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Characterization of eosinophil development and lineage expansion through transcriptomic and high-dimension flow cytometric approaches

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1. Abstract

Eosinophils are specialized granulocytes predominantly considered for their diagnostic value in type 2 immune disorders, but have also been attributed roles in immune homeostasis, microbial defense, metabolism, or anticancer protection. Despite the increasing use of biological therapies targeting eosinophils through their dependency on Interleukin-5 (IL-5), the biological activities, ontogeny and mechanisms of lineage expansion of eosinophils are less resolved than those of other immune cells. We integrated single-cell proteomics and transcriptomics with a novel IL-5Rα reporter (IL5RAporter) mouse model to comprehensively resolve eosinophil development. This approach reconciled human and murine eosinophilopoiesis and facilitated further study of the eosinophil lineage. We observed that the eosinophil lineage expands via a transit amplification mechanism enabled and promoted by IL-5 bioavailability. Eosinophil lineage transit amplification was characterized by increased cycling activity, prolonged proliferative capacity, and delayed maturation of committed eosinophil progenitors. Conversely, deletion or neutralization of IL-5 attenuated eosinophil progenitor transit amplification without compromising maturation, challenging previous assumptions. Further comparison of residual eosinophils in IL-5-depleted murine or human hosts indicated that IL-5 depletion does not impair eosinophil maturation. Overall, this work provides valuable resources and insights into eosinophil ontogeny, the effects of precision therapeutics, and the regulation of eosinophil development in health and disease.

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2. Introduction

2.1 What is an eosinophil?

Eosinophils are leukocytes, more precisely a particular member of the granulocyte group. In the 1870's, Paul Ehrlich established staining procedures for blood granulocytes. He described cells he called "eosinophils" because of the red tint that their granules harbor in brightfield microscopy following staining with the acidic dye eosin (Kay, 2015). Those remarkably specialized immune cells originate, develop, and mature in the bone marrow before transiting and migrating via the blood to different locations under physiological or pathological conditions (Rothenberg & Hogan, 2006). While eosinophils are present in relatively low amounts in the blood of healthy individuals, their numbers can drastically increase under certain circumstances such as infection by helminths and other parasites which first brought eosinophils in front of the scene, as well as in malignancies, and a plethora of allergy-related diseases now grouped under the term eosinophil associated diseases (EADs) (Wechsler et al., 2021). EADs are currently capturing most of the attention because of their elevated prevalence and socioeconomic burden worldwide (Braman, 2006; Loftus & Wise, 2015). The biological roles of eosinophils are still heavily debated and they have been alternatively seen as protectors and troublemakers, while it is still unclear if they are true actors or mere bystanders in certain conditions (Mitre & Klion, 2021; Syeda et al., 2023). Eosinophil granules are loaded with cytotoxic proteins as well as inflammatory and immunomodulatory cytokines chemokines and mediators (Davoine & Lacy, 2014). Although this very high cytotoxicity may grant them the ability to protect us against various types of infections, it also hints that they may be major culprits in EADs. The suspected detrimental role of eosinophils in EADs led to the development of monoclonal antibody-based eosinophil-targeting and -depleting precision therapies (Klion et al., 2020). Currently used "anti-eosinophil" precision therapies include recombinant monoclonal antibodies targeting the main eosinophil cytokine interleukin-5(IL-5), namely reslizumab and mepolizumab, or antibodies that bind to the IL-5Rα subunit of the IL-5 receptor and lead to eosinophil depletion through antibody-dependent cell cytotoxicity, called benralizumab (E. A. Jacobsen et al., 2021). The implementation of these anti-eosinophil biologics further stimulated

the eosinophil research field, which also saw eosinophils being increasingly credited with an abundance of homeostatic functions, from organ development and tissue repair and remodeling to energy metabolism and immunoregulation (Marichal et al., 2017).

