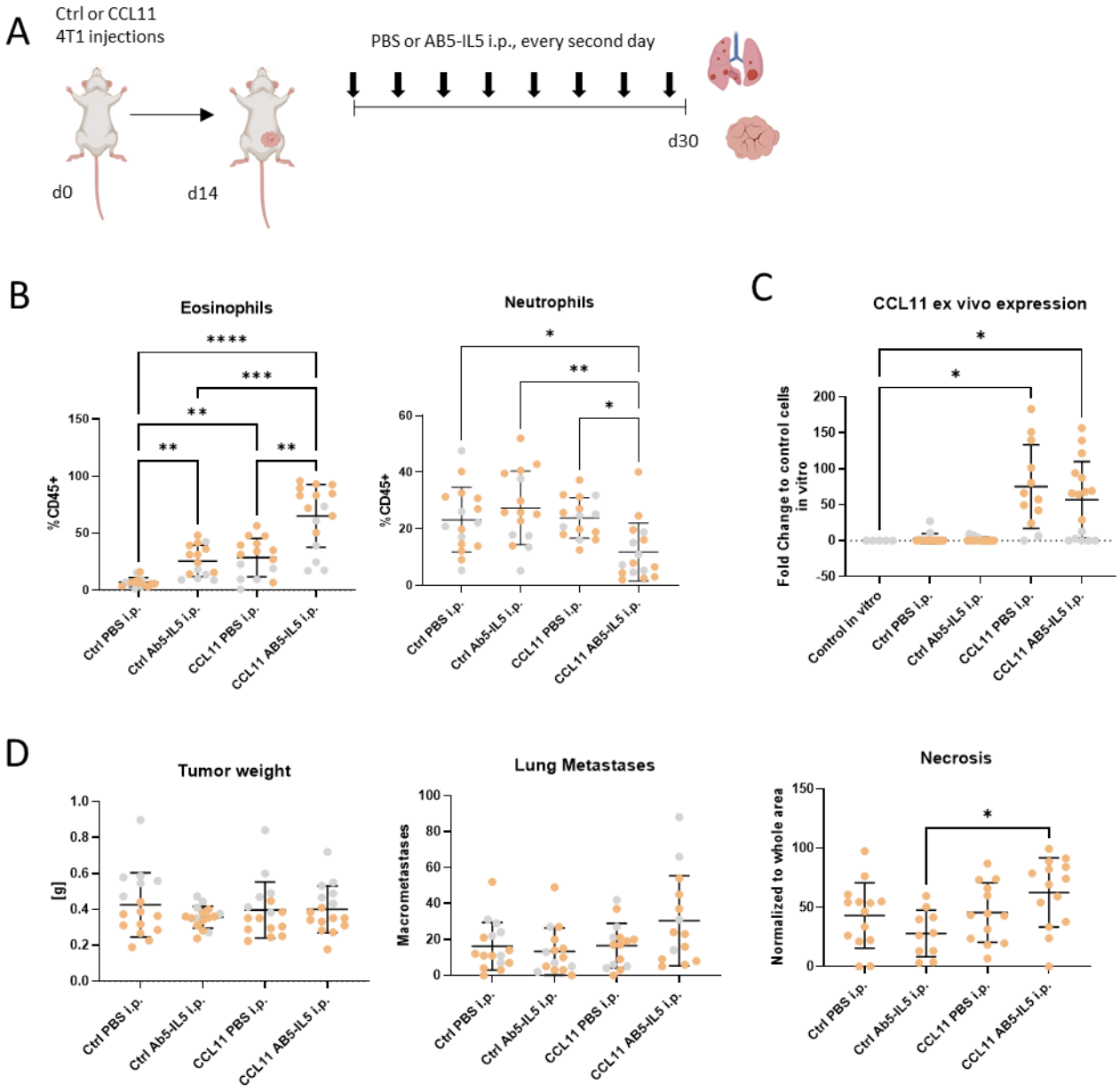


Figure18: Late on-set eosinophilia does not affect primary tumor growth or metastasis

WT Balb/c mice were injected with Ctrl or CCL11 expressing 4T1 cells and PBS or 50ug AB5-IL5 was administered systemically every second day. Immune cells were analyzed by flow cytometry. Tumors were weighted and analyzed by qPCR for CCL11 expression and lung metastases were counted. RPL19 was chosen as housekeeping gene. Necrosis was analyzed by histology. Data is pooled from two independent experiments. Experiment1: Grey dots. Experiment2: Beige dots.

A: Experimental workflow.

B: Analysis of TAE and TAN tumor infiltration by flow cytometry. Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6g^{neg\text{-}med}SiglecF^{+}SSC^{high}$ (Brown-Forsythe one-way Welch ANOVA). Neutrophils were defined as $CD45^{+}Cd11b^{+}Ly6G^{high}$ (ordinary one-way ANOVA).

C: CCL11 *ex vivo* tumor expression determined by qPCR (Brown-Forsythe one-way Welch ANOVA).

D: Tumor weights, lung macrometastases and necrosis analysis (One-way ANOVA). Necrotic area was analyzed visually using an H&E stain and normalized to total area of the cross-section analyzed.

WT: Wild type. i.p.: Intraperitoneally. Ctrl: Control. TAE: Tumor associated eosinophil. TAN: Tumor associated neutrophil.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p≤0.0001 = ****

Adding CSF2 as an additional activator to late onset tumor eosinophilia decreases tumor weight but does not change metastases

I saw in my previous results with the 4T1 breast cancer model that late onset eosinophilia did not affect primary tumor growth and lung metastases. I hypothesized that this weak phenotype could be due to the lack of local eosinophil activation. To test this hypothesis, I decided to treat mice with an additional activator of eosinophils, colony-stimulating factor2 (CSF2, also known as GM-CSF) located to the tumor microenvironment by coupling the cytokine to the same antibody moiety as IL5 (AB5-CSF2). In the literature, CSF2 has been shown to activate macrophages, neutrophils and eosinophils under different conditions[125] and importantly, it promoted anti-tumor immunity in colon cancer through eosinophils.[149]

I injected wild type (WT) Balb/c mice with control or CCL11 expressing 4T1 cancer cells orthotopically and began systemic administration of PBS, AB5-IL5 or AB5-IL5 and AB5-CSF2 together at day 14 (the latter referred to as control AB5-CSF2 + AB5-IL5 or CCL11 AB5-CSF2 + AB5-IL5 respectively). At day 30, immune infiltration of tumor associated eosinophils (TAEs) as well as tumor associated neutrophils (TANs), CCL11 *ex vivo* tumor expression, tumor weight, lung metastases and primary tumor necrosis were analyzed (Fig19 A).

As before, tumor eosinophilia increased in CCL11 AB5-IL5 tumors compared to control PBS tumors and control AB5-CSF2 + AB5-IL5 tumors. Addition of AB5-CSF2 treatment to AB5-IL5 significantly decreased TAEs in CCL11 tumors compared to AB5-IL5 alone (Fig19 B, left panel). SiglecF and CD69 expression levels are used as activation markers of eosinophils[146,410,413,415]. Similarly, high granularity is attributed to activated

eosinophils that have not yet degranulated whereas low granularity implies either immaturity, low activation or already occurred degranulation[411]. SiglecF expression was similar in all groups except in control AB5-CSF2 + AB5-IL5 tumors where it decreased significantly (Fig19 B, middle panel). Granularity was highest in CCL11 PBS (Fig19 B, right panel). In all groups, very few eosinophils expressed CD69 (data not shown), thus I decided not to use it further as an activation marker for TAEs in the 4T1 mouse model of breast cancer.

TAN numbers did not change between the different groups (Fig19 C, left panel). SiglecF^{+} TANs were most frequent in CCL11 AB5-IL5 tumors (Fig19 C, middle panel). All of these SiglecF^{+} TANs displayed the same high side scatter (SSC) as TAEs (see Fig17). SiglecF^{+} TANs in CCL11 AB5-IL5 tumors had the highest SiglecF expression (Fig19 C, right). Because of their unusually high granularity, one must consider if these cells represent a new subset of neutrophils that more closely resembles eosinophils. Of note, the SiglecF^{high} neutrophils described by Engblom et al.[402] had a lower SSC than eosinophils.

CCL11 expression remained high throughout *in vivo* growth in CCL11 expressing tumors compared to control tumors (Fig19 D). The CCL11 expressing cells that were used in the experiment had an about 60-fold increase in CCL11 compared to control transfected cells prior to injections (data not shown).

There was a significant decrease in tumor weight when AB5-CSF2 was added to AB5-IL5 treatment in CCL11 tumors (Fig19 E, left panel). Lung metastases and necrosis did not change between any of the treatment conditions (Fig19 E middle and right panel).

Finally, I wanted to know if tumor eosinophilia can influence tumor associated lymphocytes (TILs). Activated CD8 T cells and Th1 CD4 T cells produce large amounts of IFNγ and to some extend TNFα whereas Th2 CD4 T cells produce large amounts of IL4. Analyzing the production of these cytokines by TILSs allowed me to determine their activation and polarization. No changes in any of the cytokines were seen in CD8 or CD4 TILs upon PMA/Ionomycin stimulation between the different groups (data not shown). Of note, the 4T1 breast cancer mouse model is known to contain very few TILs.

To summarize, adding CSF2 as an additional activator did not significantly change tumor progression and tended to decrease TAEs, making it a non-favorable eosinophils activator for my purposes. I also observed a SiglecF^{+}SSC^{high} population of TANs that has not yet been described.

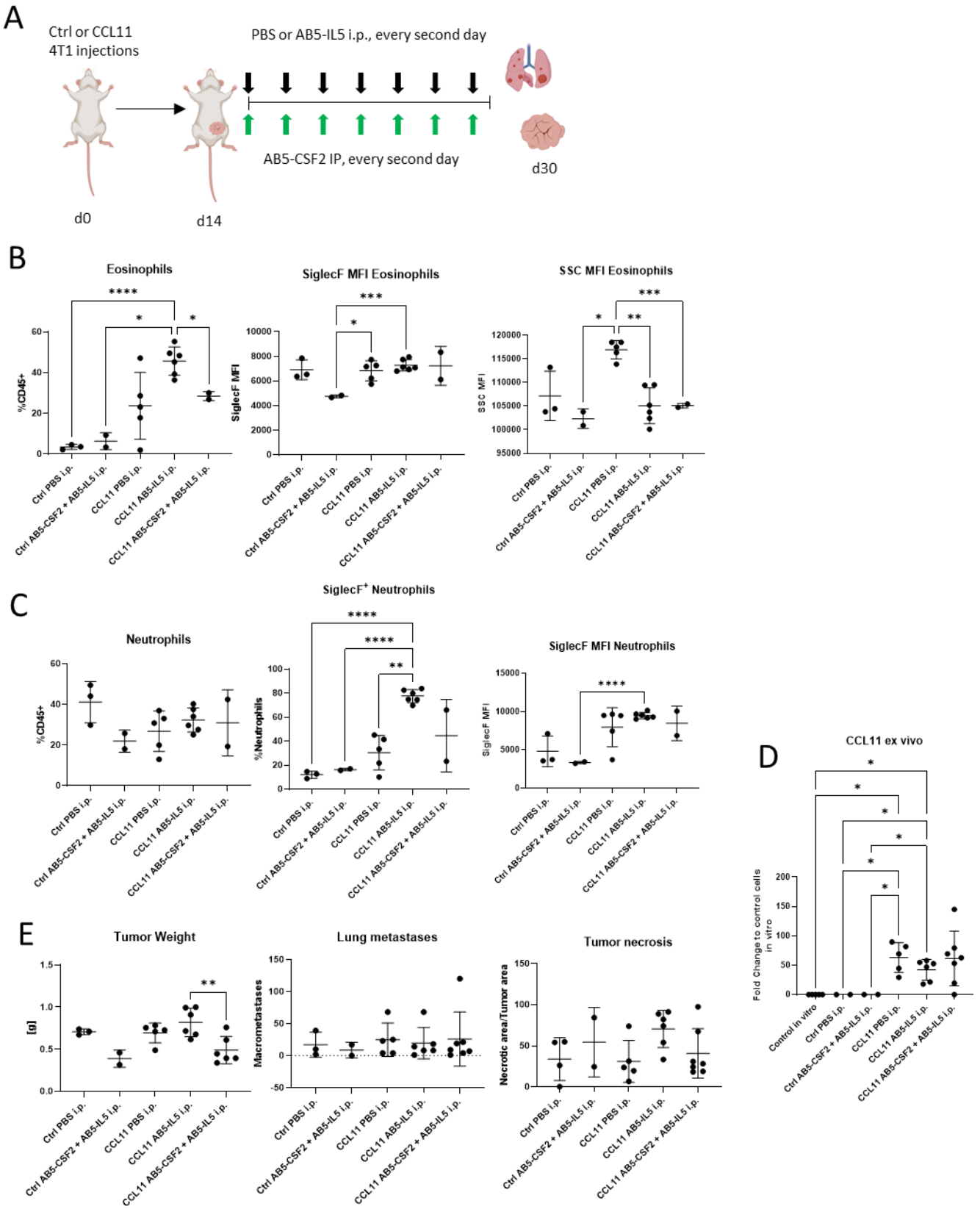

A
Ctrl or CCL11
4T1 injections
PBS or AB5-IL5 i.p., every second day
AB5-CSF2 IP, every second day
d0
d14
d30
B
Eosinophils
SiglecF MFI Eosinophils
SSC MFI Eosinophils
%CD45+
SiglecF MFI
SSC MFI
C
Neutrophils
SiglecF+ Neutrophils
SiglecF MFI Neutrophils
%Neutrophils
D
CCL11 ex vivo
Fold Change to control cells in vitro
E
Tumor Weight
Lung metastases
Tumor necrosis
[g]
Macrometastases
Necrotic area/Tumor area
Ctrl PBS i.p.
Ctrl AB5-CSF2 + AB5-IL5 i.p.
CCL11 PBS i.p.
CCL11 AB5-IL5 i.p.
CCL11 AB5-CSF2 + AB5-IL5 i.p.
Control in vitro

Figure 19: CSF activation of TAEs does not change metastatic spread.

WT Balb/c mice were injected with 4T1 WT or CCL11 expressing cells and PBS, 50ug AB5-IL5 and/or 20ug AB5-CSF2 was administered i.p. every second day. Immune cells were analyzed by flow cytometry. Tumors were weighted and analyzed by qPCR for CCL11 expression and lung metastases were counted. RPL19 was chosen as housekeeping gene. Necrosis was analyzed by histology.

A: Experimental Workflow.

B: Analysis of TAE tumor infiltration by flow cytometry. Eosinophils were defined as $Cd45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}$ SSC^{high} (Brown-Forsythe one-way Welch ANOVA). SiglecF (One-way ANOVA), SSC (Brown-Forsythe one-way Welch ANOVA) and CD69 (One-way ANOVA) expressions are presented as MFIs.

C: Analysis of TAN tumor infiltration by flow cytometry. Neutrophils were defined as $Cd45^{+}CD11b^{+}Ly6G^{high}$ (One-way ANOVA). $SiglecF^{+}$ TANs (Brown-Forsythe one-way Welch ANOVA) were analyzed. SiglecF expression is presented as MFI (Brown-Forsythe one-way Welch ANOVA).

D: CCL11 *ex vivo* tumor expression determined by qPCR (Brown-Forsythe one-way Welch ANOVA).

E: Tumor weights, lung macrometastases count and tumor necrosis analysis (One-way ANOVA). Necrotic area was analyzed visually using an H&E stain and normalized to total area of the cross-section analyzed.

i.p.: Intraperitoneally. TAEs: Tumor associated eosinophils. TANs: tumor associated neutrophils. MFI: Mean fluorescent intensity. SSC: Side scatter.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p≤0.0001 = ****

Summary late onset eosinophilia in breast cancer

When I combined all late onset eosinophilia experiments, I saw that both AB5-IL5 treatment and CCL11 expression significantly induced tumor associated eosinophilia on their own. Combining both significantly increased TAE numbers further (Fig20 A, left panel). There was no change in tumor weight and lung metastases between the groups (Fig20 A, middle and right panel).

Tumor associated eosinophils showed no correlation with tumor weight and metastases (Fig20 B, left and middle panel) and there was a positive correlation between tumor eosinophils and tumor CCL11 expression (Fig20 B, right panel). I saw no change in necrosis between the treated groups and the control groups and there was no significant correlation between TAE numbers and necrosis (Fig20 C)

Lastly, I analyzed the infiltration of tumor associated neutrophils (TANs) within the tumors as well as their SiglecF expression. I found no significant changes in TAN and $SiglecF^{+}$ TAN numbers. (Fig20 D).

Overall, late onset tumor associated eosinophilia had no effect on tumor progression and neutrophil tumor infiltration.