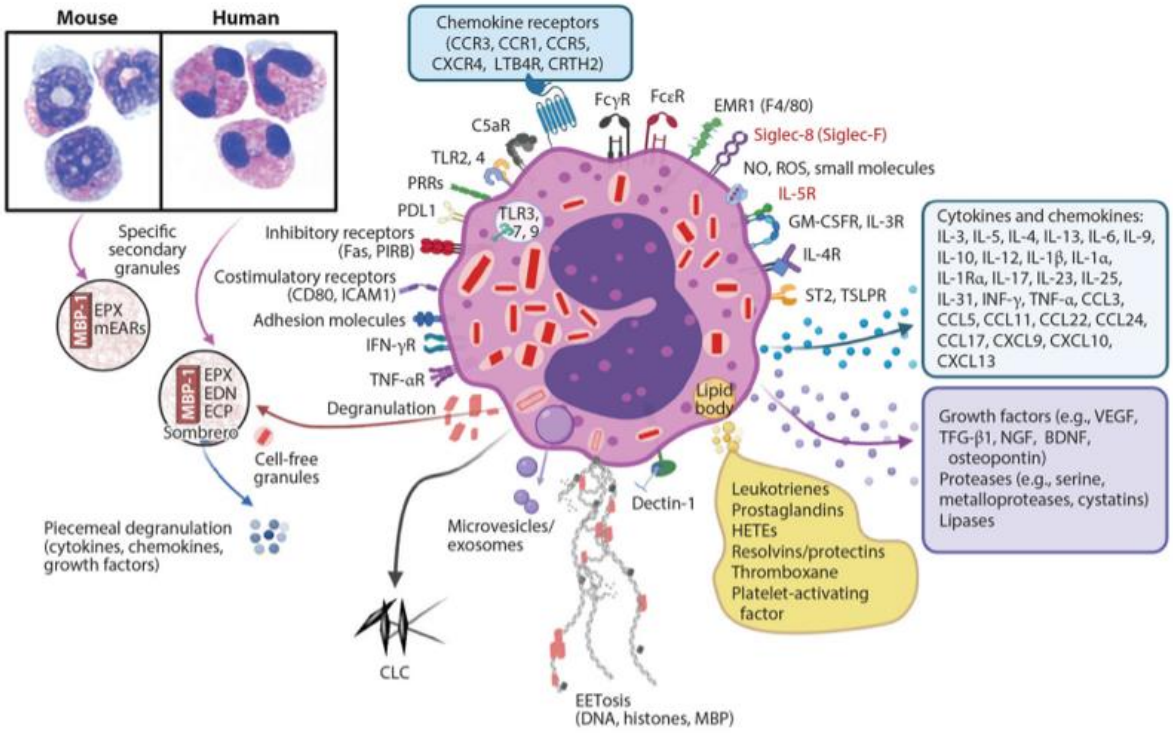


Figure 1: Main eosinophil surface markers, granule proteins and secreted factors

From (E. A. Jacobsen et al., 2021)

Abbreviations:

BDNF, brain-derived neurotrophic factor; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CLC, Charcot–Leyden crystal protein; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; FcεR, Fc-epsilon receptor; FcγR, Fc-gamma receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; HETE, hydroxyeicosatetraenoic acid; ICAM1, intercellular adhesion molecule 1; LTB4R, leukotriene B4 receptor; MBP-1, major basic protein 1; mEAR, mouse eosinophil-associated ribonuclease; NGF, nerve growth factor; NO, nitric oxide; PIRB, paired immunoglobulin-like receptor B; PDLI, programmed death ligand 1; PRR, pathogen recognition receptor; ROS, reactive oxygen species; Siglec, sialic acid–binding immunoglobulin-like lectin; ST2, suppressor of tumor 2 [also known as IL-1RL1 (interleukin-1 receptor-like 1)]; TGF-β1, transforming growth factor beta 1; TLR, Toll-like receptor; TSLPR, thymic stromal lymphopoietin receptor; VEGF, vascular endothelial growth factor.

2.1.1 Eosinophil content and main markers

This section intends to give a general overview of eosinophil biology, including their major hallmark intracellular proteins and cell surface markers.

Eosinophil identification

Eosinophils are easy to identify. The eosin dye that stains eosinophil granules in red, giving eosinophils their name, is sufficient to identify them in bright field microscopy (J. J. Lee et al., 2012). In flow cytometry, the combination of just 3 parameters suffices to identify eosinophils; eosinophils display elevated granularity, observable by the side scatter (SSC) parameter, along with surface expression of C-C motif Chemokine Receptor 3 (CCR3) and Sialic acid-binding Ig-like lectin 8 (Siglec-8) in humans or Siglec-F in mice. CCR3 is the receptor to eotaxins and other chemokines recruiting eosinophils to different tissues. High levels of CCR3 expression are restricted to eosinophils in mice but CCR3 is also expressed on basophils in humans (Uguccioni et al., 1997). In humans again, Siglec-8, which has been shown to promote eosinophil apoptosis, can also be found on mast cells (Youngblood et al., 2021). In contrast, Siglec-F is also expressed on different types of macrophages in mice (Feng & Mao, 2012). Another frequently used marker for eosinophils in flow cytometry is EGF-like module containing mucin-like hormone receptor 1 (EMR1), a surface receptor of yet unknown function. EMR1 is specific to eosinophils in humans, while its ortholog F4/80 in mice is also expressed on monocytes and macrophages (Hamann et al., 2007; Legrand et al., 2014). Other relevant markers at the surface of eosinophils are ST2, the receptor for the alarmin interleukin-33 (IL-33), a potent activator of eosinophils, as well as receptors for cytokines, especially the IL-5Rα subunit of the IL-5 receptor whish also named CD125 (Johnston et al., 2016). The receptor to IL-5, the main eosinophilopoietin, Is a heterodimer composed of two different chains: the alpha chain or IL-5rα specific for IL-5 and the beta chain, which is shared with the receptors to Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (Martinez-Moczygemba & Huston, 2003). Finally, it is also worth mentioning that an array of adhesion molecules is present at the surface of eosinophils

along with Toll-Like Receptors (TLRs) and Pattern Recognition Receptors (PRRs) that allows them to recognize foreign organisms or damage to tissues (Gigon et al., 2023).

Cytokines, chemokines, growth factors and lipid mediators

Eosinophils store and secrete a large panel of cytokines and chemokines (fig1). Major ones are Inflammatory and pyrogenic cytokines such as IL-1β, Tumor Necrosis Factor (TNF)-α, and IL-6 along with the anti-viral Interferon-γ and the immunoregulatory IL-4 (Davoine & Lacy, 2014). The production of other cytokines by eosinophils is currently debated, notably that of IL-5 and IL-13 as evidence is scarce or of poor quality. We suspect that the alleged production of IL-5 and IL-13 by eosinophils could be due to contamination of eosinophil populations by Innate Lymphoid Cells type 2 (ILC2).

Eosinophils also secrete growth factors such as vascular endothelial growth factor (VEGF) which favors angiogenesis and Transforming growth factor-β(TGF-β) that can induce fibroblast proliferation and activation (Hoshino et al., 2001; Ohno et al., 1992). Together with metalloproteases, these growth factors grant eosinophils with the ability to degrade and remodel the extracellular matrix.

The arsenal of eosinophils also includes lipid mediators, of which the pro-inflammatory Leukotrienes B4 (LTB4) and C4 are probably the most representative (Bandeira-Melo & Weller, 2003). Platelet activating factor is also worth mentioning since it allows eosinophils to play a role in coagulation (Coden & Berdnikovs, 2020; Pałgan & Bartuzi, 2015).

Granules proteins

Eosinophil secondary granules can be recognized by a crystal of major basic protein (MBP) at their center (fig1). Inside those granules, Pro-MBP is processed into its mature form which is then stored in a crystalloid core. This process is thought to protect the cell from the extreme basicity and cytotoxicity of MBP (Soragni et al., 2015). Eosinophil Derived Neurotoxin (EDN, also called RNASE2) and Eosinophil Cationic Protein (ECP, also called RNASE3) are abundant proteins

within eosinophil granules but are specific to humans. These two proteins result from a gene duplication event (Bystrom et al., 2011; Rosenberg et al., 1995). They are members of the RNAse A superfamily and both possess cytotoxic and ribonuclease activity. On their side, murine eosinophil granules contain several mouse Eosinophil Associated Ribonucleases (mEARs) with activities similar to EDN and ECP (Rosenberg, 2015). Another eosinophil granule protein that is present in human but no murine eosinophils is the Charcot-Leyden Crystal protein (CLC), also called Galectin-10 (Aegerter et al., 2021). As its name suggests, CLC can crystallize in the extracellular milieu and form spiky crystals of various structure. CLC crystals thicken the mucus and exacerbate type 2 inflammation.

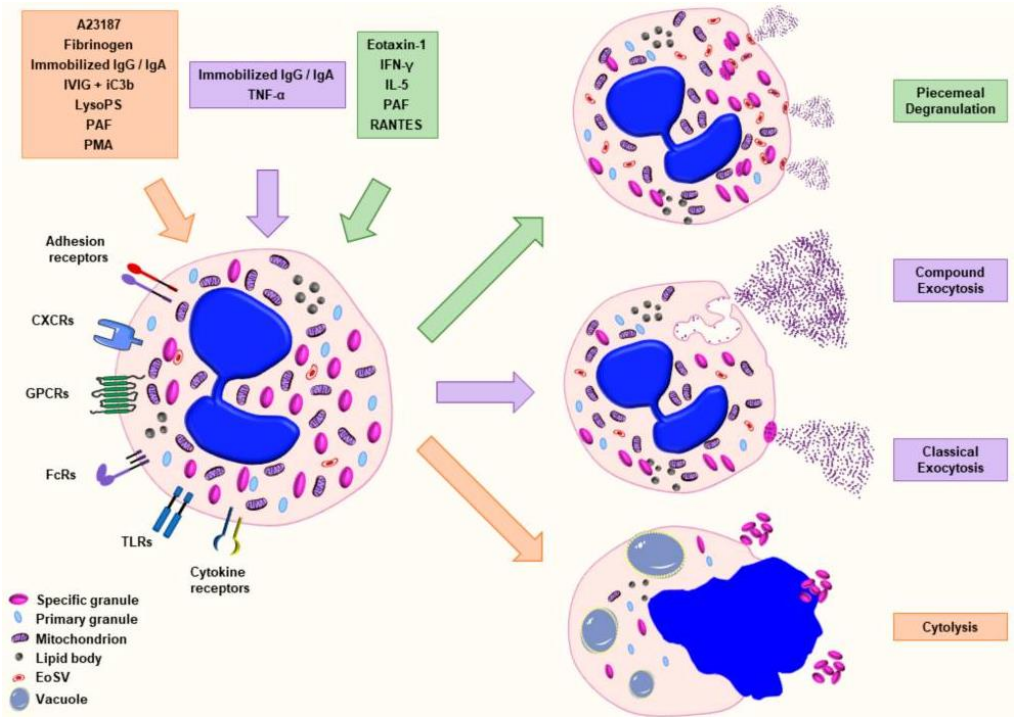


Figure 2: Different mechanisms of eosinophil degranulation

From (Fettrelet et al., 2021)