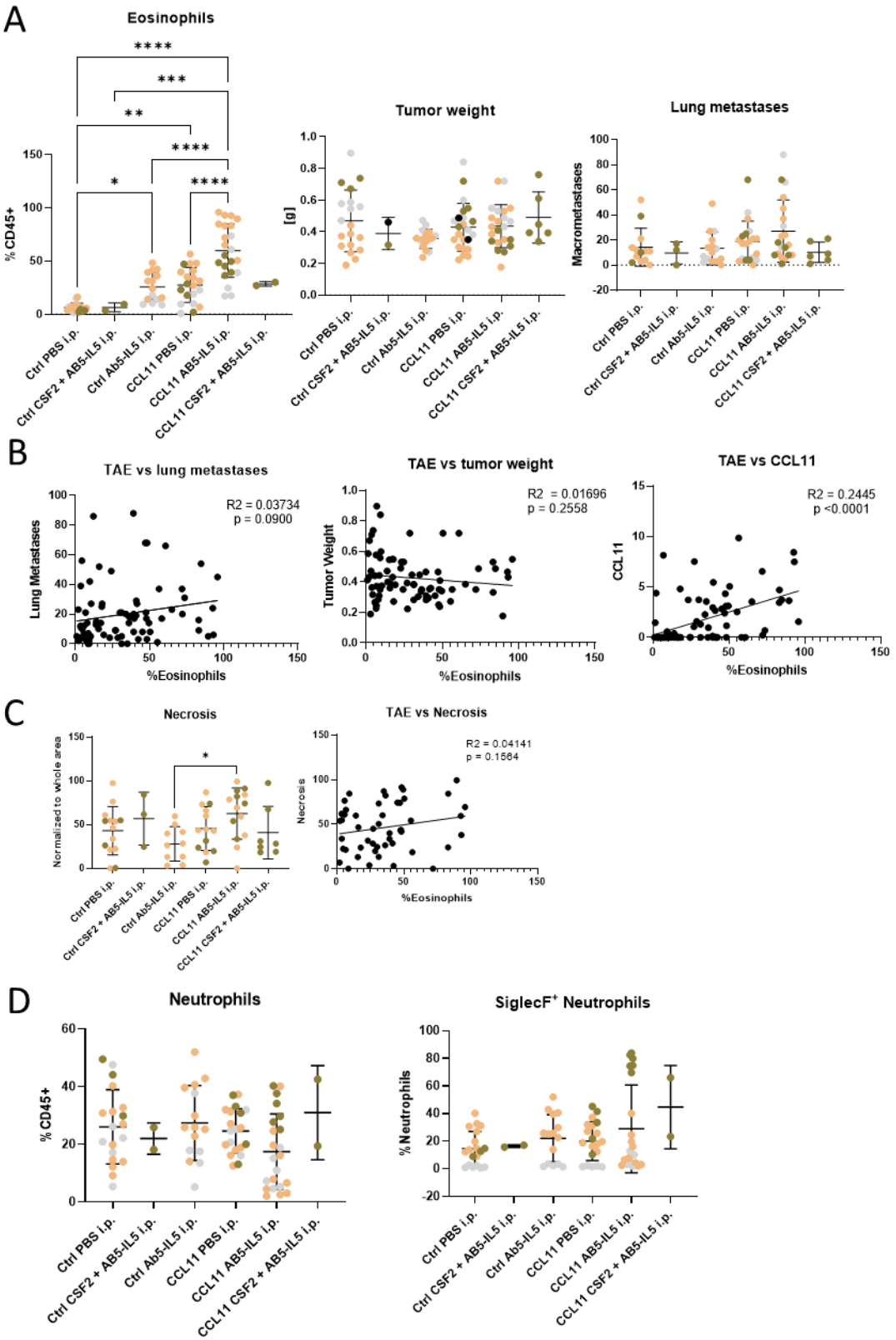

A
Eosinophils
% CD45+
Tumor weight
[g]
Lung metastases
Macrometastases
Ctrl PBS i.p.
Ctrl CSF2 + AB5-IL5 i.p.
Ctrl Ab5-IL5 i.p.
CCL11 PBS i.p.
CCL11 AB5-IL5 i.p.
CCL11 CSF2 + AB5-IL5 i.p.
B
TAE vs lung metastases
Lung Metastases
R2 = 0.03734
p = 0.0900
%Eosinophils
TAE vs tumor weight
Tumor Weight
R2 = 0.01696
p = 0.2558
TAE vs CCL11
CCL11
R2 = 0.2445
p <0.0001
C
Necrosis
Normalized to whole area
TAE vs Necrosis
Necrosis
R2 = 0.04141
p = 0.1564
D
Neutrophils
% CD45+
SiglecF+ Neutrophils
% Neutrophils

Figure20: Eosinophils show a weak tendency to be pro-metastatic when eosinophilia is induced late.

Data were pooled from three independent experiments unless otherwise stated. Each experiment is color coded: Experiment1: grey. Experiment2: beige. Experiment3: green. Correlations between several parameters were calculated, R2 and p-values are on the graphs.

A: Tumor eosinophilia (Brown-Forsythe one-way Welch ANOVA), tumor weight (Brown-Forsythe one-way Welch ANOVA), lung metastases (One-way ANOVA) and CCL11 tumor expression (Brown-Forsythe one-way Welch ANOVA).

B: Correlation between TAE and lung metastases, TAE and tumor weight and TAE and CCL11 tumor expression (Simple linear regression).

C: Tumor necrosis of two independent experiments (left, one-way ANOVA) and correlation between TAE and tumor necrosis (right, simple linear regression).

D: Percentage of TANs and SiglecF$^+$ TANs (One-way ANOVA).

Ctrl: Control. CSF2: Colony-stimulating factor 2. i.p.: Intraperitoneal. TAE: Tumor associated eosinophils. TAN: Tumor associated neutrophil.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p≤0.0001 = ****.

Strong early onset tumor associated eosinophilia decreased primary tumor weight but does not change metastases

Late onset eosinophilia did not strongly affect tumor progression in my 4T1 mouse model of cancer even with the addition of AB5-CSF2 as an activator. This model is highly aggressive and lung metastases occur early on, so I decided to induce eosinophilia early on during tumor development. I added activators to the system to try and activate TAEs *in situ*, with the hope of causing them to display an anti-tumorigenic phenotype. The additional activators I decided to use were immunoglobulin-E (IgE) and interferon-γ (IFNγ). Eosinophils have been shown to express both low and high affinity Fc-epsilon receptor (FcεR) as well as IFNγ-receptor (IFNγ-R)[603].

To achieve FcεR clustering, I engineered an anti-CD90.1-IgE antibody which would specifically target the tumor cells made to express CD90.1 on their surface. Binding of several anti-CD90.1-IgE antibodies to cancer cells would lead to clustering of FcεR on immune cells. IFNγ has been shown to activate eosinophils and make them strongly pro-inflammatory. By linking the cytokine to the AB5 ScFv (AB5-IFNγ) I wanted to cause local accumulation within tumors.

I injected CCL11/CD90.1 expressing 4T1 cells orthotopically into wild type (WT) Balb/c mice. Of note, no control cells were injected. At day 3, PBS, AB5-IL5, a-CD90.1-IgE, AB5-IFNγ and the combination of the latter three were administered systemically. Tumors and lungs were collected, immune infiltration, CCL11 and CD90.1 *ex vivo* expression, tumor weight and lung macrometastases number and size were analyzed (Fig21 A).

Analysis of TAEs showed that AB5-IL5 treatment increased tumor eosinophilia compared to PBS treatment, whereas anti-CD90.1-Ige or AB5-IFNγ treatment did so only in combination with AB5-IL5 (Fig21 B). This indicated that even in the presence of local activators, AB5-IL5 is required to increase circulating eosinophils which could then infiltrate the tumor. Highly eosinophilic tumors contained over 60% of eosinophils, a phenotype I did not observe in any of the consequent experiments.

TAN analysis showed that there was a significant decrease upon the combination of all three treatments compared to aCD90.1-IgE treatment (Fig21 C, left panel). As before, I observed a population of SiglecF$^+$ TANs with high granularity (Fig21 C, right panel and data not shown). CCL11 *ex vivo* expression was similar in all tumors as expected (Fig21 D, right panel).

Analysis of CD90.1 expression showed that almost 90% of cells expressed CD90.1 before injection, as measured by binding of the injected anti-CD90.1-IgE antibody compared to control transfected cells (Fig21 E, red histogram vs blue histogram), but almost no cancer cells expressed CD90.1 at the end of the experiment as measured by a commercial anti-CD90.1 antibody (Fig21 F, left panel). The few remaining cells expressing CD90.1 showed strongly decreased expression as compared to a control stain (Fig21 F, histogram). It seems that during *in vivo* tumor progression, CD90.1 was either downregulated or CD90.1$^+$ 4T1 cancer cells were outcompeted by CD90.1$^-$ cells. FcεR expression was also measured in eosinophils *ex vivo*. Due to technical problems, none of the CCL11 AB5-IL5 tumors could be acquired. Only a very small number (on the average 1.6% in all groups) of eosinophils expressed FcεR (data not shown), implying that this method of activation is not suited.

CCL11 AB5-IL5 and CCL11 AB5-IL5+aCD90.1-IgE+AB5-IFNy treatment led to decreased tumor weight compared to PBS treatment whereas single treatments had no effect (Fig21 G, left panel). Neither metastatic seeding nor metastatic growth, as determined by lung metastasis size, were affected (Fig 21 G middle and right panel)

In summary, CD90.1 expression in 4T1 tumors was not stable *in vivo* and only few eosinophils express the FcεR. Due to that, activation of eosinophils *in situ* through FcεR clustering is unlikely to succeed in this model. Early onset AB5-IL5 treatment with and without additional activators significantly decreased primary tumor weight but did not change metastatic spread and growth, indicating that eosinophils do not have an effect on overall tumor progression.

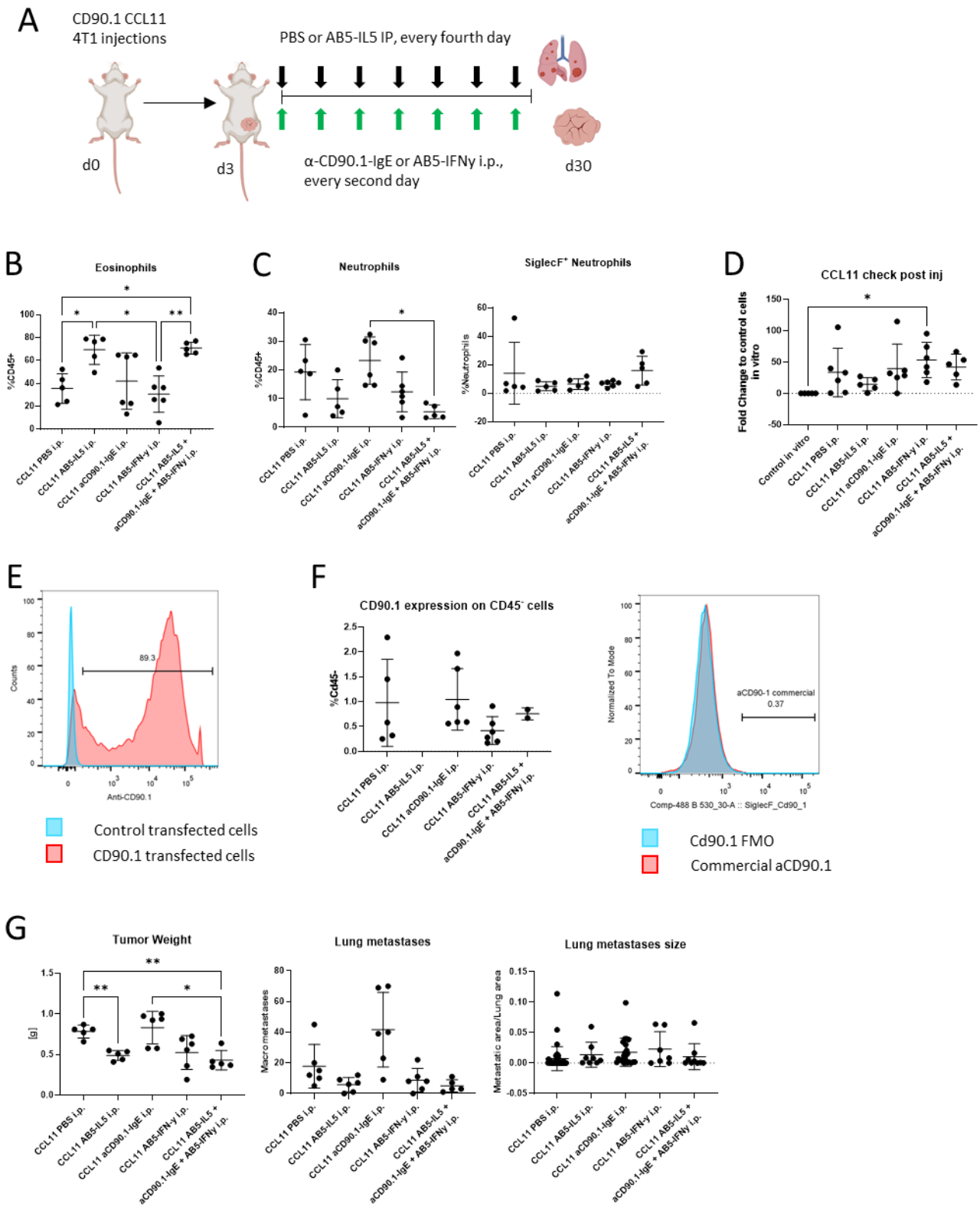

A
CD90.1 CCL11
4T1 injections
PBS or AB5-IL5 IP, every fourth day
d0
d3
α-CD90.1-IgE or AB5-IFNγ i.p.,
every second day
d30
B
Eosinophils
%CD45+
C
Neutrophils
%CD45+
SiglecF+ Neutrophils
%Neutrophils
D
CCL11 check post inj
Fold Change to control cells in vitro
Control in vitro
CCL11 PBS i.p.
CCL11 AB5-IL5 i.p.
CCL11 aCD90.1-IgE i.p.
CCL11 AB5-IFNγ i.p.
CCL11 AB5-IL5 + aCD90.1-IgE + AB5-IFNγ i.p.
E
Counts
89.3
Anti-CD90.1
Control transfected cells
CD90.1 transfected cells
F
CD90.1 expression on CD45- cells
%Cd45-
Normalized To Mode
aCD90-1 commercial
0.37
Comp-488 B 530_30-A :: SiglecF_Cd90_1
Cd90.1 FMO
Commercial aCD90.1
G
Tumor Weight
[g]
Lung metastases
Macro metastases
Lung metastases size
Metastatic area/Lung area

Figure21: Strong early onset tumor eosinophilia decreases tumor weight.

WT Balb/c mice were injected orthotopically with CD90.1 CCL11 expressing tumor cells. PBS, 50ug AB5-IL5, 10 ug aCD90.1-IgE, 100ug AB5-IFNy or a combination of the last three were administered i.p. every fourth day. Immune cells were analyzed by flow cytometry. Tumors were weighted and analyzed by qPCR for CCL11 expression and CD90.1 expression by flow cytometry. RPL19 was chosen as housekeeping gene. Lung metastases were counted and lung metastasis size was determined on sections through histology.

A: Experimental workflow.

B: Analysis of TAE tumor infiltration by flow cytometry. Eosinophils were defined as $Cd45^{+}CD11b^{+}Ly6G^{neg-med}SiglecF^{+}SSC^{high}$ (Brown-Forsythe one-way Welch ANOVA).

C: Analysis of TAN tumor infiltration by flow cytometry. Neutrophils were defined as $Cd45^{+}CD11b^{+}Ly6G^{high}$ (Brown-Forsythe one-way Welch ANOVA). $SiglecF^{+}$ TANs were enumerated (One-way ANOVA).

D: CCL11 expression of tumors was determined *ex vivo* via qPCR (One-way ANOVA).

E: Flow cytometry analysis of CD90.1 surface expression on CD90.1 transfected 4T1 cells prior to injection (One-way ANOVA). CD90.1 expression was assessed using the injected anti-CD90.1-IgE antibody.

F: CD90.1 expression on tumor cells measured by a commercial antibody was measured *ex vivo* using flow cytometry (left, Brown-Forsythe one-way Welch ANOVA). A representative histogram for CD90.1 expression is shown (right).

G: Tumor weights (One-way ANOVA), lung macrometastases (Brown-Forsythe one-way Welch ANOVA) and metastatic size normalized to lung area (One-way ANOVA). For tumor weights and lung metastasis, each dot represents one mouse. For metastatic size, each dot represents one lung metastasis.

i.p.: Intraperitoneally. TAEs: Tumor associated eosinophils. TANs: Tumor associated neutrophils.

Significant changes are denoted by *. $p \leq 0.5$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****

Moderate tumor associated eosinophilia does not affect tumor progression in the presence of IL33

I wanted to know if strong eosinophilia could be induced not only in CCL11 expressing tumors but also in control tumors because in a clinical setting, not all patients would have tumors expressing high CCL11. Additionally, I wanted to know if treatment with lower amounts of AB5-IL5 would have the same effect as with higher amounts which would translate into lowering cost. Finally, I added IL33 as an additional activator as this cytokine has been shown to activate eosinophils both directly and indirectly[324].

Wild type (WT) Balb/c mice were injected with control or CCL11 cancer cells additionally expressing a control plasmid or IL33 (hereto forth referred to as IL33 or CCL11/IL33 tumors respectively) and PBS or 50ng of AB5-IL5 was administered systemically starting at day3 (Fig22 A).

Only CCL11/IL33 AB5-IL5 tumors had significantly more TAEs compared to all control tumor groups (Fig22 B, left panel). There was no change in TAE numbers between CCL11 tumors, regardless of additional

treatment. Although SiglecF expression varied between groups (Fig22 B, middle panel), there was no change in TAE granularity as measured by side scatter (Fig22 B, right panel).

Tumor associated neutrophils (TANs) significantly decreased in CCL11/IL33 AB5-IL5 tumors compared to control PBS tumors and IL33 AB5-IL5 tumors (Fig22 C, left panel). SiglecF^{+} TANs increased in most of the CCL11 tumors compared to their control tumor counterparts (Fig22 C, middle panel) and their expression of SiglecF varied between the treatments (Fig22 C, right panel)

There was no change in tumor weight, lung or liver metastases (Fig22 D, left, middle and right panel, respectively). Metastases in other organs such as the heart and mammary glands were not observed.

To summarize, strong eosinophilia could not be induced in control or IL33 tumors and lower amounts of AB5-IL5 induced moderate eosinophilia in CCL11/IL33 tumors only. Of note, I did not achieve the same strong eosinophilia in tumors expressing CCL11 as in the previous setting. Tumors with the highest numbers of TAE displayed decreased neutrophilia and I again observed variable amounts of SiglecF^{+} TANs in all groups.