Since CLC seems to play a particularly important role in human eosinophil-associated diseases and there is no equivalent in mouse, genetically modified mouse models have been generated in which epithelial cells or eosinophils produce human CLC. Potentially therapeutic antibodies have also been generated that are able to dissolve the CLC crystals ex vivo (Persson et al., 2019). Finally, in both humans and mice a myeloid peroxidase (MPO)-related enzyme, Eosinophil Peroxidase (EPX), participates to eosinophil cytotoxicity by catalyzing oxidoreduction reactions and allowing eosinophils to generate high levels of ROS (Silveira et al., 2019). EPX, but also other eosinophils granule proteins are often measured in blood or sputum as biomarkers for EADs (M. Tang et al., 2024).

Eosinophil degranulation

Eosinophil possess an impressive panel of 4 different mechanisms of degranulation: classical exocytosis, compound exocytosis, piecemeal degranulation and cytolysis (fig2). Classical exocytosis happens via a fusion of the eosinophil granule membrane with the plasma membrane thereby releasing the granule content while compound exocytosis consists in a fusion of granules together combined with exocytosis releasing a massive amounts of granule content in a short period of time. Piecemeal degranulation is a less aggressive process in which specialized vesicles, called Eosinophil Sombrero Vesicles (EOSVs) progressively select, transport and release parts of the secondary granule content (Neves et al., 2024). The last process, cytolysis is a non-apoptotic form of cell death and a very inflammatory process and in which the plasma membrane of the eosinophil ruptures and intact granules and CLC are released (Erjefält et al., 1999).

Like neutrophils, eosinophils are also able to release extracellular trap-like (ETs) structures consisting of DNA associated with granule proteins and histones. Eosinophil ETs (EETs) released by so-called EETosis can trap microorganisms (Germic et al., 2021; Wechsler et al., 2021).

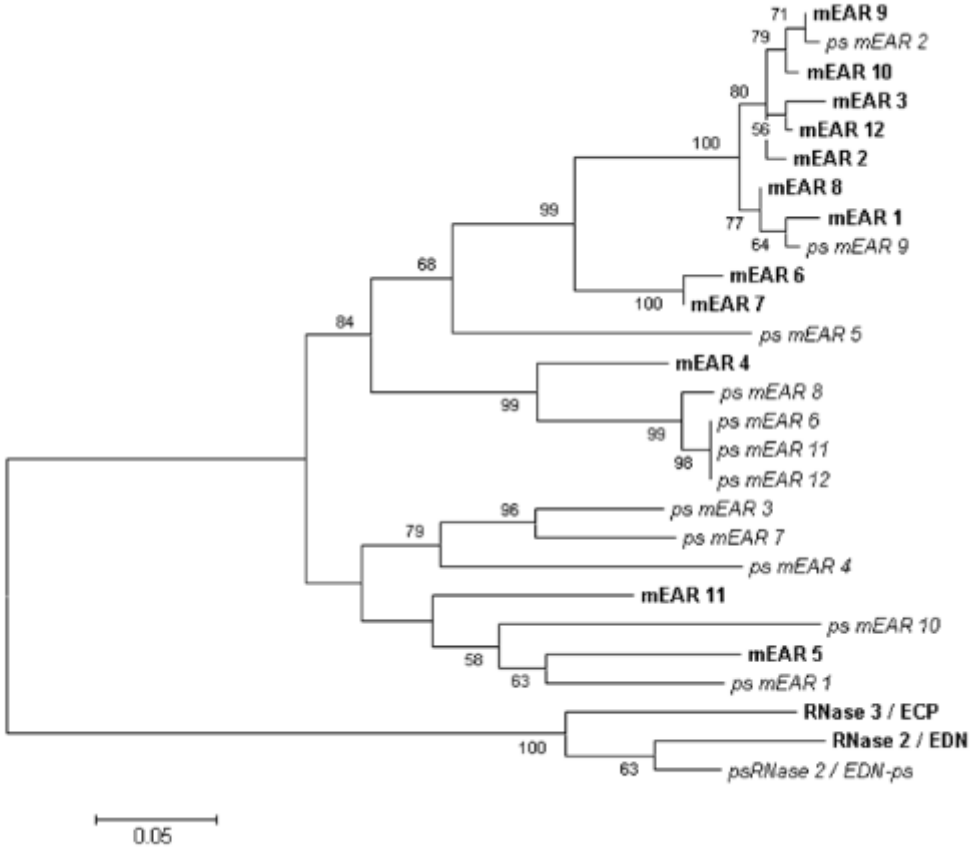


Figure 3: Phylogenetic tree documenting relationships among the mouse eosinophil-associated ribonuclease genes and pseudogenes.