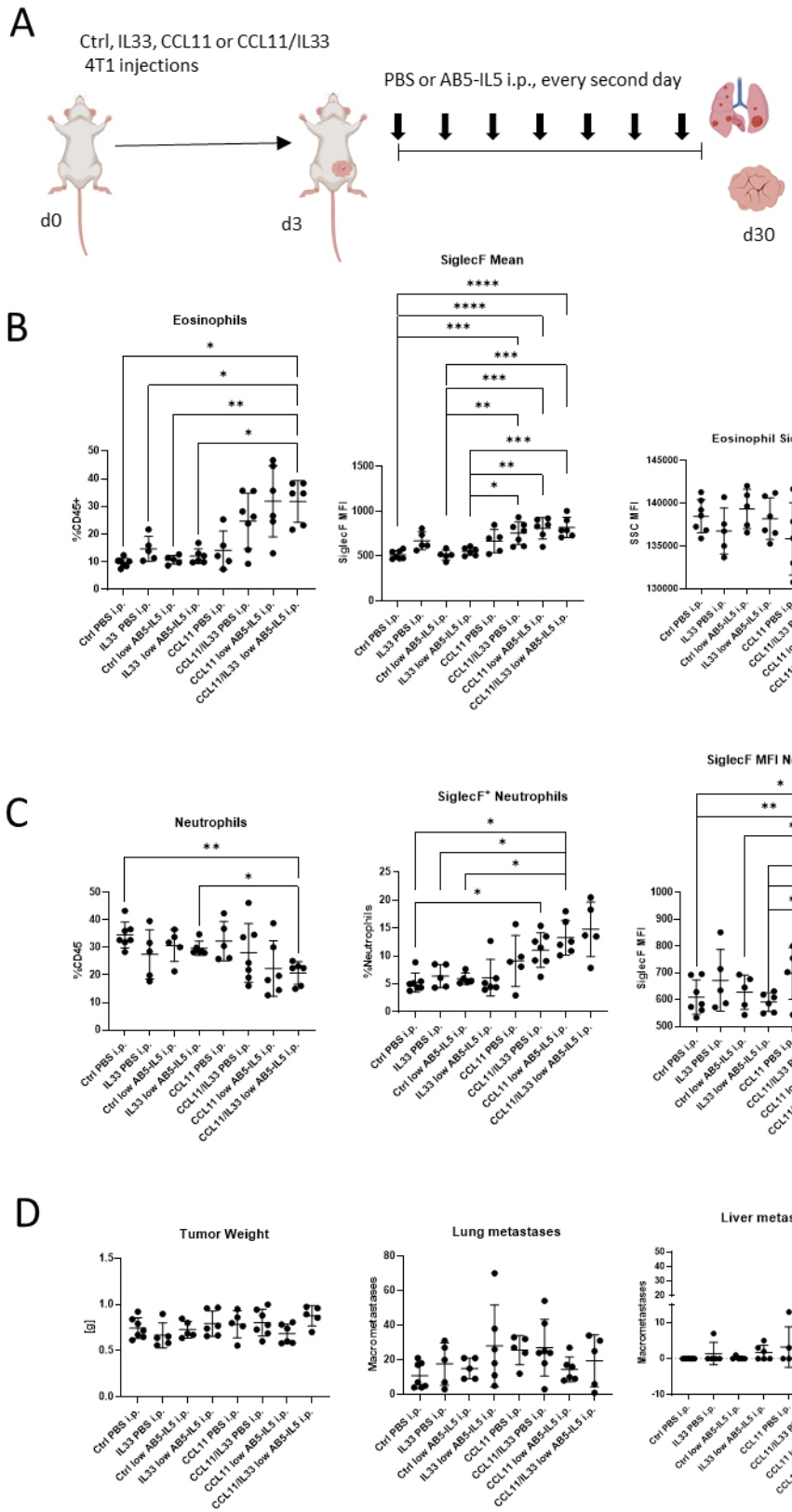

A
Ctrl, IL33, CCL11 or CCL11/IL33
4T1 injections
PBS or AB5-IL5 i.p., every second day
d0
d3
d30
B
Eosinophils
%CD45+
SiglecF Mean
SiglecF MFI
Eosinophil Side Scatter
SSC MFI
C
Neutrophils
%CD45
SiglecF+ Neutrophils
%Neutrophils
SiglecF MFI Neutrophils
SiglecF MFI
D
Tumor Weight
[g]
Lung metastases
Macrometastases
Liver metastases
Macrometastases
Ctrl PBS i.p.
IL33 PBS i.p.
Ctrl low AB5-IL5 i.p.
IL33 low AB5-IL5 i.p.
CCL11 PBS i.p.
CCL11/IL33 PBS i.p.
CCL11 low AB5-IL5 i.p.
CCL11/IL33 low AB5-IL5 i.p.

Figure22: CCL11 and IL33 do not affect tumor progression or tumor eosinophilia.

WT Balb/c mice were injected with ctrl, IL33, CCL11 or CCL11/IL33 4T1 tumor cells orthotopically and treated with PBS or 50ng AB5-IL5 i.p. every second day. Immune cells were analyzed by flow cytometry. Tumors were weighted. Macrometastases in the lungs, livers and other organs were counted.

A: Experimental workflow.

B: Analysis of TAE tumor infiltration by flow cytometry. Eosinophils were defined as $Cd45^{+}CD11b^{+}Ly6G^{neg-med}SiglecF^{+}SSC^{high}$. (Brown-Forsythe one-way Welch ANOVA). SiglecF MFI and SSC MFI were measured (One-way ANOVA).

C: Analysis of TAN tumor infiltration by flow cytometry. Neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$ (One-way ANOVA). $SiglecF^{+}$ TANs were enumerated (One-way ANOVA). SiglecF MFI was measured in $SiglecF^{+}$ TANs (One-way ANOVA).

D: Tumor weights, lung macrometastases (One-way ANOVA) and liver macrometastases (Kruskal-Wallis non-parametric test).

WT: Wild type. i.p.: Intraperitoneal. Ctrl: Control. TAEs: Tumor associated eosinophils. TANs: Tumor associated neutrophils. MFI: Mean fluorescent intensity. SSC: Side Scatter.

Significant changes are denoted by *. $p \leq 0.5$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p < 0.0001$ = ****.

Lung eosinophilia does not decrease metastatic spread but can influence the lung immune environment.

My results so far indicated that breast cancer associated eosinophilia had no effect on primary tumor growth nor spontaneous metastatic seeding in the 4T1 breast cancer model, although several papers showed that blood and tissue eosinophilia is beneficial in breast cancer patients (see introduction). I wanted to know if established lung eosinophilia can affect metastatic seeding of 4T1 breast cancer cells.

I pre-treated wild type (WT) Balb/c mice intraperitoneally (i.p.) with PBS or AB5-IL5 to induce systemic eosinophilia, and by extension lung eosinophilia, and injected them intravenously (i.v.) with WT 4T1 cells. PBS or AB5-IL5 treatment was stopped after cancer cell injection. I analyzed the lungs via flow cytometry before cancer cells injection ("pre-Tumor") and 3 weeks after cancer cell injection ("post-Tumor"). In the post-tumor setting, lung macrometastases were counted (Fig23 A).

There was no significant change in lung metastases between the PBS and AB5-IL5 pretreated group (Fig23 B). Before cancer cell injection there was a significant increase in lung eosinophils in AB5-IL5 pre-treated mice compared to PBS pre-treated mice which increased even more after tumor injection (Fig23 C, compare first two columns to last two columns). This phenomenon, namely that i.v. injected breast cancer increased lung eosinophilia without additional AB5-IL5 treatment, has been shown by other researchers as well[408]. I should point out that PBS treated lungs did had no increase in eosinophilia upon 4T1 cancer cell i.v. injections. Lung eosinophils in healthy, AB5-IL5 treated mice displayed a high SiglecF expression level and low granularity compared to PBS treated mice (Fig23 C, middle and right panel, respectively), indicating that

they were most likely degranulated. SiglecF expression of lung eosinophils in metastasis bearing mice decreased after tumor injection and so did their granularity (Fig23 C, middle and right panel, respectively). This implies that either the tumor cells have an inhibitory effect on eosinophil activation in the lung, that tumor cells induce eosinophil degranulation, that AB5-IL5 directly or indirectly activates eosinophils in the lungs or that there is a combination of all three. It should be noted that almost all lung eosinophils expressed medium to high levels of F4/80, a phenomenon known to occur in the lung[404,604] (data not shown).

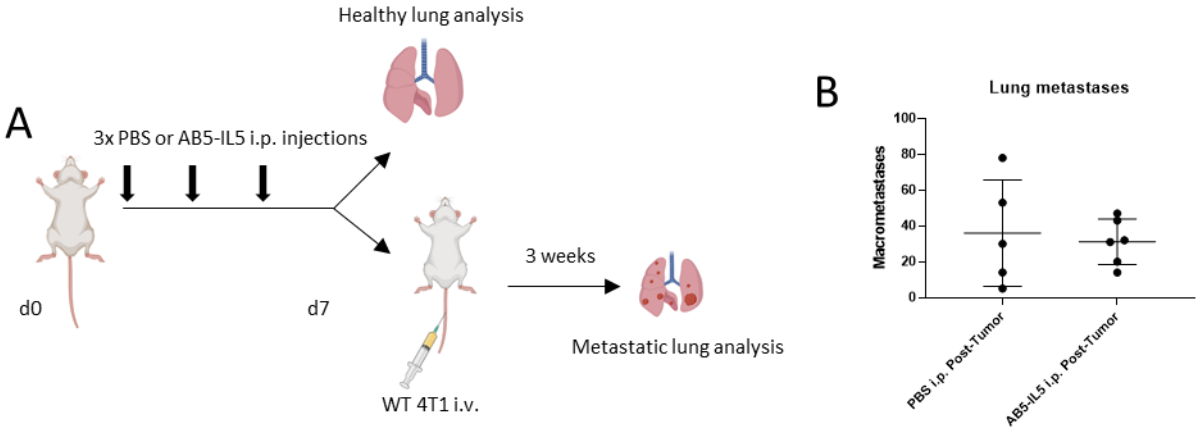
Recently, two subsets of eosinophils were discovered in the lungs of mice, induced and resident eosinophils (iEos and rEos respectively). iEos were characterized by their high expression of CD62L and increased infiltration and activation upon allergic stimulation, whereas rEos showed no CD62L expression, no change in recruitment and no activation upon stimulation.[404] I analyzed these subpopulations in healthy and metastatic lungs and saw that AB5-IL5 treatment significantly increased iEos and reduced rEos in healthy but not metastatic lungs (Fig23 D).

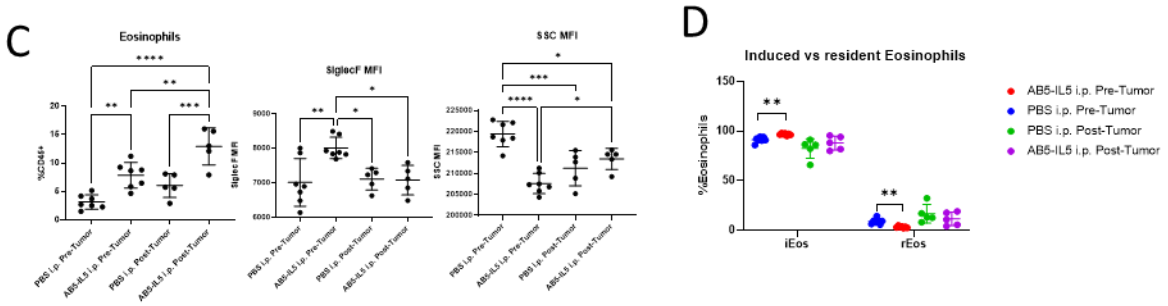
Neutrophils increased in PBS treated metastatic lungs compared to both treatment groups in tumor free lungs (Fig23 E, left panel). Their SiglecF$^+$ subset and its SiglecF expression were strongly increased in AB5-IL5 pre-treated healthy lungs but almost completely disappeared after metastatic seeding (Fig23 E, middle and left panel).

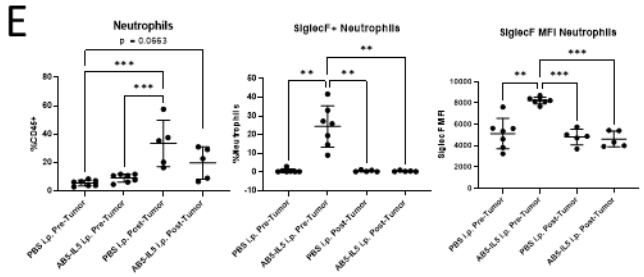
I also analyzed lung infiltration of dendritic cells (DCs) as well as their activation status. Overall and CD40$^+$ DCs were not changed in healthy or metastatic lungs, regardless of AB5-IL5 treatment (Fig23 F). I.v. cancer cell injection caused significant loss of MHCII^{high} DCs in favor of MHCII^{med}, MHCII^{low} and MHCII^{neg} DCs (Fig23 G, left panel).

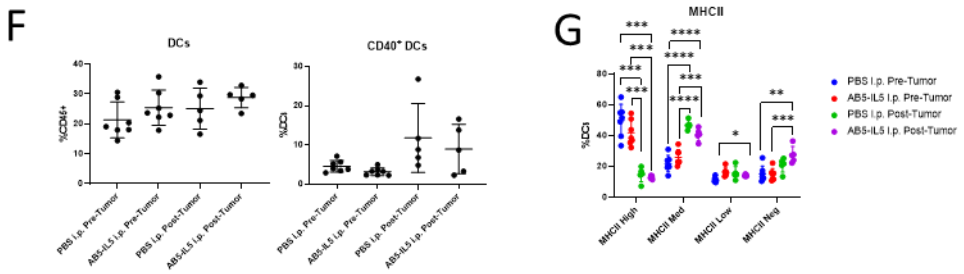
Lastly, I analyzed the effects lung eosinophilia and metastases had on lung T cells. There was no change in CD8 T cells although there was a trend for decrease upon tumor injection (Fig23 H, left panel). PD1$^+$ CD8 T cells accumulated strongly in tumor bearing lungs compared to healthy lungs when the mice were pre-treated with PBS but not AB5-IL5 (Fig23 H, right panel). Eosinophils are known to interact with CD4 T cells in the lungs, especially during airway inflammation (see introduction). Metastatic lungs had increased CD4 T cell infiltration compared to healthy lungs in both PBS and AB5-IL5 treatment settings (Fig23 I). There was no change in PD1$^+$ CD4 T cells (data not shown) but I observed a significant decrease in Tregs in metastatic lungs and healthy control lungs compared to healthy AB5-IL5 treated lungs (Fig23 I), indicating an anti-inflammatory role of eosinophils in tumor free mice.

In summary, AB5-IL5 pre-treatment significantly increased lung eosinophils but this established lung eosinophilia prior to i.v. cancer cell injection did not affect metastatic spread to the lungs. There was an increase in induced eosinophils and a decrease in resident eosinophils upon AB5-IL5 treatment in healthy lungs and a similar trend was seen in metastatic lungs. AB5-IL5 pre-treatment increased Tregs in healthy but not in tumor bearing lungs. Overall, lung associated eosinophils significantly changed the immune environment in the lung but these changes did not affect metastatic spread.


A
Healthy lung analysis
3x PBS or AB5-IL5 i.p. injections
d0
d7
WT 4T1 i.v.
3 weeks
Metastatic lung analysis
B
Lung metastases
Macrometastases
PBS i.p. Post-Tumor
AB5-IL5 i.p. Post-Tumor


C
Eosinophils
SiglecF MFI
SSC MFI
D
Induced vs resident Eosinophils
%Eosinophils
iEos
rEos
AB5-IL5 i.p. Pre-Tumor
PBS i.p. Pre-Tumor
PBS i.p. Post-Tumor
AB5-IL5 i.p. Post-Tumor


E
Neutrophils
SiglecF+ Neutrophils
SiglecF MFI Neutrophils


F
DCs
CD40+ DCs
G
MHCII
MHCII High
MHCII Med
MHCII Low
MHCII Neg
PBS i.p. Pre-Tumor
AB5-IL5 i.p. Pre-Tumor
PBS i.p. Post-Tumor
AB5-IL5 i.p. Post-Tumor

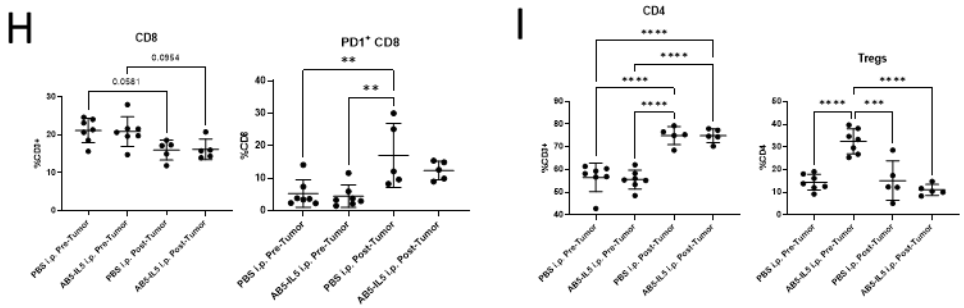

H
CD8
PD1+ CD8
I
CD4
Tregs

Figure23: Lung eosinophilia does not alter metastatic spread but can influence the lung immune environment.

WT Balb/c mice were injected with PBS or 50ug AB5-IL5 i.p. on three consecutive days. WT 4T1 cells were injected i.v. and treatment was stopped. Healthy and metastatic lungs were analyzed by flow cytometry.

A: Experimental workflow.

B: Lung macrometastases (Unpaired two-tailed t-test).

C: Analysis of TAE lung infiltration by flow cytometry. Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6g^{neg\text{-}med}SiglecF^{+}SSC^{high}$ (One-way ANOVA). SiglecF MFI and SSC MFI of TAEs were analyzed (One-way ANOVA).

D: iEos and rEos (two-way ANOVA) were measured in the lungs. iEos were defined as $CD45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}SSC^{high}$ $CD62L^{neg}$ and rEos were defined as $CD45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}SSC^{high}$ $CD62L^{+}$.

E: Analysis of TAN lung infiltration by flow cytometry. Neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$ (Brown-Forsythe one-way Welch ANOVA). $SiglecF^{+}$ TANs were enumerated (Brown-Forsythe one-way Welch ANOVA). SiglecF MFI was measured in $SiglecF^{+}$ TANs (One-way ANOVA).

F: Analysis of DC lung infiltration by flow cytometry. DCs were defined as $CD45^{+}CD11c^{+}$ (Brown-Forsythe one-way Welch ANOVA). The expression of CD40 (Brown-Forsythe one-way Welch ANOVA) was measured on DCs.

G: MHCII expression on DCs was analyzed (Two-way ANOVA).

H: Analysis of CD8 T cell lung infiltration by flow cytometry. CD8 T cells were defined as $CD45^{+}CD3^{+}CD8^{+}$ (One-way ANOVA). PD1 expression on CD8 TILs was measured (One-way ANOVA).

I: Analysis of CD4 T cell lung infiltration by flow cytometry. CD4 T cells are defined as and $CD45^{+}CD3^{+}CD4^{+}$ (One-way ANOVA). Tregs were defined as $CD45^{+}CD3^{+}CD4^{+}CD25^{+}FoxP3^{+}$ (One-way ANOVA).

WT: Wild type. i.p.: Intraperitoneal. i.v.: intravenously. TAEs: Tumor associated eosinophils. iEos: Induced eosinophils. rEos: Resident eosinophils. TANs: Tumor associated neutrophils. MFI: Mean fluorescent intensity. SSC: Side scatter. DCs: Dendritic cells.

Significant changes are denoted by *. $p \leq 0.5$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p < 0.0001$ = ****.

Eosinophilia in E0771 tumors does not affect primary tumor growth but tends to increase lung metastases.

The results I obtained in the 4T1 mouse model of breast cancer indicated that eosinophils had no effect on tumor progression in this model. It is possible that this was due to either the cell line or the mouse strain. I decided to investigate the role of eosinophils in breast cancer in another mouse strain, namely C57BL/6J. I injected WT C57BL/6J mice with CCL11 overexpressing or control transfected E0771 cancer cells orthotopically. Tumors were palpable at d7 and PBS or AB5-IL5 was administered systemically every fourth day until d30, at which time mice were sacrificed. Tumors were weighted, homogenized and analyzed for immune infiltrating leukocytes via flow cytometry, CCL11 *ex vivo* tumor expression was measured via qPCR and lung macrometastases were counted (Fig24 A). Lung metastases counts were pooled from two independent experiments which are color coded.

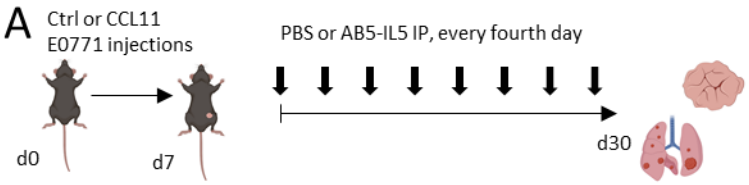
Tumor associated eosinophil (TAE) numbers significantly increased in CCL11 AB5-IL5 tumors compared to control PBS and CCL11 PBS tumors (Fig24 B, left panel). SiglecF expression was significantly increased in control AB5-IL5 and CCL11 AB5-IL5 tumors compared to control PBS tumors (Fig24 B, middle-left panel). Granularity showed the opposite, namely a decrease in control AB5-IL5 tumor and CCL11 AB5-IL5 tumors compared to control PBS tumors (Fig24 B, middle-right panel) indicating that the TAEs were activated and had degranulated. This hypothesis was strengthened when I observed a strong negative correlation between SiglecF expression and granularity in TAEs across the groups (Fig24B, right panel).

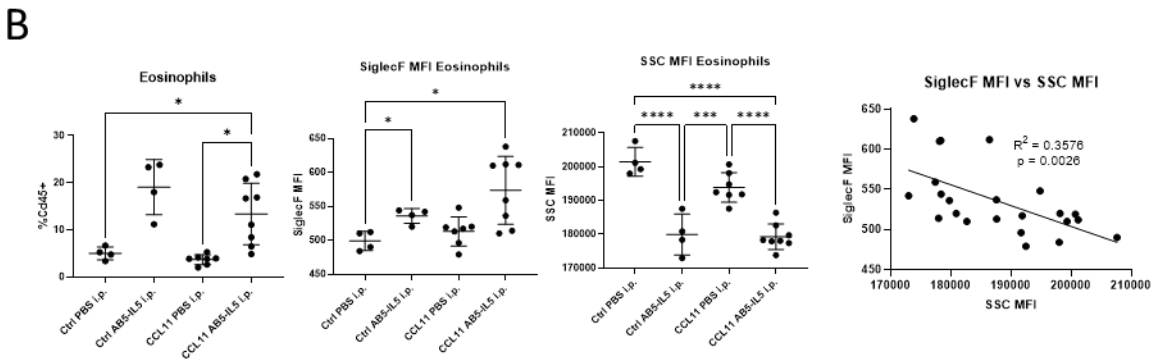
CCL11 expression was low in control tumors but significantly increased in CCL11 expressing tumors (Fig24 C, left panel). Primary tumor weights did not differ between the groups and lung macrometastases were observed (Fig24 C, middle and right panel).

To see if TAEs might affect other immune populations, tumor associated neutrophils (TANs) and tumor associated myeloid suppressor cells (MDSCs) were analyzed. MDSCs significantly decreased in control AB5-IL5 tumors and CCL11 AB5-IL5 tumors compared to control PBS tumors (Fig24 D, left panel). There was no change in TAN accumulation between the groups (Fig24 D, middle-left panel). In all groups, there were few TANs expressing SiglecF and the expression levels did not change with the varying treatments (Fig24 D, middle-right and right panel).

Finally, lung metastases had an almost significant positive association with TAEs and a negative correlation with CCL11 tumor expression (Fig24 E, left and middle-left panel). There was no correlation between lung metastases and SiglecF or SSC MFI (Fig24 E, middle-right and right panel) indicating that in this model, the activation status of eosinophils does not affect lung metastases

In conclusion, eosinophils accumulated in CCL11 expressing E0771 tumors upon AB5-IL5 treatment and showed increased activation upon AB5-IL5 treatment, regardless of tumor CCL11 expression. CD69 cannot be reliably used as an activation marker for TAEs as its expression did not correlate with SiglecF expression or granularity (data not shown). TAEs did not affect primary tumor growth and tended to increase lung metastases in the E0771 mouse model of breast cancer.


A
Ctrl or CCL11
E0771 injections
d0
d7
PBS or AB5-IL5 IP, every fourth day
d30


B
Eosinophils
%Cd45+
SiglecF MFI Eosinophils
SiglecF MFI
SSC MFI Eosinophils
SSC MFI
SiglecF MFI vs SSC MFI
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p = 0.0026
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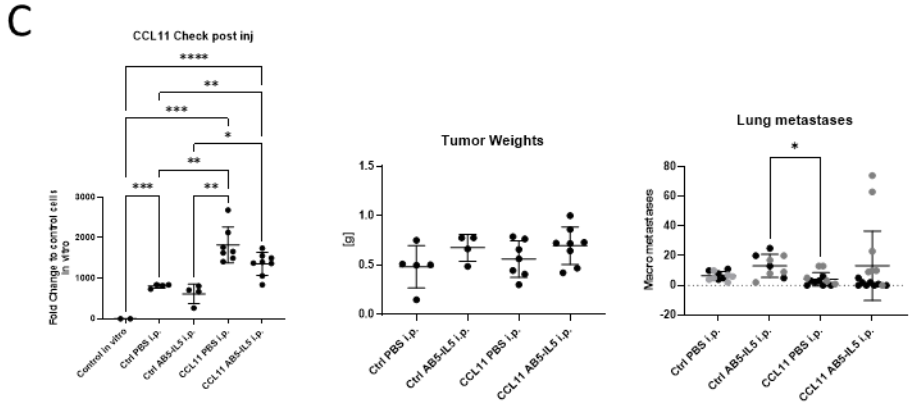
Ctrl AB5-IL5 i.p.
CCL11 PBS i.p.
CCL11 AB5-IL5 i.p.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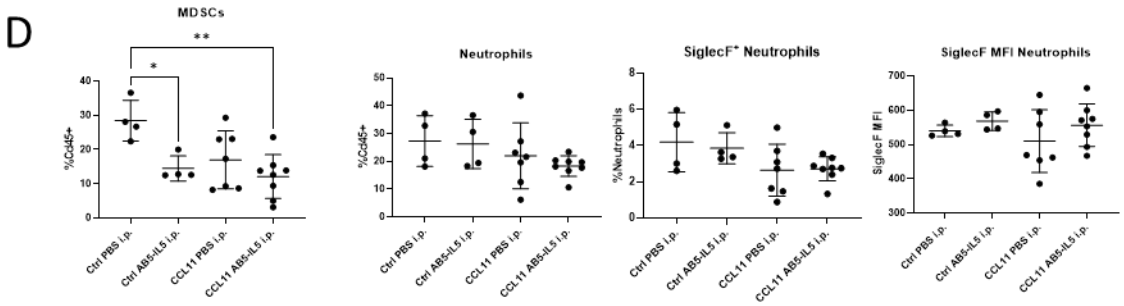
CCL11 Check post inj
Fold Change to control cells in vitro
Control in vitro
Tumor Weights
[g]
Lung metastases
Macro metastases


D
MDSCs
%Cd45+
Neutrophils
SiglecF+ Neutrophils
%Neutrophils
SiglecF MFI Neutrophils
SiglecF MFI

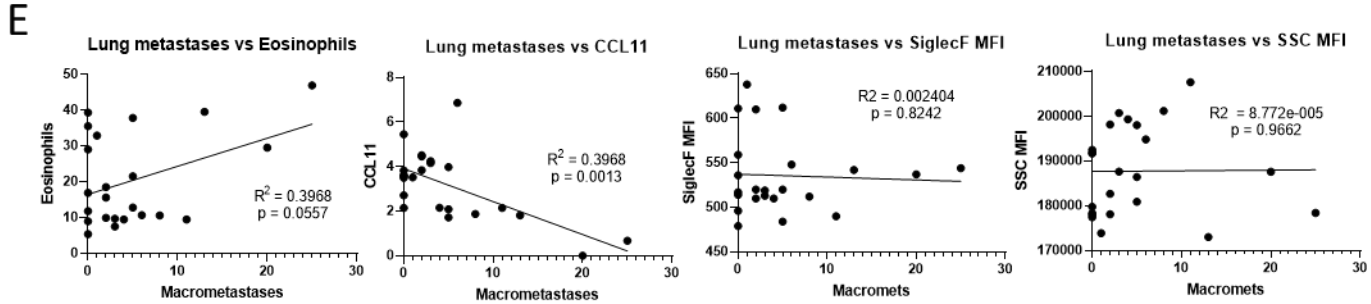

E
Lung metastases vs Eosinophils
Eosinophils
R2 = 0.3968
p = 0.0557
Macrometastases
Lung metastases vs CCL11
CCL11
R2 = 0.3968
p = 0.0013
Macrometastases
Lung metastases vs SiglecF MFI
SiglecF MFI
R2 = 0.002404
p = 0.8242
Macromets
Lung metastases vs SSC MFI
SSC MFI
R2 = 8.772e-005
p = 0.9662
Macromets

Figure24: Eosinophilia in E0771 tumors does not affect primary tumor growth.

WT C57BL/6J mice were injected with control or CCL11 E0771 cells orthotopically and treated with PBS or 50ug AB5-IL5 every fourth day. Immune cells were analyzed by flow cytometry. Tumors were weighted and macrometastases in the lungs were counted. Correlations between several parameters were calculated, R2 and p-values are on the graphs

A: Experimental workflow.

B: Analysis of TAE tumor infiltration by flow cytometry. Eosinophils were defined as $CD45^{+}Cd11b^{+}Ly6G^{neg-med}SiglecF^{+}SSC^{high}$ (Brown-Forsythe one-way Welch ANOVA). Analysis of SiglecF expression (Brown-Forsythe one-way Welch ANOVA) and granularity (One-way ANOVA) on TAEs. Correlation between SiglecF expression and granularity of TAEs (Simple linear regression).

C: *Ex vivo* CCL11 expression of tumors measured by qPCR, tumor weights and lung macrometastases (One-way ANOVA). Results for lung metastases were pooled from two independent experiments, Experiment1: black. Experiment2: grey.

D: Analysis of MDSC and TAN tumor infiltration by flow cytometry. MDSCs were defined as $CD45^{+}CD11b^{+}SiglecF^{-}SSC^{low}Ly6G^{low}Ly6C^{high}$ (One-way ANOVA). Neutrophils were defined as $Cd45^{+}CD11b^{+}Ly6G^{high}$ (One-way ANOVA). $SiglecF^{+}$ TANs were enumerated and SiglecF expression was measured (One-way ANOVA).

E: Correlation between Lung metastases and TAEs, tumor CCL11 expression, TAE SiglecF expression and TAE granularity (Simple linear regression).

i.p.: Intraperitoneal. Ctrl: Control. MFI: Mean Fluorescent intensity. SSC: Side scatter. MDSCs: Myeloid derived suppressor cells. TAEs: Tumor associated eosinophils. TANs: Tumor associated neutrophils.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

AB5-IL5 is sufficient to induce tumor eosinophilia in a MMTV-PyMT mouse model of breast cancer

As I did not see a strong effect of TAEs on tumor progression in the E0771 breast cancer model, I wanted to know if this lack of impact was tumor model rather than strain dependent. Wild type (WT) C57BL/6J mice were injected with primary MMTV-PyMT cancer cells, which did not overexpress CCL11, orthotopically. Once tumors were palpable at day7, AB5-IL5 was administered i.p. for 25 days at which point the tumors were removed and analyzed by flow cytometry. After the tumorectomies I ceased treatment and lungs were analyzed for macrometastases (Fig25 A).

There was no significant increase in primary tumor weight (Fig25 B). No lung macrometastases were seen, even 67 days after primary tumor injection, although the primary tumors injected had spontaneously metastasized within the donor mice (data not shown).

AB5-IL5 treatment significantly increased and activated TAEs compared to PBS treatment as was seen by increased SiglecF expression and decreased granularity (Fig25 C, from left to right). AB5-IL5 injections did not affect TANs and there was a small sub-population of highly granular, $SiglecF^{+}$ neutrophils (Fig25 D left and middle panel) although their SiglecF expression levels were lower than those of TAEs (Fig25 D, right

panel, compare y-axes with C, SiglecF MFI Eosinophils). Macrophage infiltration into the tumor did not change upon AB5-IL5 treatment (Fig25 E).

The MMTV-PyMT mouse model is defined as an immune cell infiltrated breast cancer model, it is especially enriched with tumor infiltrating lymphocytes (TILs) compared to other mouse models used, such as the 4T1 mouse model (data not shown). To see if TAEs can affect TILs, CD8 and CD4 T cells were analyzed. There was no change in overall CD8 and CD4 T cells (Fig25 F) nor were their memory and effector subsets affected by the presence of tumor eosinophils (data not shown).

Taken together, these results showed that AB5-IL5 can increase TAE numbers in an orthotopically injected primary MMTV-PyMT mouse models without CCL11 expression. AB5-IL5 administration caused increased activation of TAEs but there was no effect on other myeloid populations analyzed. Finally, TILs were not affected by eosinophils. Because the tumors did not metastasize, I was not able to make any conclusions about how eosinophils might affect metastatic seeding.

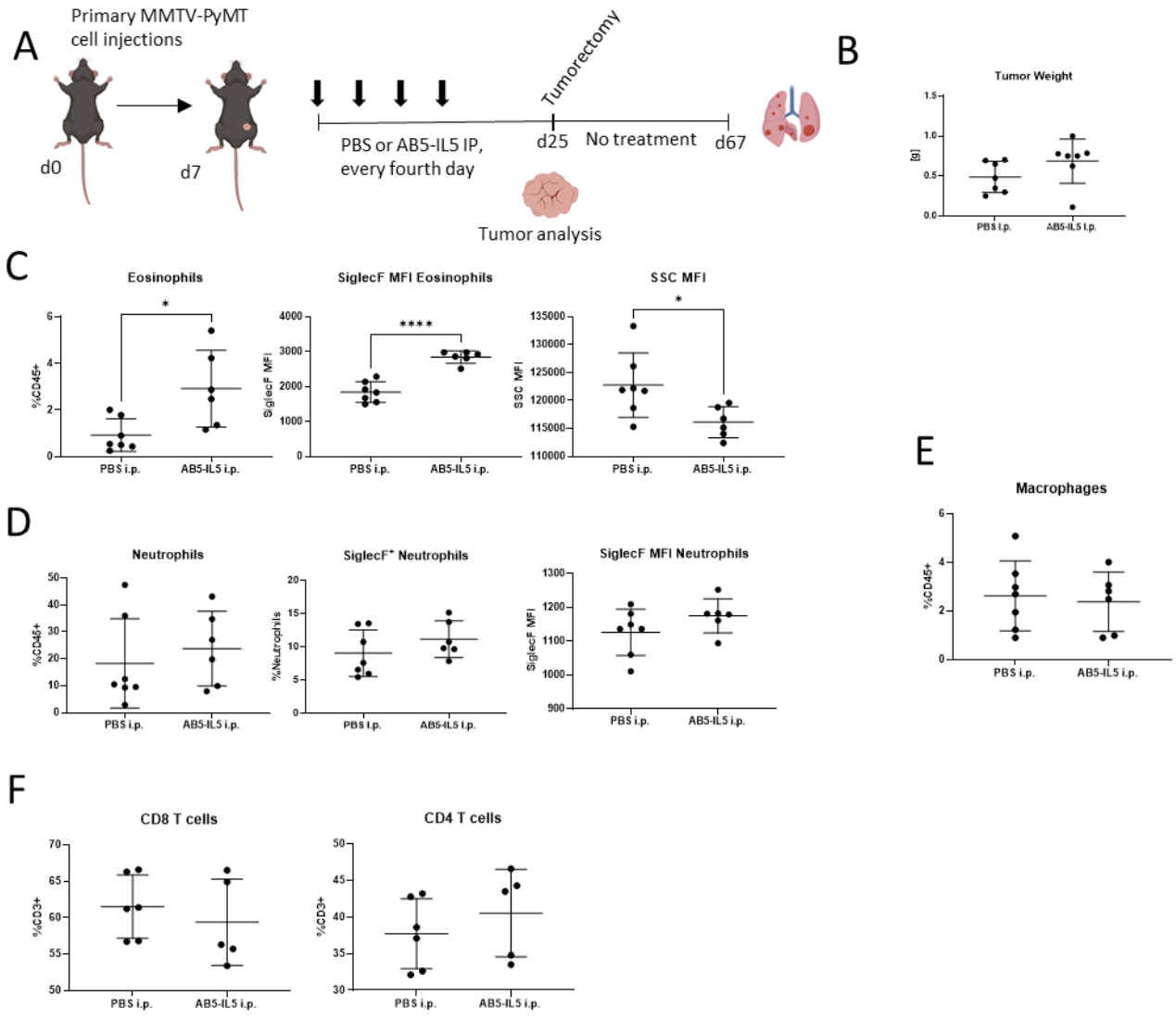


Figure25: Eosinophilia does not affect primary tumor growth in a MMTV-PyMT mouse model of breast cancer.

WT C57BL/6J mice were injected with primary MMTV-PyMT cell orthotopically and tumors were treated every fourth day with PBS or 50ug AB5-IL5. Tumors were removed via tumorectomy and analyzed via flow cytometry. Lungs were analyzed for macrometastases.

A: Experimental workflow.

B: Primary tumor weights at tumorectomy (Unpaired two-tailed t-test). There were no lung macrometastases.

C: Analysis of TAE tumor infiltration by flow cytometry. Eosinophils were defined as $CD45^{+}CD11b^{+Ly6Gneg\text{-}med}SiglecF^{+}SSC^{high}$ (Unpaired two-tailed t-test). SiglecF expression and granularity of TAEs were assessed (Unpaired two-tailed t-test).

D: Analysis of TAN tumor infiltration by flow cytometry. Neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$ (Unpaired two-tailed t-test). $SiglecF^{+}$ TANs were enumerated and SiglecF expression was measured (Unpaired two-tailed t-test).

E: Analysis of TAM tumor infiltration by flow cytometry. Macrophages were defined as $CD45^{+}CD11b^{+}SiglecF^{-}F4/80^{+}$ (Unpaired two-tailed t-test).