From (Rosenberg, 2015)

RNAses

To say that eosinophils contain RNAses is quite an understatement. This is why these deserve another mention in their own subsection. To make a dubious but very visual analogy, we could say that eosinophils store RNAses merely a bit less than adipocytes store lipids. Indeed, eosinophils harbor an enormous variety and quantity of RNAses (Wilkerson et al., 2016). These RNAses harbor anti-microbial activities that have been linked to their ribonuclease activity (Eid & Borish, 2021). EDN activity was tested in vitro and was shown to have similar RNA degradation potential has bovine pancreatic RNase A (Slifman et al., 1986). In some cases, the deletion of this catalytic activity has been shown to drastically reduce their anti-microbial properties (Dyer & Rosenberg, 2006). In the proteome of human eosinophils, two of the 10 most abundant proteins are RNAses and EDN/RNASE2 is only second to Actin in this ranking (Wilkerson et al., 2016). Human eosinophils possess additional representatives of the RNase A superfamily such as Angiogenin or RNASE 5 and the epithelial-derived cytotoxin or RNASE 7.A total of 13 genes belonging to the RNase A family has been found on the human chromosome 14q11.2 (Cho et al., 2005).

Mice also possess an impressive panel of mEARS alongside a lot of pseudogenes reflecting the strong evolutionary pressure present on eosinophil associated RNAses (fig3). These murine eosinophil associated RNases also exhibit cytotoxic, anti-microbial and particularly anti-viral properties. Ribonuclease activity has been tested and observed in vitro for some of them. We can notably mention mEAR6, expressed specifically in response to pathophysiologic stress, and mEAR2 that have a catalytic activity similar to human EDN (McDevitt et al., 2001; Nitto et al., 2004). The catalytic activity of mEAR11, which is highly inducible by IL-33 stimulation, is lower but it has a potent chemoattractant activity for F4/80(+)CD11c(-) tissue macrophages (Yamada et al., 2015).

Eosinophil RNAses are still seldom-studied and a lot remains to be discovered or clarified about their functions. As a final element of importance regarding eosinophil studies, RNAses are suspected to cause most of the technical difficulties in analyzing eosinophils in Single cell RNA sequencing (ScRNAseq) (E. A. Jacobsen et al., 2021) .

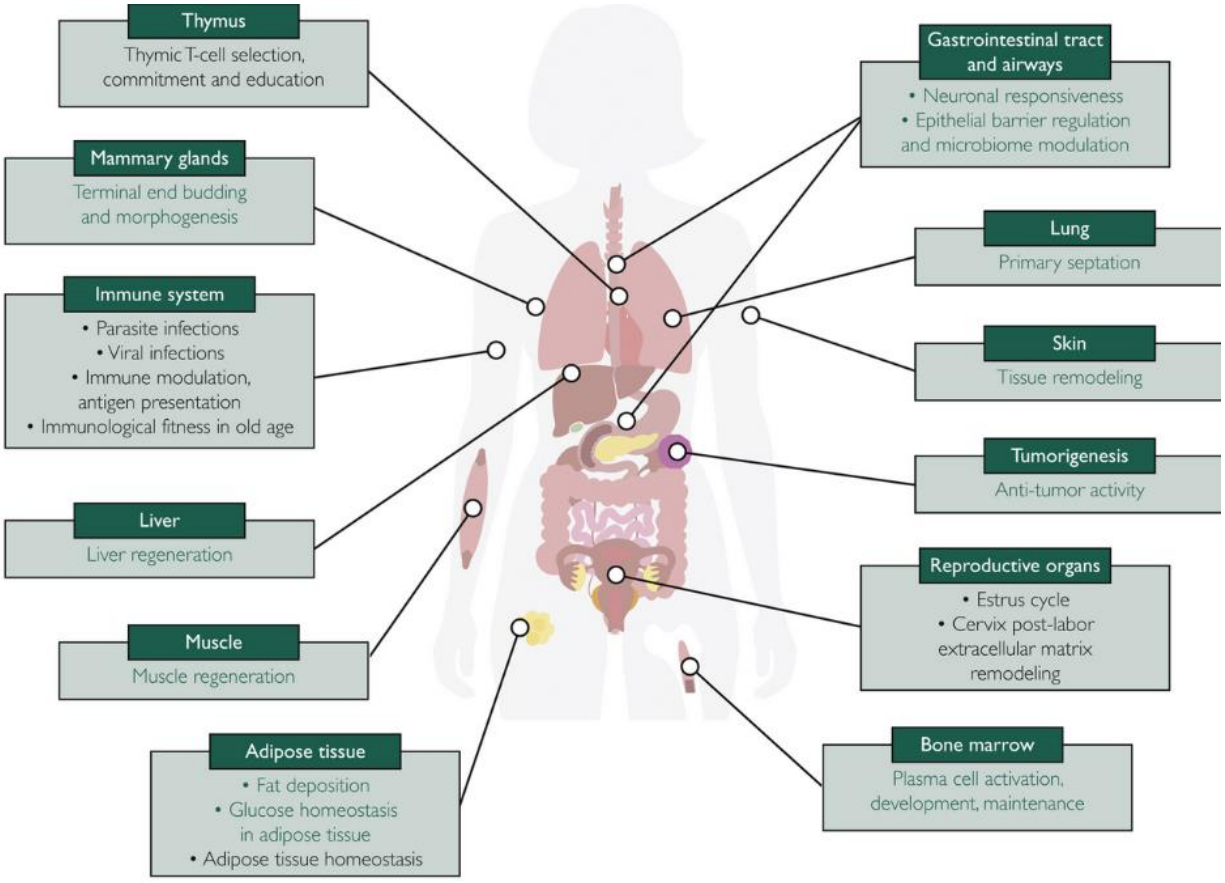


Figure 4: Proposed eosinophil roles in homeostasis and tissue development.