F: Analysis of TIL tumor infiltration by flow cytometry. CD8 (left) and CD4 (right) T cells are defined as $CD45^{+}CD3^{+}CD8^{+}$ $CD45^{+}CD3^{+}CD4^{+}$ respectively (Unpaired two-tailed t-test).

i.p.: Intraperitoneal. TAEs: Tumor associated eosinophils. TANs: Tumor associated neutrophils. TILs: Tumor infiltrating lymphocytes. MFI: Mean fluorescence intensity. SSC: Side scatter.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

Tumor associated eosinophilia does not affect tumor progression in the MMTV-PyMT breast cancer model

In my previous experiment I was unable to analyze the effect of eosinophils on lung metastases in an orthotopic MMT-PyMT tumor model because the tumors did not spontaneously metastasize. Thus, I repeated the experiment with a different primary tumor as well as an MMTV-PyMT cell line established in our laboratory. Additionally, I wanted to know if eosinophils impact tumor progression in the absence of an adaptive immune system.

I injected both wild type (WT) C57BL/6J and immunocompromised Rag2/Il2rg mice orthotopically with MMTV-PyMT cancer cells. Once tumors were palpable at day7, I began PBS or AB5-IL5 administrations intraperitoneally (i.p.) followed by a tumorectomy once tumor reached an average size of $5mm^3$ after which I ceased AB5-IL5 treatment (Fig26 A). The results presented are pooled from three independent experiments, each experiment is color coded. I analyzed the immune composition of the tumors in one out of the three experiments whereas tumor weight and lung macrometastases were analyzed in all three. In only one of the experiments did I observe liver metastases.

Due to the design of the experiment, there was no change in primary tumor weights between the different treatments and mouse strains, nor did I observe a change in lung macrometastases (Fig26 B, left and middle

panel). Liver macrometastases were only observed in AB5-IL5 treated, immunocompromised mice in one out of three experiments (Fig26 B, right panel).

Tumor eosinophils tended to increase upon AB5-IL5 treatment, although the change was not significant (Fig26 C, left panel). Rag2/Il2rg mice treated with AB5-IL5 had significantly more active eosinophils, as measured by their SiglecF expression, compared to the other groups (Fig26 C, middle panel). Granularity remained the same in all conditions (Fig26 C, right panel).

Several publications have shown that eosinophils can function as antigen presenting cells (APCs) (see introduction). I thus analyzed the percentage of eosinophils expressing co-activation markers CD40, CD80 and CD86 as well as MHCII.

There were few $CD40^+$ and $CD80^+CD86^+$ TAEs and their percentages did not change between the groups (Fig26 D, left and middle panel). PBS treated immunocompromised mice had increased $MHCII^+$ TAEs compared to WT mice and AB5-IL5 treated immunocompromised mice (Fig26 D, right panel).

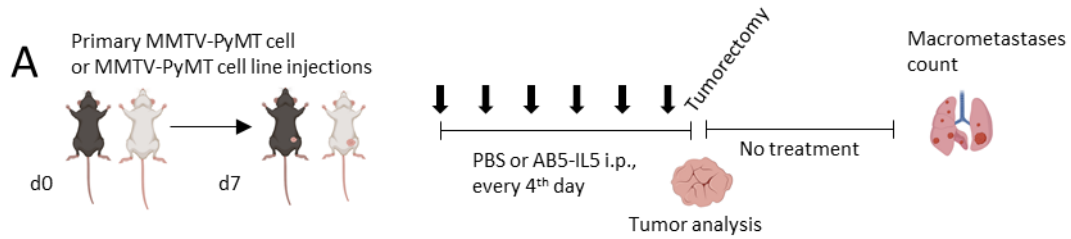
Analyses of induced and resident TAEs (iEos and rEos respectively) in tumors showed that iEos did not differ between the groups whereas rEos significantly decreased in AB5-IL5 treated immunocompromised mice compared to PBS treated WT mice (Fig26 E, left panel). Analyses of SiglecF expression and granularity in each of these subsets showed that, as expected, iEos had increased levels of SiglecF MFI levels compared to rEos regardless of mouse strain and treatment (Fig26 E, middle panel). Granularity of rEos significantly decreased in immunocompromised mice compared to iEos but did not change between the subsets in WT mice (Fig26 E, right panel).

I also analyzed the effect TAE had on tumor associated neutrophils (TANs), tumor associated myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs) and tumor associated dendritic cells (DCs).

Overall TANs and $SiglecF^+$ TANs, all of the latter were SSC^{high} (data not shown), did not change between groups (Fig26 F). MDSCs showed a decreasing trend upon AB5-IL5 treatment in both WT and immunocompromised mice, although these changes were not significant (Fig26 G). The frequency of TAMs and their different subsets remained the same between the groups (Fig26 H). DC infiltration and activation did not change under the different conditions (Fig26 I). Because of technical difficulties, I was not able to analyze the lymphocyte populations within the tumors.

To conclude, I saw that AB5-IL5 did not change lung metastases in the MMTV-PyMT breast cancer model but could potentially increase liver metastases in immunocompromised mice.

I also showed that TAEs have the potential to act as APC due to their expression of MHCII and co-stimulatory molecules but whether this happens *in vivo* is not clear. Finally, I confirmed that induced eosinophils are more activated than resting eosinophils.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d7
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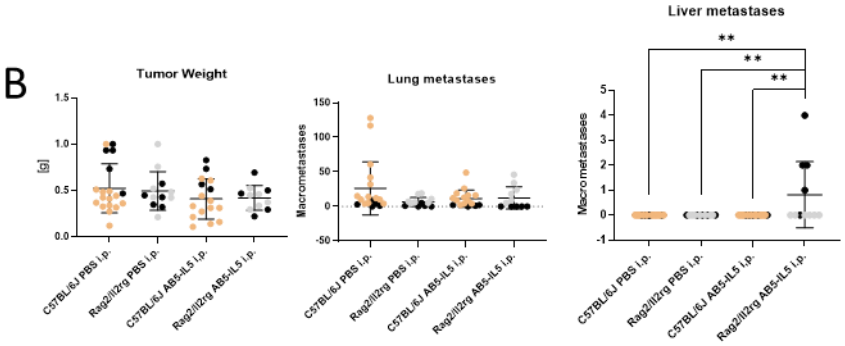
No treatment
Tumor analysis
Macrometastases
count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Macrometastases
Liver metastases
Macrometastases
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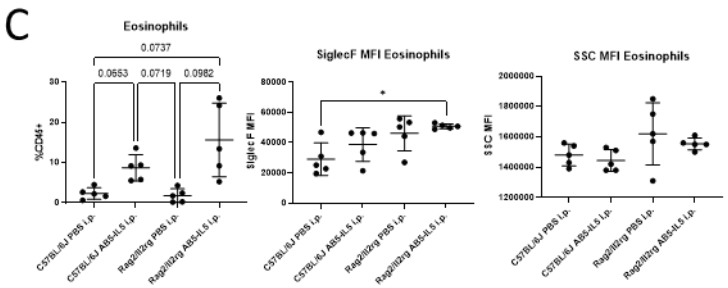
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Eosinophils
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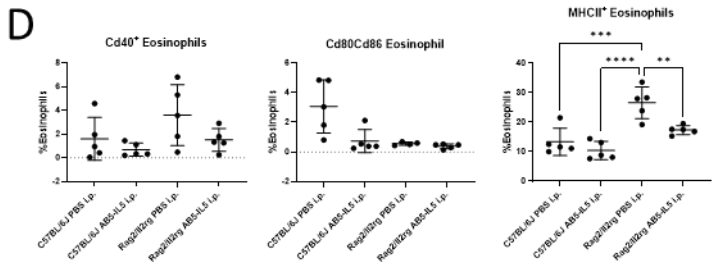
SiglecF MFI Eosinophils
SiglecF MFI
SSC MFI Eosinophils
SSC MFI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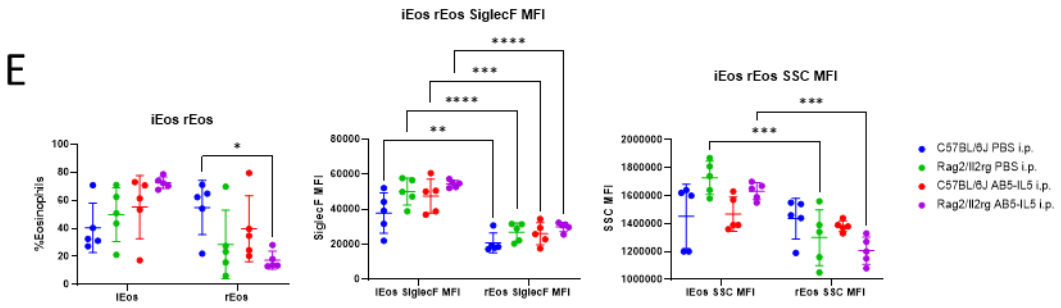
Cd40+ Eosinophils
Cd80Cd86 Eosinophil
MHCII+ Eosinophils
%Eosinophils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iEos rEos SiglecF MFI
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iEos rEos SSC MFI
SSC MFI
iEos SSC MFI
rEos SSC MFI
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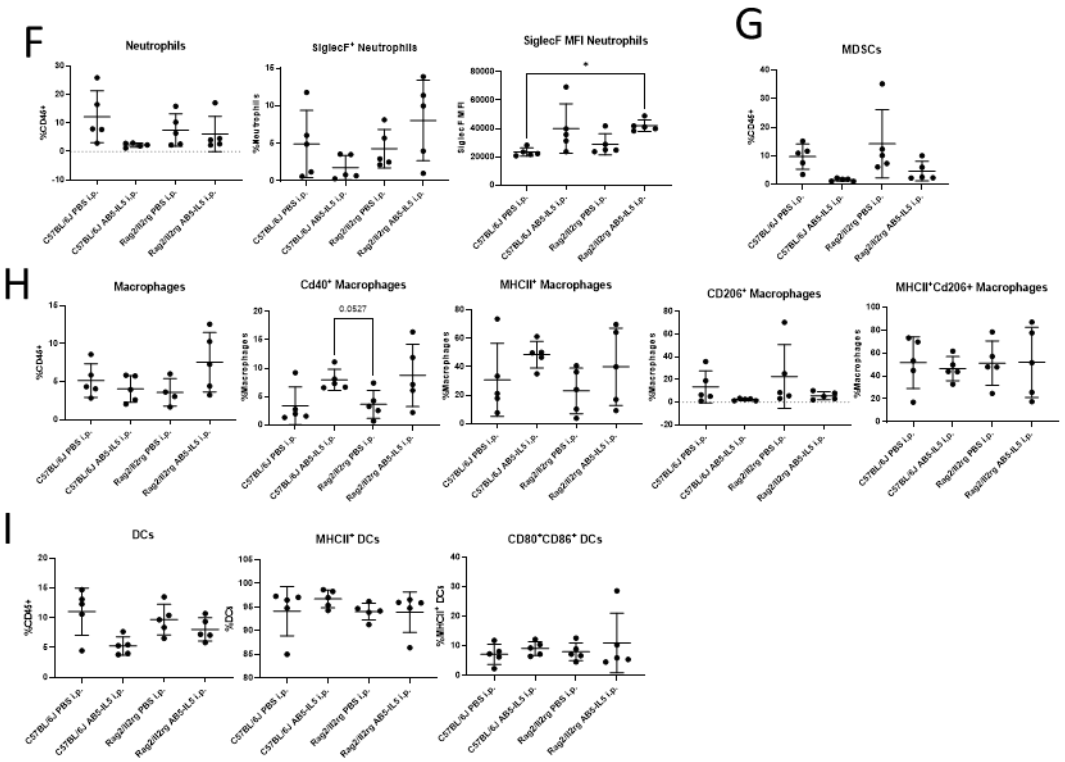
F
Neutrophils
SiglecF+ Neutrophils
SiglecF MFI Neutrophils
G
MDSCs
H
Macrophages
Cd40+ Macrophages
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MHCII+ Macrophages
CD206+ Macrophages
MHCII+Cd206+ Macrophages
I
DCs
MHCII+ DCs
CD80+CD86+ DCs
C57BL/6J PBS i.p.
C57BL/6J AB5-IL5 i.p.
Rag2Il2rg PBS i.p.
Rag2Il2rg AB5-IL5 i.p.

Figure26: Tumor eosinophilia does not affect breast cancer progression in the MMTV-PyMT mouse model.

WT C57BL/6J or immunocompromised Rag2/Il2rg mice were injected orthotopically with primary MMTV-PyMT cancer cells and treated with PBS or 50ug AB5-IL5 i.p. every fourth day. After tumorectomy, treatment was ceased and tumors were weighted and analyzed by flow cytometry. Lungs and livers were analyzed for macrometastases. Results for tumor weight, lung metastases and liver metastases were pooled from three independent experiments. Flow cytometry results are from one experiment. The results are color coded. Experiment1: black. Experiment2: grey. Experiment3: beige.

A: Experimental workflow.

B: Tumor weights at tumorectomy (One-way ANOVA). Lung and liver macrometastasis after euthanasia (Kruskal-Wallis non-parametric test).

C: Analysis of TAE tumor infiltration by flow cytometry. Eosinophils were defined as $CD45^{+}CD11b^{+}Ly6g^{neg\text{-}med}SiglecF^{+}SSC^{high}$ (Brown-Forsythe one-way Welch ANOVA). SiglecF MFI and SSC MFI of TAEs were measured (One-way ANOVA).

D: Analysis of $CD40^{+}$ (One-way ANOVA), $CD80^{+}CD86^{+}$ (Brown-Forsythe one-way Welch ANOVA) and $MHCII^{+}$ (One-way ANOVA) TAEs.

E: Analysis of iEos and rEos (Two-way ANOVA). iEos were defined as $CD45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}SSC^{high}$ $CD62L^{neg}$ and rEos were defined as $CD45^{+}CD11b^{+}Ly6G^{neg\text{-}med}SiglecF^{+}SSC^{high}$ $CD62L^{+}$.

F: Analysis of TAN tumor infiltration by flow cytometry. Neutrophils were defined as $CD45^{+}CD11b^{+}Ly6G^{high}$ (One-way ANOVA). $SiglecF^{+}$ TANs were enumerated and SiglecF MFI was measured in $SiglecF^{+}$ TANs (One-way ANOVA).

G: Analysis of MDSC tumor infiltration by flow cytometry. MDSCs were defined as $CD45^{+}CD11b^{+}SiglecF^{-}SSC^{low}Ly6G^{low}Ly6C^{high}$ (One-way ANOVA).

H: Analysis of TAM tumor infiltration by flow cytometry. Total macrophage numbers and their polarization was analyzed. Macrophages were defined as $CD45^{+}CD11b^{+}F4/80^{+}$ (One-way ANOVA). M1 macrophages were defined as $CD45^{+}CD11b^{+}F4/80^{+}MHCII^{+}CD206^{-}$ (One-way ANOVA) and M2 macrophages were defined as $CD45^{+}CD11b^{+}F4/80^{+}MHCII^{-}CD206^{+}$ (One-way ANOVA).

I: Analysis of DC tumor infiltration by flow cytometry. Total DC numbers and their activation was analyzed. Dendritic cells were defined as $CD45^{+}CD11c^{+}$ (One-way ANOVA). The expression of co-activators MHCII, CD80 and CD86 were measured on DCs (One-way ANOVA).

WT: Wild type. i.p.: Intraperitoneal. TAEs: Tumor associated eosinophils. rEos: Resident eosinophils. iEos: Induced eosinophils. TANs: Tumor associated neutrophils. MDSCs: Myeloid derived suppressor cells. TAMs: Tumor associated macrophages. DCs: Dendritic cells.

Significant changes are denoted by *. $p \leq 0.5$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p < 0.0001$ = ****.

Results summary

I tested the effects of tumor associated eosinophils in three different breast cancer models. My results showed that in none of them eosinophils affected lung metastases, whether spontaneous or i.v. injected. I consistently observed a population of $SiglecF^{+}$ TANs that had the same granularity as TAEs. These neutrophils were most prominent in breast cancer models in the Balb/c background, such as the 4T1 model

and the EMT6 model (data not shown for the latter). Pooling all the experiments in the Balb/c background, I observed that TAEs had a strong anti-correlation with TANs in both 4T1 and EMT6 breast cancer models (R2 = 0.25, p > 0.0001 and R2 = 0.6, p = 0.016) and a positive correlation with SiglecF^{+} TANs (R2 = 0.05, p = 0.027 and R2 = 0.76 and p = 0.005). I saw only a modest and inconsistent effect on the rest of the immune environment of primary cancers but a significant change of the immune infiltration within the lungs.

I concluded that in the mouse models I used, eosinophils do not affect breast cancer progression. Table 15 is a summary of the effect of TAEs on TANs, tumor weight and lung metastases compared to the control group and table 16 is a summary of the mean percentage of TAE, mean tumor weights and mean number of lung metastases in the different experiments presented. In both tables, X marks groups that were not included in the respective experiment

TABLE 15: SUMMARY OF OUTCOMES IN DIFFERENT EXPERIMENTS

Treatment	Mouse Strain	Cell line	Experiment	Outcome compared to control tumors treated with PBS
Control tumor + AB5-IL5	Balb/c	4T1	Exp1:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp2:	X
			Exp3:	X
			Exp4:	Neutrophils→, Tumor weight→, Lung metastases→
CCL11 tumor + PBS			Exp1:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp2:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp3:	X
			Exp4:	Neutrophils→, Tumor weight→, Lung metastases→
CCL11 tumor + AB5-IL5			Exp1:	Neutrophils↓, Tumor weight→, Lung metastases→
			Exp2:	X
			Exp3:	X
			Exp4:	Neutrophils→, Tumor weight→, Lung metastases→
Control tumor + AB5-IL5	C57BL/6J	E0771	Exp5:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp6:	Lung metastases→
CCL11 tumor + PBS			Exp5:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp6:	Lung metastases→
CCL11 tumor + AB5-IL5			Exp5:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp6:	Lung metastases→
WT + AB5-IL5			Exp7:	Neutrophils→, Tumor weight→

		Primary MMTV-PyMT	Exp8:	Neutrophils→, Tumor weight→, Lung metastases→
		PyMT cell line	Exp10:	Tumor weight→, Lung metastases→
Rag2/Il2rg + AB5-IL5	NMRI	Primary MMTV-PyMT	Exp8:	Neutrophils→, Tumor weight→, Lung metastases→
			Exp9:	Tumor weight→, Lung metastases→

TABLE 16: SUMMARY OF THE MEAN PERCENTAGE OF TAES, MEAN TUMOR WEIGHTS AND MEAN NUMBER OF LUNG METASTASES

Treatment	Mouse Strain	Cell line	Experiment	%Eosinophils Mean	Tumor weight [mg] Mean	Lung metastases [#] Mean
Control tumor + PBS	Balb/c	4T1	Exp1:	7	425	16
			Exp2:	3	707	17
			Exp3:	X	X	X
			Exp4:	10	744	11
Control tumor + AB5-IL5			Exp1:	26	357	13
			Exp2:	X	X	X
			Exp3:	X	X	X
			Exp4:	11	728	15
CCL11 tumor + PBS			Exp1:	29	396	17
			Exp2:	24	693	25
			Exp3:	36	784	18
			Exp4:	14	786	26
CCL11 tumor + AB5-IL5			Exp1:	65	400	30
			Exp2:	46	820	20
			Exp3:	69	489	6
			Exp4:	32	683	15
Control tumor + PBS	C57BL/6J	E0771	Exp5:	5	482	8
			Exp6:	X	X	6
Control tumor +			Exp5:	19	675	16

AB5-IL5			Exp6:	X	X	11
CCL11 tumor + PBS			Exp5:	4	560	2
			Exp6:	X	X	7
CCL11 tumor + AB5-IL5			Exp5:	13	696	1
			Exp6:	X	X	27
WT + PBS		Primary MMTV-PyMT	Exp7:	1	490	X
			Exp8:	2	813	3
		PyMT Cell line	Exp10:	X	419	35
WT + AB5-IL5		Primary MMTV-PyMT	Exp7:	3	687	X
			Exp8:	9	660	1
		PyMT cell line	Exp10:	X	326	15
Rag2/Il2rg + PBS	NMRI	Primary MMTV-PyMT	Exp8:	2	423	1
			Exp9:	X	546	10
Rag2/Il2rg + AB5-IL5			Exp8:	16	435	0
			Exp9:	X	406	23

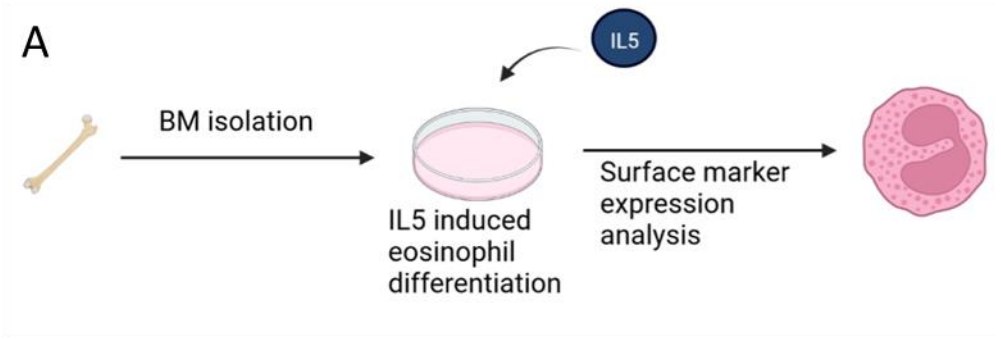
In vitro differentiation and characterization of eosinophils

My results so far showed that tumor associated eosinophils do not affect tumor progression or the tumor lymphocytic environment *in vivo*. I wanted to know if this was also the case *in vitro*. First, I optimized a reliable *in vitro* differentiation protocol for bone marrow derived eosinophils (BMDEos) and characterized these cells. Then I characterized the impact of activation on BMDEos surface marker expression and their capacity to affect T cells activation. Finally, I co-cultured activated eosinophils with different cancer cells lines *in vitro* to analyze their capacity to kill tumor cells.

In vitro derived eosinophils have a different surface marker expression profile than *in vivo* derived tumor associated eosinophils.

I characterized eosinophil surface marker expression during *in vitro* differentiation without additional activators. (Fig27 A). I saw that initially, CD11b was expressed in about 50% of analyzed cells which increased to approximately 75% at day8. Strangely, there was a significant decrease in CD11b expression at day15, when the *in vitro* cultured bone marrow cells where supposedly an almost pure population of eosinophils (Fig27 B, left panel). Curiously, SiglecF was only expressed in about 4% BMDEos at day 15 (Fig27 B, middle-left panel). Because I had seen clear Ly6G expression on TAEs, I tested this surface markers expression on *in vitro* differentiated cells. I saw that at day8 and day15, about 6% of cells expressed Ly6G (Fig27 B, middle-right panel). I had observed F4/80 expression in TAEs *ex vivo* (data not shown), a phenotype also reported by others[438]. I saw that between 30 and 60% of *in vitro* cultured cells expressed F4/80, depending on the time points at which the cells were analyzed. (Fig27 B, right panel).

Because of the lack of SiglecF expression in my BMDEos, I wanted to verify that these cells were in fact eosinophils. I spun aliquots of the flow cytometry analyzed cells upon glass slides and stained the cells with H&E. A representative image of cells acquired on day 15 is shown. I indeed had an almost pure population of eosinophils characterized by strong eosinophilic stain and doughnut shaped nuclei (Fig27 C, bottom inset). There was very mild contamination of monocytes (Fig27 C, right inset, red square) although this could not account for the low percentage of $SiglecF^{+}$ cells. The cytospins were quantified by choosing 7 fields of vision and counting the number of eosinophils and non-eosinophil cells (Fig27 C, bottom right panel). Thus, I found that *in vitro* differentiated eosinophils displayed a very different surface marker expression profile compared to TAEs and lung associated eosinophils.


A
IL5
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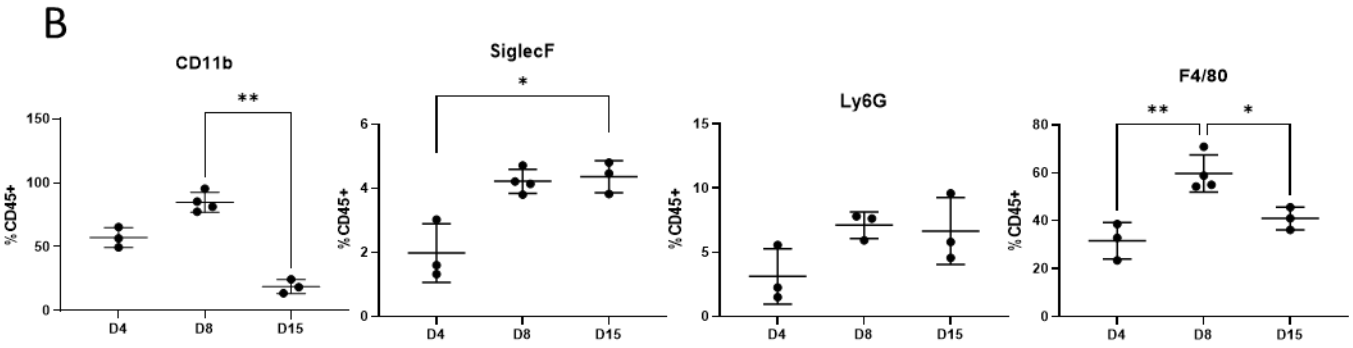
IL5 induced
eosinophil
differentiation
Surface marker
expression
analysis


B
CD11b
SiglecF
Ly6G
F4/80
% CD45+
D4
D8
D15

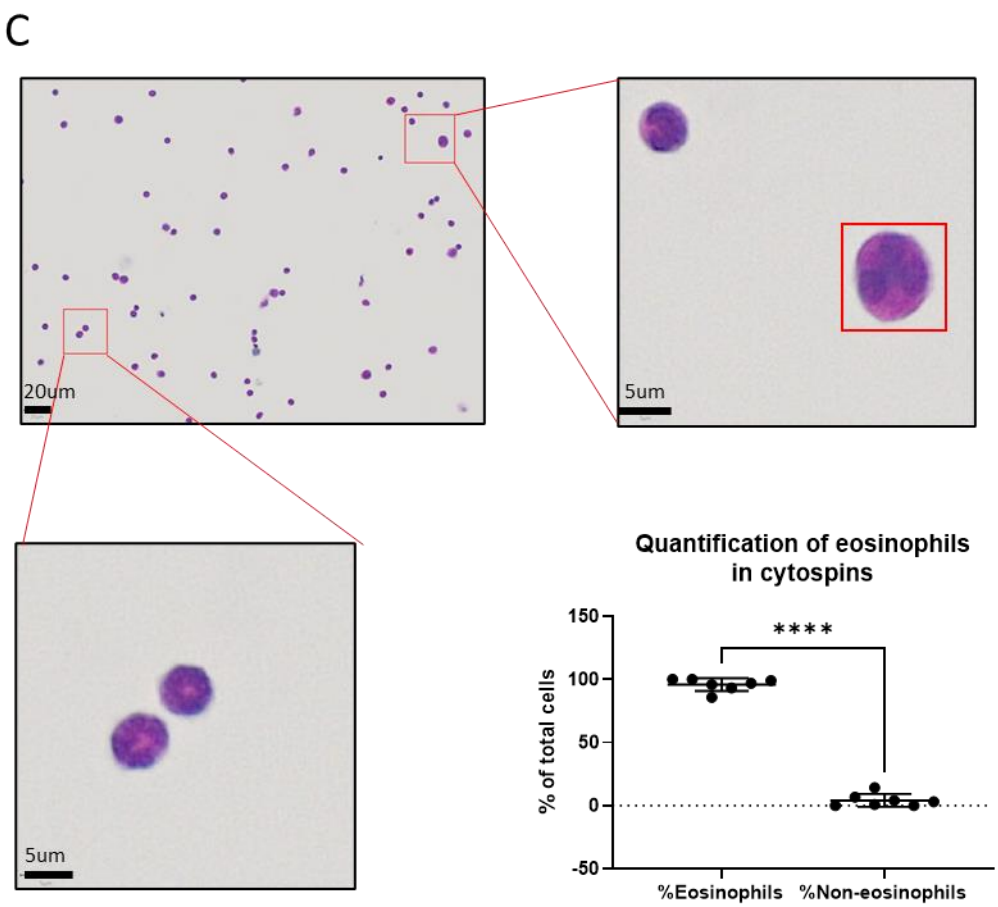

C
20um
5um
5um
Quantification of eosinophils
in cytospins
% of total cells
%Eosinophils
%Non-eosinophils

Figure27: *In vitro* differentiated eosinophils differ from *in vivo* eosinophils.

Bone marrow derived cells were cultured with eosinophil differentiation factors for 15 days. The expression several surface markers measured on cultured cells on day4, day8 and day15 via flow cytometry. Aliquots of cells analyzed by flow cytometry were taken for cytospins and H&E characterization.

A: Experimental workflow. Created with BioRender.com

B: Expression of CD11b, SiglecF, Ly6G and F4/80 on eosinophils during *in vitro* differentiation (One-way ANOVA).

C: Representative image of H&E staining of *in vitro* differentiated eosinophils. Quantification is shown in the bottom right corner (Two-tailed t-test). Each dot represents one biological replicate.

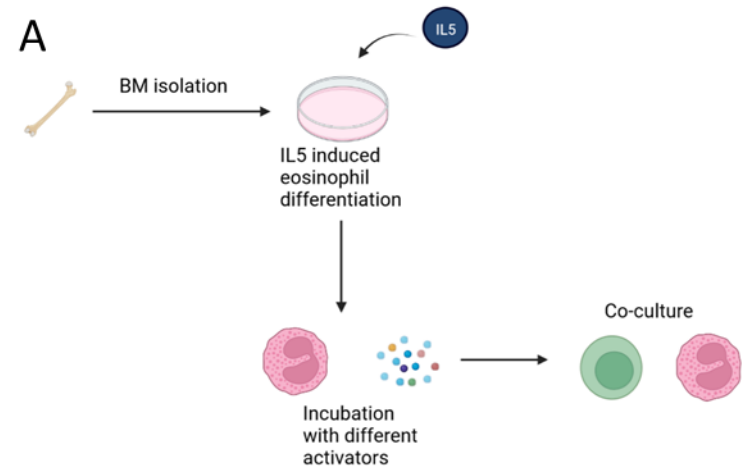
Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

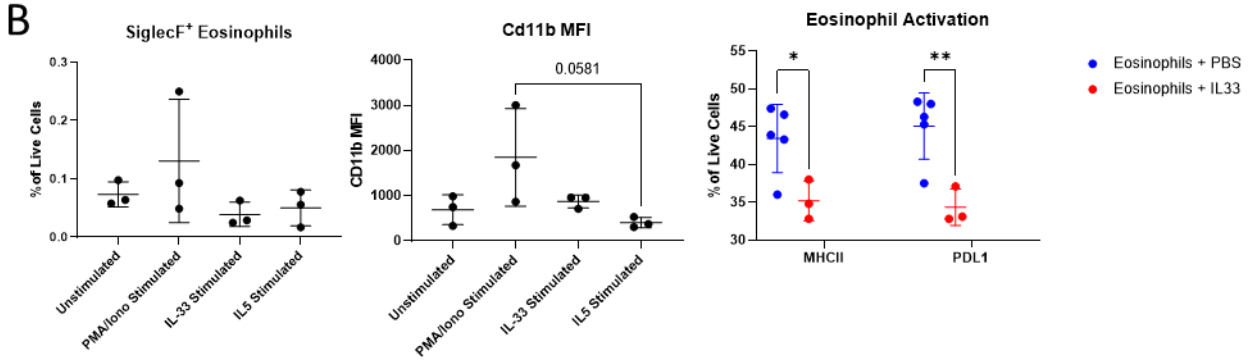
Eosinophils can acquire an antigen-presenting phenotype *in vitro* and affect T cell activation

For activation, eosinophils were treated *in vitro* with IL33 and their expression levels of SiglecF, CD11b, MHCII and PDL1 were analyzed. (Fig28 A). Of note, the side scatter did not vary between the different activation conditions (data not shown). SiglecF expression remained very low, even in the presence of IL33 (Fig28 B, left panel). Because of this low SiglecF expression, I was not able to reliably calculate the MFI of SiglecF. Obviously, SiglecF, it is not an ideal marker for activation *in vitro*. Similarly, CD11b expression did not increase upon stimulation with PMA/Iono, IL33 or IL5 (Fig28 B, middle panel). Eosinophils downregulated both MHCII and PDL1 upon IL33 treatment (Fig28 B, right panel). Eosinophils are known to exert direct effect on T cells by presenting antigen and recently, a PDL1 expressing eosinophil population was identified within the intestine[38]. I wanted to know if IL33 activated eosinophils are able to activate antigen specific CD8 T cells as measured by CD69 expression on T cells.

I co-cultured basal (Eos), activated (A_Eos), ovalbumin SL-8 peptide pulsed (P_Eos) and activated and SL-8 peptide pulsed (A/P_Eos) eosinophils with purified SL-8 specific OT-1 T cells in the presence or absence of antigen (SL8). I found that eosinophils without antigen were not able to increase $CD69^+$ T cells but did so significantly in the presence of the antigen peptide SL8. Eosinophils pulsed with SL8 prior to T cell co-culture significantly increased $CD69^+$ T cells compared to eosinophils not presenting the peptide (Fig28 C). Basal and IL33 activated eosinophils also increased T cell proliferation during bead stimulation compared to stimulated T cells alone in 4/4 and 3/4 biological replicates respectively (Fig28 D).

Overall, I showed that eosinophils can acquire an antigen presenting phenotype *in vitro* and that these *in vitro* differentiated eosinophils are able to affect CD8 T cell activation.


A
IL5
BM isolation
IL5 induced
eosinophil
differentiation
Co-culture
Incubation
with different
activators


B
SiglecF+ Eosinophils
% of Live Cells
Unstimulated
PMA/Iono Stimulated
IL-33 Stimulated
IL5 Stimulated
Cd11b MFI
CD11b MFI
0.0581
Eosinophil Activation
% of Live Cells
MHCII
PDL1
Eosinophils + PBS
Eosinophils + IL33

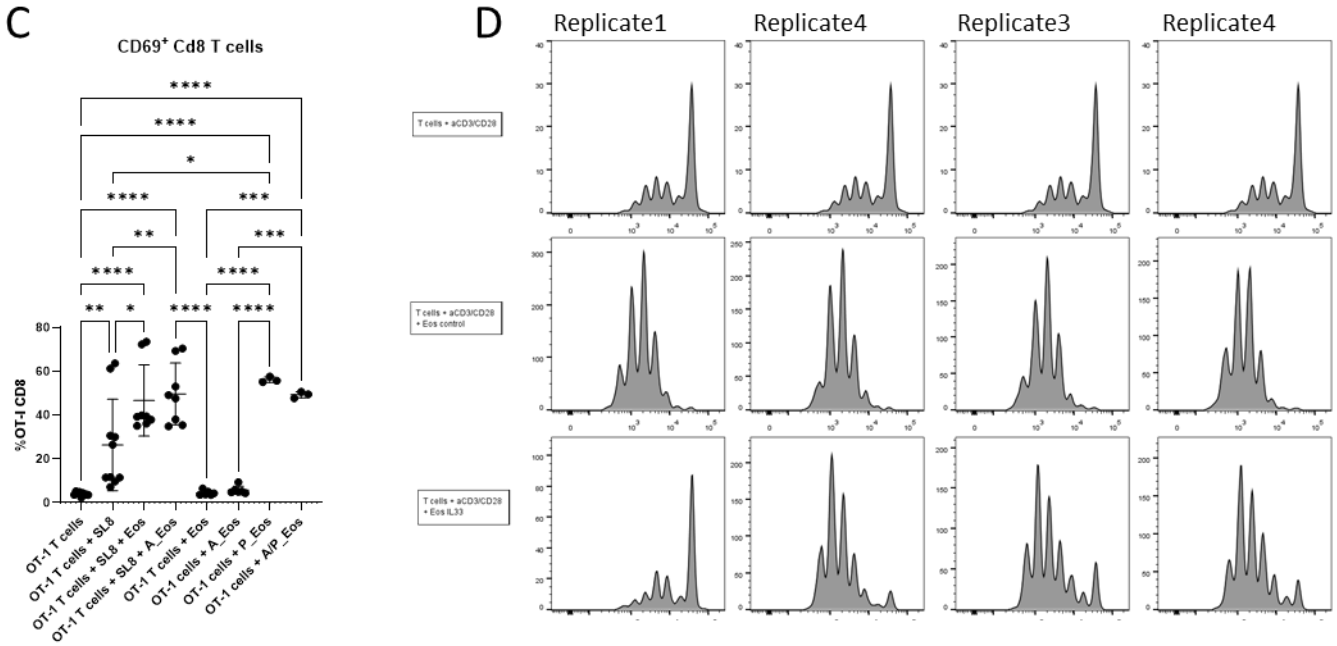

C
CD69+ Cd8 T cells
%OT-I CD8
OT-1 T cells
OT-1 T cells + SL8
OT-1 T cells + SL8 + A_Eos
OT-1 T cells + Eos
OT-1 cells + A_Eos
OT-1 cells + P_Eos
OT-1 cells + A/P_Eos
D
Replicate1
Replicate4
Replicate3
Replicate4
T cells + aCD3/CD28
T cells + aCD3/CD28 + Eos control
T cells + aCD3/CD28 + Eos IL33

Figure28: Eosinophils present MHC1-restricted peptides and can activate CD8 T cells *in vitro.*

Eosinophils were differentiated *in vitro* and stimulated with PMA/Iono, IL33 or IL5. Expression of activation markers were measured via flow cytometry. Eosinophils were stimulated and/or pulsed with SL8 peptide (A_Eos, P_Eos and A/P_Eos respectively) Activated eosinophils were co-cultured with SL8 specific OT-I CD8 T cells and the effect on the T cells was analyzed. **A**: Experimental workflow. Created with BioRender.com

B: Effect of *in vitro* stimulation on *in vitro* differentiated eosinophils. Percentages of SiglecF+ eosinophils and their CD11b expression levels were measured (One-way ANOVA). $MHCII^+$ and $PDL1^+$ eosinophil percentages were calculated (Two-way ANOVA).