Note: In different reviews these proposed eosinophil roles are represented on human body. However, they may have been better represented on a mouse since most of these functions were investigated in mice and few translational data is available.

From (Wechsler et al., 2021)

2.2 Roles of eosinophils:

2.2.1 Homeostasis (metabolism, immune homeostasis, tissue repair)

The recent increase in attention towards eosinophils led to some interesting observations and discoveries regarding their potential functions in the steady state. Indeed, eosinophils have been described to be involved in the maintenance and regulation of different parameters of homeostasis (fig4). These notably comprise tissue repair and remodeling in several instances (uterus at pregnancy and post-partum, thymus, lung and mammary gland buds development), glucose metabolism in adipose tissue, plasma cell maintenance and class switching of B cells towards IgA production and potential modulation of t-cell maturation in the thymus (Albinsson et al., 2023; Chu et al., 2011; Cosway et al., 2025; E. H. Lee et al., 2018). However, it is important to be noted that those observations have been made using mouse models and that the roles of eosinophils in human homeostasis are still unclear. Notably mouse and human eosinophil display different cytokine and lipid mediators secretion abilities, which could explain their increased participation to certain functions in homeostasis in mice (Archambault et al., 2020). Moreover, patients treated with eosinophil depleting therapies or even benralizumab-treated eosinophil "KO" humans do not seem to display significant impairment in homeostasis due to their absence of eosinophils (Jackson & Pavord, 2023; E. A. Jacobsen et al., 2021).

Tissue development, repair and remodeling:

In mice, eosinophils have been shown to participate, along with macrophages, in mammary gland development, favoring ductal outgrowth into the mammary fat pad and terminal end bud formation (Gouon-Evans et al., 2000). At puberty, eosinophils are recruited towards growing terminal end buds via eotaxin. In eotaxin-deficient mice, eosinophil recruitment was abolished and duct and bud formation significantly reduced. In parallel, eosinophils are also recruited in the uterus with a peak during estrus (Marichal et al., 2017).They have also been proposed to be involved in uterus remodelling during menstruation and preparation for pregnancy. Furthermore, eosinophils could also be involved in tissue regeneration after acute injury. In

mice subjected to upperbody sublethal irradiation, eosinophils are recruited to the damaged thymus via CCL11, also known as eotaxin-1, and its receptor CCR3 at their surface (Cosway et al., 2025). This transient thymic eosinophilia is associated with thymus regeneration and is impaired in ΔdblGATA mice that do not harbor eosinophils. ILC-2s within the thymus have been described to participate in this process via their IL-5 production. Injection of IL-5 around the time of irradiation improved thymic regenerative capacity. Similar observations have been made regarding liver or skeletal muscle regeneration, which can be boosted by eosinophils in mice (Heredia et al., 2013; Xie et al., 2025). Once again in these models, eosinophil-deficient mice see a decrease in regenerative capacity while eosinophil repleted mice experience an improvement or restoration of this regenerative capacity. These regenerative activities and their contribution in macrophage stimulation and polarization have been linked to the production of IL-4 by murine eosinophils (Goh et al., 2013). Finally, eosinophil have been shown to infiltrate damaged lung and skin tissue in mice where they release growth factors that stimulates fibroblast proliferation and angiogenesis (Coden & Berdnikovs, 2020; Radonjic-Hoesli et al., 2021). In humans, a recruitment of eosinophils in burned skin tissue has also been observed but their functions are less clear than in mouse models. Since IL-4 is involved in most of these potential eosinophil functions, it could be hypothesized that they are more prevalent and apparent in mice due to a greater production and secretion of IL-4 by murine eosinophils. This difference in IL-4 production would explain a greater impact of eosinophils in homeostasis and development in mice than in humans.

Glucose metabolism in adipose tissue:

An unexpected role of eosinophils in metabolism has been revealed using mouse models fed with a high fat diet. Eosinophils have been found to be to the main producers of IL-4 in murine adipose tissue. Eosinophil-derived IL-4 induces the polarization of macrophages into alternatively activated macrophages, which contribute to glucose homeostasis (Wu et al., 2011). Interestingly, increased eosinophil numbers associated with helminth infection also decreased insulin resistance in infected mice. In line with these observations, eosinophil-deficient ΔdblGATA mice display decreased alternatively activated macrophages numbers, increased liver

fat deposition following high fat diet and lower glucose tolerance. This regulatory role of eosinophils in adipose tissue has recently been linked to eosinophil autophagy since a mouse model in which specifically eosinophils are lacking the autophagy-associated protein 5 displays altered adipose tissue cellular architecture, increased body weight and impaired glucose tolerance (Hosseini et al., 2023).