C: Effect of co-culture of differentially manipulated eosinophils with T cells on T cell CD69 expression (Brown-Forsythe one-way Welch ANOVA).

D: Effect of Effect of co-culture of differentially manipulated eosinophils on T cell proliferation. Each dot represents one biological replicate.

PMA: Phorbol myristate acetate. Iono: Ionomycin. A_Eos: IL33 treated eosinophils. P_Eos: SL8-pulsed eosinophils. A/P_Eos: IL33 treated, SL8 pulsed eosinophils.

Significant changes are denoted by *. p≤0.5 = *, p≤0.01 = **, p≤0.001 = ***, p<0.0001 = ****.

Eosinophils preferentially kill intestinal and melanoma cancer cells *in vitro*

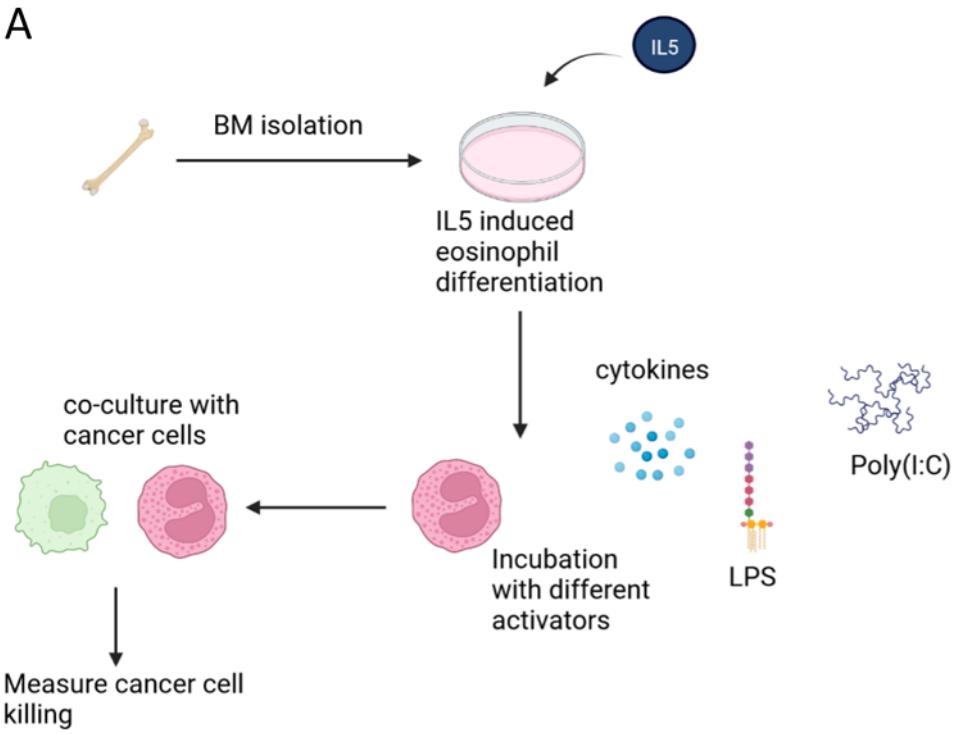
Tumor associated eosinophils did not affect breast cancer progression *in vivo* in any of my experiments and I wanted to know if I could confirm these results *in vitro*. I differentiated eosinophils from bone marrow cells *in vitro* and then activated them with different molecules known to cause eosinophil activation. I then washed these activated eosinophils and co-cultured them with four different cancer cells lines and calculated killing (Fig29 A).

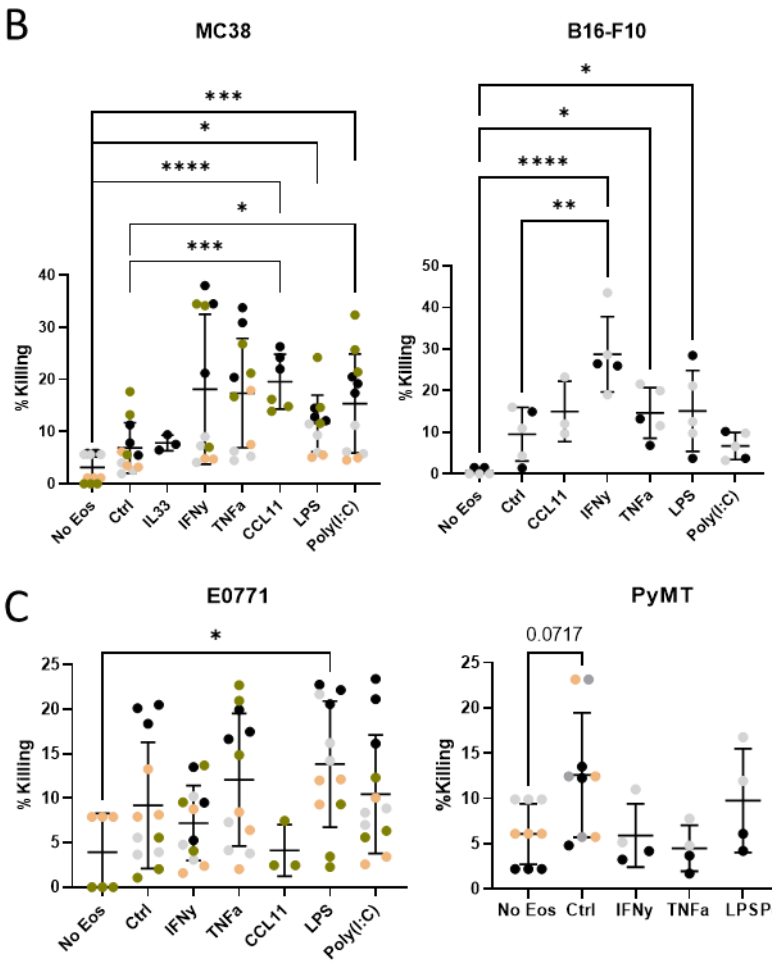
I saw that CCL11, LPS and Poly (I:C) activated eosinophils significantly increased killing of MC38 colon carcinoma cells. Only CCL11 and LPS activated eosinophils in a way to significantly kill tumor cells compared to unstimulated eosinophils (Fig29 B, left panel).

Melanoma B16-F10 cells were efficiently killed when co-cultured with eosinophils activated with IFNγ, TNFα and poly (I:C) compared to cancer cell monoculture (Fig29 B, right panel). IFNγ activation also significantly increased tumor cell killing by eosinophils compared to no activation.

Because this project is about breast cancer, I tested two breast cancer cell lines. In E0771 co-culture with eosinophils, only LPS significantly increased cancer cell killing by eosinophils compared to cancer cell monoculture. None of the other treatments significantly increased the tumor killing capacity of eosinophils *in vitro* (Fig29 C, left panel). I also used an in-house established cell line derived from primary PyMT tumors. There was no difference in cancer cell death when adding eosinophils to the culture, regardless of prior activation (Fig29 C, right panel).

Overall, *in vitro* differentiated eosinophils seem to be efficient at killing colon cancer and melanoma cells after activation but not breast cancer cells which might explain why I never saw a strong phenotype *in vivo*.


A
IL5
BM isolation
IL5 induced
eosinophil
differentiation
cytokines
Poly(I:C)
co-culture with
cancer cells
Incubation
with different
activators
LPS
Measure cancer cell
killing


B
MC38
B16-F10
% Killing
No Eos
Ctrl
IL33
IFNγ
TNFa
CCL11
LPS
Poly(I:C)

C
E0771
PyMT
% Killing
%Killing
0.0717
No Eos
Ctrl
IFNγ
TNFa
CCL11
LPS
Poly(I:C)

Figure29: *In vitro* differentiated eosinophils are not efficient at directly killing breast cancer cells.

Eosinophils were differentiated *in vitro* and stimulated with IFNy, TNFa, CCL11 LPS or Poly (I:C) and then co-cultured o/n with four different cancer cell lines. Tumor killing by eosinophils was analyzed. Results were pooled from two to four experiments. Each experiment is color coded: Exp1: Black, exp2: Grey, exp3: Beige, exp4: green.

A: Experimental workflow. Created with BioRender.com

B: *In vitro* killing by eosinophils of MC38 colon cancer cells (Brown-Forsythe one-way Welch ANOVA) and B16-F10 melanoma cells (One-way ANOVA).

C: *In vitro* killing by eosinophils of E0771 and PyMT breast cancer cells (One-way ANOVA). Each dot represents one biological replicate.

Ctrl: Control. IFNy: Interferon-y. TNFa: Tumor necrosis factor a. LPS: Lipopolysaccharide. Poly (I:C): Polyinosinic:polycytidylic acid.

Significant changes are denoted by *. $p \leq 0.5$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p < 0.0001$ = ****.

Discussion

General

The goal of my thesis was to characterize how eosinophils affect breast cancer. Eosinophils have been shown to be correlated with patient survival in breast cancer by others but no causative link has been proven[352,536,543,544,553,554,557–559,561,563,564,566–570,605–611]. In order to try and find if and how eosinophils can change breast cancer progression, I used different breast cancer models where I established a reliable method to induce tumor associated eosinophilia by expressing the main eosinophil chemoattractant CCL11 in tumor cells and treating the mice with IL5 coupled to an antibody moiety (termed AB5-IL5) in order to both increase time in circulation and tumor-targeting, which resulted in increased eosinophilia. I established a reliable gating strategy to identify eosinophils and distinguish them from neutrophils and I analyzed the effect of eosinophils on primary tumor growth, metastatic spread and the tumor immune environment.

Identification of eosinophils

Eosinophils were initially identified through histology by Paul Ehrlich in 1874[35] in humans. He observed that they had a stronger eosin stain than other immune cells due to their eosinophilic granules and a bilobed nucleus. Mouse eosinophils have the same intense eosin stain but in general display a doughnut shaped nucleus. Today, eosinophils are identified using either flow cytometry, histological staining, including antibody staining, and gene expression analysis.

In this section I will discuss the difficulties in defining one surface marker set to identify eosinophils and distinguish them from other immune cells (especially neutrophils and macrophages) via flow cytometry, present the caveats of using gene expression to define eosinophils in patient samples and how histological analysis can be used to confirm the identity of these cells. I will describe how I dealt with the existing issues, explain why, to this day, there is no core signature for eosinophils and what guidelines researchers should follow in order to ensure that the cells they are analyzing are indeed eosinophils.

Identification of eosinophils via flow cytometry

A general difficulty at the beginning of my project was to define a gating strategy to identify eosinophils via flow cytometry. This was due to the fact that different groups had characterized the surface marker profile of eosinophils in different organs, mainly the gut, the lungs, the circulation and the bone marrow, and then generalized it to the entire eosinophil population. Yet we know now that depending on their location and activation status, eosinophils can display a variety of surface markers[612–614]. For a complete list of surface markers used to identify eosinophils in different organs and at different levels of activation, see table 4.

The most common markers used in a variety of tissues, including breast cancer models[615–617], are CD11b[618], SiglecF[618–620] and high granularity which translates into a high side scatter[621]. These markers were confirmed in eosinophils from different tissues and I consider them to reliably identify eosinophils. But identification

alone is not sufficient, differentiation between eosinophils and other immune cells is just as crucial as and turned out to be more difficult than I initially estimated, especially in regard to neutrophils. SiglecF, the ortholog of the human Siglec-8 glycoprotein was discovered and characterized in the early 2000s[622] and thought to be expressed exclusively in eosinophils[623] but later publications showed that neutrophils are also able to express high levels of SiglecF under certain circumstances[624,625]. This required the addition of another marker to my set, which most logically was Ly6G, a molecule thought for a long time to be expressed exclusively on neutrophils and, importantly, not eosinophils. I quickly realized that when excluding all $Ly6G^{+}$ cells from my eosinophil gate, my flow cytometry results did not match my histology results, namely histological analyses indicated significantly more eosinophils within the tumor tissue than flow cytometry. I further observed a significant increase in $Ly6G^{medium}$ cells upon eosinophilia induction. I hypothesized that eosinophils express Ly6G, which turned out to be the case (see figure 15). There are some reports which confirmed my results in eosinophils from different tissues[393,401,626]. Furthermore, careful study of flow cytometry plots in papers describing an absence of Ly6G on eosinophils revealed that, despite their text claiming the opposite, their eosinophil population was not $Ly6G^{negative}$, but rather $Ly6G^{negative}$ to $Ly6G^{medium}$ [598,627,628]. It thus seems that the dogma of eosinophils not expressing Ly6G is false and in order to distinguish between eosinophils and neutrophils, SiglecF and Ly6G need to be used carefully and by defining thresholds which allow to distinguish these myeloid populations under all experimental conditions. Adding side scatter as an additional marker should always be done to distinguish between these cells, as I do not know of any reports showing neutrophils with the same high granularity as eosinophils. In order to avoid underestimating eosinophils, I chose to define them as $Ly6G^{neg\text{-}med}$ whereas neutrophils were defined as $Ly6G^{high}$.

Another difficulty I had with my gating strategy, as shown by my cytospins in figure 15 was a contamination of monocytic cells. These cells could have most likely also been excluded had I used high side scatter in that experiment. Monocytes are known to express high levels of Ly6C, so this could also be added, yet several reports showed Ly6C expression in eosinophils[401] hence it might not be an ideal marker to distinguish between eosinophils and monocytes.

A recent paper showed that there is a fluidity in phenotype in mouse bone marrow cells. They observed a population with both neutrophilic and eosinophilic surface markers which upon isolation could differentiate into neutrophils, eosinophils or monocytes[488]. These results might explain why distinguishing between these three different immune populations is not always as easy as initially thought. Finally, SiglecF was not a good marker for in vitro differentiated eosinophils in my experiments, as these cells, confirmed to be eosinophils by histology, barely expressed it (see figure 27).

Because of these caveats with SiglecF, I decided to investigate additional markers for eosinophils. IL5R and CCR3 were used by some researchers to identify eosinophils and distinguish them from other immune cells[600,601]. IL5R, although expressed in eosinophils, was also expressed in alveolar macrophages and neutrophils[601]. The former is known to express this receptor, whereas the latter is typically not, although I

found one other study which demonstrated that neutrophils in the olfactory neuroepithelium also displayed high IL5R expression[629]. I have tried to use IL5R as a marker in my experiments but was unable to do so, due to the fact that the antibody did not bind to any cells. Because of its expression on other immune cells, it would not have furthered my goal to uniquely marking eosinophils. Additionally, I would not have been able to distinguish between eosinophils and (alveolar) macrophages using IL5R due to the fact that the macrophage "specific" marker F4/80 has also been shown to be expressed in eosinophils[630,631]. CCR3, although ascribed to eosinophils specifically, is not used often in publications and when it is, usually not all eosinophils express it[600,601], a phenomenon I also observed. My results demonstrated that a majority of neutrophils express CCR3 (see figure 16). Although other reports did not observe this marker on neutrophils in the lungs[601], neutrophils have been shown to upregulate CCR3 under certain conditions[487]. Furthermore, human blood basophils and T cells have also been shown to express CCR3[373,374].

Overall, it is clear that in order to differentiate between eosinophils and other immune cells, specifically neutrophils, monocytes and macrophages, via flow cytometry, care is required. Using the established gating strategies in published reports might lead to misidentifications because of the apparent promiscuity of surface marker thought to be specific to only one type of immune cells[626]. This holds true not only for SiglecF and Ly6G, but also for IL5R, CCR3 and F4/80. I chose to use CD11b, Ly6G$^{neg\text{-}med}$, SiglecF and a high side scatter to identify eosinophils in my thesis, but I am aware that different experimental conditions (tissues, activation states) may require another marker set.

Identification of eosinophils via RNA

The identification of eosinophils via gene expression analysis is difficult since single cell RNA sequencing (scRNAseq) datasets are scarce due to the fact that the usual preparations for scRNAseq destroy eosinophils. To date, there exists only one paper which was able to use this technique on tissue associated murine eosinophils[632]. Hence other scRNAseq datasets do not contain any information about these cells. Papers which investigated the effect of breast cancer associated eosinophilia patient survival[633–635] used bulk sequencing data and analyzed it via a method called CIBERSORT[636]. CIBERSORT is based on a pre-designated gene expression signature for each type of immune cell and can be used to identify which immune cells are present in bulk sequencing data as well as estimate their percentage within that tissue. Their eosinophil signature is based solely on circulating eosinophils, either naïve or *in vitro* activated[637]. My own data, as well as data from others, have clearly demonstrated that there are subtypes of eosinophils expressing a variety of markers and gene signatures, depending on their surroundings. This was nicely demonstrated by Gurtner et al.[632] in their scRNAseq paper. They showed that eosinophils can adopt at least six different phenotypes according to their gene expression: Circulating eosinophils within the blood, progenitors and immature eosinophils within the bone marrow, basal eosinophils in the spleen and the stomach as well as activated eosinophils withing the intestine. Each of these subsets had a different gene expression. Additionally, they showed that infections increased certain subsets whereas it decreased other. A paper by Mesnil et al.[638] showed that within the lungs there are two different subtypes of eosinophils

(resident and induced eosinophils) which also displayed a very different gene expression signature. Thus, using a fixed gene expression signature to identify eosinophils, especially a signature based only on one subset of eosinophils not relevant for the studied tissue, will not allow for reliable enumeration of eosinophils in tumors, much less allow the researchers to make any conclusions about the activation status of these cells.

To summarize, identifying eosinophils via bulk sequencing using CIBERSORT is not reliable. The training set of this method must be updated, based on eosinophils isolated from several different tissues. I did not use gene expression analyses to identify eosinophils in tissues, but when I analyzed several eosinophil specific genes in bulk tumors via qPCR, such as EPX, SiglecF, IL5R and CCR3 (data not shown) I saw that these genes were barely expressed, even in eosinophilic tumors, and could not use their expression to conclude whether or not eosinophils were present in the tumors. This may be due to known discrepancies between RNA and protein expression for proteins with long half-lives.

An additional difficulty in identifying eosinophils via gene expression is that canonical markers, such as their granule proteins eosinophil peroxidase (EPX) or major basic protein (MBP), are not expressed at the mRNA level throughout their development. Granulopoiesis, the process in which eosinophilic granules are formed and filled with granule proteins, occurs during the progenitor stage[389]. The EPX and MBP genes (*EPX* and *PRG2* respectively) are only expressed in progenitor eosinophils and are downregulated in mature cells, i.e. in eosinophil which have left the bone marrow[632]. Thus, canonical eosinophil genes cannot be used to identify mature eosinophils.

Overall, reliable identification of eosinophils via gene expression analysis is not yet possible. More scRNAseq experiments are required to identify an eosinophil core signature or specialized signatures for each tissue. The main difficulties are that the mainstream scRNAseq process destroys eosinophils and that these cells consist of several different subsets, depending on their location and the health status of the organism, making it difficult to assign them only to one gene expression signature.

Identification of eosinophils via histology

The identification of eosinophils via histology is the oldest method. Using a common hematoxylin/eosin (H&E) stain, pathologists have identified eosinophils based on their strong eosin stain and the shape of their nucleus. As mentioned above, human eosinophils have a bi-lobed nucleus whereas murine eosinophils were traditionally thought of as having a doughnut shaped nucleus. It is important to know that a paper in 2016 by Valencia et al.[639] demonstrated that one cannot solely rely on nuclear shape to identify eosinophils and especially not to distinguish them from neutrophils. They showed that upon *in vivo* allergen challenge, eosinophils displayed a segmented nucleus, a phenotype usually ascribed to neutrophils. I used both H&E staining as well as Sirius red staining to identify eosinophils. Both of these methods are easy to implement. H&E has the advantage that it allows an experienced researcher to also make conclusions about other cells present in the samples, whereas Sirius red only marks eosinophils, which makes their quantification simpler,

especially through image analyses methods. Finally, antibody staining with anti-EPX or anti-MBP are also used in some papers, allowing for easy identification. During my thesis I tested several commercial anti-EPX and anti-MBP antibodies and none of them worked. The only EPX and MBP antibodies which did work were provided by Elizabeth Jacobsen[640] and identification of eosinophil subsets was difficult, due to the limited number of antibodies which can be combined in histology at once and because most other antibodies do not work on fixed sections.

Unfortunately, only a minority of papers that analyze eosinophils via flow cytometry or gene expression analyses use a histological stain to confirm the identity of the cells and when they do, their images sometimes contradict their claim of having a pure eosinophil population. Histology is a reliable method to identify eosinophils in tissues as well as the circulation and has the added advantage of spatial analysis in solid tissues. It should be noted that in the early stages of eosinophil differentiation, the granule proteins which cause the strong eosin stain are not yet present in large quantities and that upon granulation, eosinophils lose most of their granules. Thus, in these two circumstances histological analyses is more difficult, whether it is done by chemical staining or antibody staining.

Conclusion about eosinophil identification

As discussed above, there are several issues with each method used to identify eosinophils. Flow cytometric analysis allows for relative fast identification and cell count and can also be used to identify several subtypes of eosinophils at once. It is commonly used in pre-clinical models but there is no report which uses flow cytometry to identify eosinophils in breast cancer to assess their impact on tumor progression. Gene expression methods are not yet precise enough to use for eosinophil identification in bulk sequencing data and there is no established pipeline which allows for scRNAseq. Histology is in my opinion the most reliable method, especially antibody-based staining of EPX and MBP and the Sirius red stain, but if eosinophils are still in the progenitor state or have degranulated, reliable identification becomes more difficult. To my knowledge, neither of these proteins has been found in other cell types and Sirius red specifically reacts with the granules of eosinophils (and Paneth cells, which can easily be distinguished from eosinophils, data not shown). I mainly used flow cytometry to identify eosinophils in tissues but I confirmed my gating strategy using histology, which should become a standard in future research to ensure the identity of the cells analyzed.

Role of eosinophils in tumor mouse models

The role of eosinophils in breast cancer is still not clear. There are three different types of datasets which can be analyzed to try and define this role: Patient blood eosinophil count, patient tissue eosinophil count and results from mouse model experiments.

Studies analyzing the effect of blood eosinophilia in breast cancer, i.e. comparing breast cancer patient survival between groups with high vs normal amounts of eosinophils within the circulation, indicate that

blood eosinophilia tends to be beneficial[641–643] although one report found a decrease in disease-free survival (DSF) in Her2+ breast cancer when blood eosinophilia was present[644]. Other papers have shown that an increase in blood eosinophilia upon breast cancer therapy, not necessary a high basal level of blood eosinophils, is beneficial for the patient[645,646]. Finally, there is one report that showed no effect of blood eosinophilia on breast cancer patients' survival[647]. I induced blood eosinophilia in all my experiments by treating the mice with AB5-IL5 yet saw no changes in tumor progression compared to the control group. The effect of blood eosinophilia in patients analyzed by cancer subtype was shown to be most beneficial in the Luminal A subtype[643] and I used triple negative breast cancer models. Hence, it is possible that more aggressive tumor models are not strongly affected by blood eosinophilia.

Reliable characterization of tumor associated eosinophilia (TAE) is more difficult, as discussed above. In general, there are only a handful of papers that analyzed TAE in breast cancer. The only papers which looked at TAE in correlation with patient survival, of which to my knowledge exist only three, used CIBERSORT to identify eosinophils. As discussed above, this method has several caveats and the results must be taken with a grain of salt. All three papers agree that eosinophils are a rare immune population within breast cancer. Two of them have shown that TAE is beneficial for the patients (at least in some subtypes of breast cancer)[634,635] and one showed the opposite[633]. Two more papers used histological analyses to establish the number of eosinophils in breast cancer patient samples. Neither of them looked at the association between TAE and survival, but both indicate that TAE is correlated with increased tumor aggressiveness, although no causal link was shown[648,649]. I was able to induce TAE, and confirmed this by both flow cytometry and histology, but I saw no effect on breast cancer progression, in contrast to human data. But because the identification of eosinophils in these datasets is not reliable, it is possible that, contrary to their results, eosinophilia has no causative effect on breast cancer progression. My own analyses of available patient datasets, which was based on the assumption that breast tumors expressing high levels of CCL11 have more eosinophils than those with low CCL11 expression, indicated no clear role of these cells when the patients were not divided according to breast cancer subtype (see figure10). Upon analyzing the subtypes, I found that eosinophils were consistently beneficial for patients with basal breast cancer. This might partially explain the discrepancy observed in their overall effect on other patient datasets as none of them were separated according to breast cancer subtype. Furthermore, no correlation between blood and breast cancer eosinophilia has ever been established in human datasets. There is one study[408] which used a small dataset of 22 patients where they showed that this correlation was not present, but larger studies are needed to confirm or reject this result. Of note, there is one paper which showed a positive association between blood and cancer tissue eosinophilia in patients with melanoma[650].

To summarize, human data about the role of eosinophils in breast cancer is not conclusive. This can be due to the methods used to identify eosinophils, the subtypes of breast cancer analyzed or because eosinophil do not directly affect breast cancer and other factors must be considered in addition, such as the presence of other immune cells, the activation status of the eosinophils or the overall health of the patients.

There are some, although few, reports which analyze the effect of TAE on breast cancer in mouse models. Here one has to distinguish between correlative and causative data. To my knowledge there are only three papers using breast cancer mouse models which show a causative effect of TAE on breast cancer progression.

Grisuaru-Tal et al.[616] used both orthotopic tumor and i.v. injected metastases models to show that eosinophils spontaneously increase within the affected tissue, i.e. the primary tumor in the mammary gland and the lungs respectively. The former was shown in both Balb/c mice with 4T1 cancer cells and C57BL/6J mice with MMTV-PyMT cancer cells, whereas the latter only in the MMTV-PyMT model. Upon loss of TAE, using either eosinophil deficient mice or SiglecF-antibody mediated ablation, they observed increased metastatic burden after i.v. injections. Although the exact mechanism is not clear, it seems that eosinophils might increase the activation of tumor infiltrated lymphocytes (TILs), which could lead to the observed phenotype. My data in the i.v. injected 4T1 lung metastasis model did not show a spontaneous accumulation of eosinophils in metastasis bearing lungs (see figure 23). Grisaru-Tal et al. only showed a causative effect of eosinophils in the MMTV-PyMT i.v. injected model and not in the 4T1 mouse model or an orthotopic MMTV-PyMT model which I used, so I cannot compare my data directly with theirs because it is well known that the tumor type and microenvironment play a crucial role in cancer and there is a significant difference between the 4T1 breast cancer model and the MMTV-PyMT breast cancer model as well as the lungs and the mammary gland. I did not see a change in the number of metastases in eosinophilic lungs compared to control treated ones, nor a difference in $PD1^+$ CD8 T cells, or in their memory or effector subsets (see figure 23 and data not shown). Although I did not use an eosinophil deficient mouse model as a control, my data on i.v. injected 4T1 breast cancer cells indicate that eosinophils do not play a role in metastatic seeding in this model. However, the direct comparison is difficult, since it remains possible that an increase of eosinophils (as in my model) doesn't produce enhanced tumor phenotypes whereas a complete depletion may show an effect.

A spontaneous accumulation of eosinophils within metastatic lungs was also demonstrated by Cederberg et al.[617]. They injected E0771 cells i.v. into mice and analyzed metastatic burden. They saw that eosinophilic mice (IL5Tg, see introduction) displayed smaller lung metastases than WT mice. They also observed that eosinophil deficient mice had larger lung metastases than WT mice. Upon depletion of eosinophils via SiglecF-antibody injections, they observed the same phenomenon. Their data proved that eosinophils have a causative effect in reducing tumor burden in the E0771 i.v. injected mouse model of breast cancer. I injected E0771 breast cancer cells orthotopically (and did not observe significant changes upon TAE) rather than i.v. and cannot directly compare my results to theirs.

Zheng et al. demonstrated that both primary orthotopic E0771 and MMTV-PyMT tumors in C57BL/6J mice did not change upon SiglecF-antibody mediated eosinophil ablation. My experiments showed the same, namely that the primary tumors of none of the models used, including E0771 and MMTV-PyMT, were affected by an increase in TAE.

In conclusion, it seems that in mouse models of breast cancer, TAE has a beneficial effect when the cancer cells are injected i.v. Their role in orthotopic models is less clear. The available data is sparse and only exists for MMT-PyMT and E0771 orthotopic models, where it indicates no effect on primary tumor growth, a result confirmed by my own data. Its effect on spontaneous metastases from primary tumors has not been analyzed yet in any of the models so I cannot compare my data with others. This lack of information is one of the reasons I decided to use an orthotopic model, another being that it reflects the clinical situation better than i.v. injected models. Although I was able to induce TAE in four different mouse models (4T1 and EMT6 in Balb/c and E0771 and MMTV-PyMT in C57BL/6J) I saw no effect of TAE on breast cancer progression, neither on primary tumor weight nor metastatic spread. I should note that because I did not use eosinophil deficient mouse models, I can neither confirm nor deny a role for eosinophils in the tested models, but rather a lack of effect of induced TAE in breast cancer.

There are several reasons why I did not see an effect of TAE on breast cancer. One possibility is that, at least in the models used, eosinophils have no effect on primary tumor growth as confirmed by others[615,616], nor spontaneous metastatic seeding, as suggested by my data but never analyzed by others. Because there is no human data which has proven a causative effect of TAE on breast cancer progression, especially when considering that data which identified eosinophils via gene expression in patient samples cannot be trusted, this is indeed one possibility. Eosinophils are strongly understudied in breast cancer, and more studies are required to confirm or deny this hypothesis, something my work contributes to.

Another reason for the absence of an effect of TAE on breast cancer might be the type of eosinophil infiltrating the tumor and/or the microenvironment. In colon cancer, eosinophils have been shown by several researchers to have an anti-tumorigenic effect, both in pre-clinical[149,397,571] and clinal data[573,576,577,651]. It would be very interesting to compare eosinophils from colorectal and breast cancer in order to see if the lack of effect in breast cancer might be due to some intrinsic property of the cells or if the microenvironment dictates their effect on tumor progression.

Interplay between eosinophils and neutrophils

I observed an anticorrelation between tumor associated eosinophil (TAE) infiltration and tumor associated neutrophil (TAN) infiltration in cancer models in the Balb/c background. This anticorrelation has also been observed by other researcher in different contexts. Jacobsen et al.[652] demonstrated that in an OVA-induced asthma model in iPHIL mice (see introduction), eosinophils significantly accumulated within the lungs whereas neutrophil accumulation remained low. Upon eosinophil ablation through diphtheria toxin (DT) administration, lung neutrophilia significantly increased. Once DT was no longer administered, lung eosinophilia returned and lung neutrophilia was suppressed. Similarly, Wang et al.[653] observed that depletion of eosinophils, using either anti-SiglecF-antibody treatment or two different eosinophil deficient mouse models, caused significant accumulation of neutrophils within the liver upon injury. Arnold et al.[654] analyzed the interplay between eosinophils and neutrophils in bacterial induced gastrointestinal

inflammation. They found that bacterial infection in wild type (WT) mice caused increased accumulation of eosinophils within the gastrointestinal (GI) tract. Upon depletion of these cells, neutrophil numbers significantly increased. It should be noted that this anticorrelation was only observed in infected mice, healthy mice had the same number of neutrophils within the GI regardless of the presence or absence of eosinophils.

A possible mechanism for this anticorrelation between eosinophils and neutrophils was shown to be through IL4 and/or IL13. Eosinophils are known to release large amounts of IL4 and IL13 upon activation (see introduction). Woytschak et al[655]. showed that upon treatment with G-CSF of healthy mice, there was a significant increase in bone marrow (BM) and splenic neutrophils, whereas circulating neutrophils only showed a tendency to increase. Addition of IL4 to the G-CSF treatment reduced neutrophilia in the BM and the spleen back to base level, whereas it reduced blood neutrophils even more than at base level. Impellizzieri et al.[656] demonstrated that eosinophils might block accumulation of neutrophils via IL4 (and IL13) by inhibiting their migration towards the tissue. Upon addition of increasing amounts of IL4 or IL13, the authors showed that human neutrophils had decreased migration towards CXCL8, one of the main chemoattractant for neutrophils.

These papers suggest that eosinophils, in my case TAEs, can suppress the accumulation of TANs into the tumor microenvironment (TME) via secretion of IL4 and IL13. It should also be noted that eosinophils can induce a type 2 immune response, causing increased production and secretion of IL4 and IL13 by Th2 cells. This would further lead to an inhibitory effect of neutrophil migration into the tissue. Because I did not analyze the secretome of TAEs I can neither confirm nor deny that the suppression of TANs occurred through this mechanism.

TANs are known to be associated with increased tumor aggressiveness in many cancers, including breast cancer[657–659]. Despite a significant anti-correlation between TAEs and TANs in all my experiments in the 4T1 breast cancer model, including i.v. injected cancer cells, I saw no effect on breast cancer progression. At first this seemed counterintuitive as suppressing a pro-tumorigenic cell should lead to (partial) tumor suppression. But it seems that in the 4T1 breast cancer model, TANs have no effect on cancer progression as demonstrated by Tabariès et al.[660]. After i.v. injection of 4T1 cancer cells they ablated neutrophils via anti-Ly6C/Ly6G antibody but did not observe a difference in metastases. In conclusion, although TAEs suppressed TANs in the 4T1 breast cancer models but this did not translate into changes in tumor progression.

Lastly, I consistently observed a SiglecF$^+$ TAN population in breast cancer models in the Balb/c background. Expression of SiglecF on TANs had already been demonstrated in a mouse model of lung cancer by the Pittet laboratory[661,662] and by Simoncello et al.[663]. These cells have also been observed in non-cancerous models[629,664,665]. In most of these papers, the authors confirmed that these cells were neutrophils by using histological analyses as well as analyzing their granularity and I fully believe that what they termed SiglecF$^+$ neutrophils, are indeed neutrophils. But my results would suggest that what I call SiglecF$^+$ TANs might be

eosinophils, for several reasons. First, they displayed the same high granularity as eosinophils, their accumulation almost always positively correlated with TAEs and I clearly observed a few eosinophils within the Ly6G^{high} population in my cytospins (see figure 15). Unfortunately, I was not able to isolate these SiglecF^{+} TANs during FACS and analyze them via histology, thus their exact identity is unclear. It would have been interesting to see if these cells are also present in mouse models genetically deficient of eosinophils, which could have proven or disproven my hypothesis that they are in fact Ly6G^{high} eosinophils, rather than neutrophils.

Conclusion

In my thesis I set out to analyze the effect of breast cancer associated eosinophilia on tumor progression. I encountered a first hurdle upon trying to find a reliable method to identify eosinophils and differentiate them from other immune cells. I found that distinguishing eosinophils from neutrophils via flow cytometry was not straightforward and that many published papers did not necessarily provide enough evidence that their gating strategy did so. When analyzing available human data on the effect of eosinophils on breast cancer progression, I observed that different studies showed contradictory results, especially when they analyzed the effect of tissue associated eosinophilia in breast cancer. These contradictions might very well be due to the methods these papers used to identify eosinophils which were based on gene expression. I have shown that this method needs to be updated before it can be used reliable to identify eosinophils, and their subsets, in patient samples. My results showed that eosinophils did not play a role in primary tumor growth nor metastasis in four different mouse models tested. At first glance, this might be in contrast to the few pre-clinical studies published, but a close analysis revealed that I used very different methods, namely orthotopic injections in contrast to intravenous injections of breast cancer cells. I showed that in the Balb/c background, eosinophils excluded neutrophils from the primary tumors and the metastatic lungs but this had no effect on breast cancer progression. Finally, I observed a SiglecF^{+} immune cell population which initially I thought to be neutrophils although further analyses are needed to confirm their identity.

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