Plasma cell maintenance and B cell class switching:

In mouse studies, using the now classical comparison between wild type and eosinophil-deficient mice, eosinophils have been proposed to be essential to plasma cell survival and retention in the bone marrow (Chu et al., 2011). Eosinophils would sustain plasma cells via the secretion of growth factors such as the activation and proliferation-induced ligand (APRIL) and interleukin 6 (IL-6). However, more recent publications are challenging this essential role and propose that eosinophil are dispensable or might be just one of multiple cell types contributing to this process (Bortnick et al., 2018; Haberland et al., 2018). In parallel, it has been proposed that large numbers of eosinophils present in the lamina propria, which underlies the gut epithelium, could play an important role in gut homeostasis through the promotion of Immunoglobulin type A (IgA) class switching in B cells from germinal centers associated with Peyer's patches, as well as in the maintenance of IgA producing plasma cells in gut-associated lymphoid tissues (Berek, 2016; Chu et al., 2011). Using two different models of eosinophil deficient mice, Chu and colleagues observed a decrease in IgA+ plasma cells numbers and secreted IgA as well as defects in the intestinal mucous barrier and an altered gut microbiota in those mice (Chu et al., 2014).

T-cell modulation in thymus:

On top of their previously mentioned regenerative capacity, mouse studies led to the attribution of other roles to eosinophils in thymic development and function. According to the most recent review on this topic and its suggestive title: "Thymic Eosinophils: What Are You Doing Here?", the exact roles of eosinophils in the thymus microenvironment and in T cell development are still unclear (Gatti & Reynolds, 2025). However, we can notably mention that

eosinophils are found in different regions of the thymus such as the medulla and corticomedullary junctions and that they have been observed forming immunological synapses with thymocytes (Albinsson et al., 2021). In vitro, they have been reported to shift de development of CD4 CD8 double positive thymocytes towards CD4 single-positive at the expense of CD8 single-positive T-cells. Eosinophil numbers also increase in the thymic medulla during the neonatal period and express the class II major histocompatibility complex, a process that is dependent on an intact microbiota (Gatti et al., 2023). Finally eosinophils have been shown to be recruited to the thymus in models of acute thymocyte negative selection in which they are associated with apoptotic bodies throughout the thymus (Throsby et al., 2000).

2.2.2 Cancer, Eosinopenia and overall prognosis

Conflicting reports are found in the literature, with eosinophils proposed as having pro- or anti-tumoral properties depending on the immunological context and tumor microenvironment (Wagner et al., 2025). Eosinophils are indeed able to infiltrate mouse and human tumors (E. A. Jacobsen et al., 2021). Yet, eosinophil-deficient mice and patients treated with eosinophil depleting therapies so far do not display an increased risk of tumor development (E. A. Jacobsen et al., 2021). In mouse models of colorectal cancer, hepatocellular carcinoma and breast cancer, eosinophils have been observed to have a direct tumor cytotoxic effect following degranulation following their activation by IL-33 produced by the tumor (Hollande et al., 2019; Reichman et al., 2019). They have also been attributed an indirect anti-tumoral effect by increasing the infiltration of tumor specific CD8 T cells, thereby favoring immune control of the tumor (Carretero et al., 2015). In human, peripheral blood eosinophils have been described to be associated with better outcome of immune checkpoint inhibitors therapy such as anti-programmed cell death protein 1 (PD-1) antibodies or anti- cytotoxic T-lymphocyte protein 4 (CTLA-4) antibodies in melanoma patients (Simon et al., 2020). Once again, peripheral blood eosinophil counts were positively correlated with $CD8^+$ T cell infiltration of skin tumor tissues. Overall, eosinophils have been shown to be beneficial notably in colon cancer, melanoma, bladder and breast cancer while detrimental in cervical carcinoma and Hodgkin's lymphoma and some lung cancers.

The hypothesis of a beneficial role of eosinophils in certain types of cancer often arises from the observation that cancer patients with eosinopenia tend to have a worse prognosis. We have already seen that induced eosinopenia when depleting eosinophils or in eosinophil-deficient mice is not harmful. However, eosinopenia arising in the context of various non eosinophil-associated diseases could be a cause of concern. Indeed, eosinopenia tend to be a factor of worsening prognosis in various conditions such as intracerebral haemorrhage, chronic obstructive pulmonary disease, peritonitis or even COVID-19 (Bolayir et al., 2017; Cazzaniga et al., 2021; Jagdeesh et al., 2013; Mao et al., 2021; Weckler et al., 2024). The correlation of a worse prognosis in certain cancers with eosinopenia might hence not be due to a direct or indirect anti-tumoral role of eosinophils. We could rather hypothesize that eosinopenia may be related to a higher stress of the bone marrow or worse overall fitness of those patients at the point of examination, which would then be directly related with prognosis.

One should thus be careful while comparing prognosis with blood eosinophil levels. A mechanism for their action should be investigated in the various cancers types before one may conclude on a direct implication of eosinophils.

2.2.3 Infectious diseases

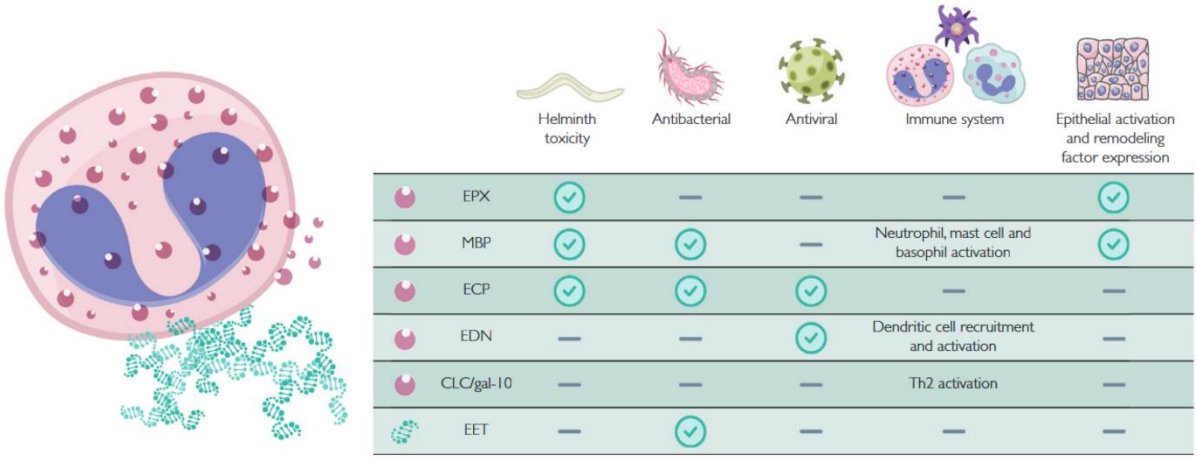


Figure 5: Roles of main eosinophil granule proteins in immune responses.
From (Wechsler et al., 2021)

Parasites

Helminth infections have shed light on eosinophils soon after their discovery and were one of the first known cause of eosinophilia. Increased blood or tissue eosinophil counts associated with helminth infection can be more frequent in travelers or tropical countries, where a substantial part of the population carries such parasites. In vitro studies first demonstrated that eosinophil accumulate around worms and target the parasites, degranulating proteins exhibiting helminthotoxic properties(fig5) (Ackerman et al., 1985). Since then, a lot of research has been performed on this topic and the roles of eosinophils in helminth infection appears to be context-dependent and is still not fully understood (Mitre & Klion, 2021). In some cases, such as infections with *Strongyloides stercoralis* and *Angiostrongylus cantonensis* eosinophils are clearly helping the host to clear the infection since eosinophil depletion leads to prolonged survival of larvae forms. However, in other cases such as infection with *Schistosoma mansoni*, although BMP and ECP are both toxic to the parasite, eosinophil depletion does not change the outcome of disease development (Huang & Appleton, 2016). In some instances, worms have

developped strategies to evade the immune system and benefit from the presence of eosinophils, which can lead to increased damage of host tissue or favor the parasite's survival, as in the case of *Trichinella spiralis* (Gebreselassie et al., 2012). A group of parasitic nematodes comprising *Necator americanus and Ancylostoma duodenale* often called "Hookworms" is responsible for a significant human health burden with an estimated 500 million people infected (Clements & Addis Alene, 2022; Loukas et al., 2016). These worms are well known to induce eosinophilia in humans with controlled infection trials bringing very robust data to this observation (Manurung et al., 2024). In our study we used a closely related murine model in which mice were infected with larvae of the nematode *Nippostrongylus brasiliensis* in order to induce eosinophilia and test eosinophil lineage expansion (Cortés et al., 2017). Finally, eosinophilia has also been observed following infection with other parasites, notably protozoans like *Toxoplasma gondii* and *Plasmodium falciparum* (Kurtzhals et al., 1998).

Bacteria

There is currently no consensus regarding the efficacy of eosinophils in combatting declared infections with significant bacterial load. However, eosinophils are equipped with antibacterial mediators and peptides as well as mechanisms to protect the host against pathogens. Eosinophils , albeit less than neutrophils, can phagocytose (Shamri et al., 2011). Eosinophils can also release nets of their own DNA coated with their granule proteins. These structures, called Eosinophil Extra cellular Traps (EETs), are able to embed bacteria thereby preventing their mobility and facilitating their clearance (fig5) (Yousefi et al., 2008). The main eosinophil granule proteins ECP and MBP have also been shown to exert bactericidal properties in vitro and eosinophils are also able to secrete the anti-microbial peptides alpha-defensins, which can perforate bacteria and fungi membranes (White et al., 1995).

Virus

The role of eosinophils regarding virus infection in human is yet another a topic that recently started to be investigated. It has been proposed that eosinophils and their secretions might promote host anti-viral defense. Eosinophil RNAses (EDN in humans and mEAR2 in mice) impede virus infectivity in vitro in the case of the respiratory syncytial virus (RSV) for humans and of Pneumonia virus of mice (PVM) for rodents(Rosenberg, 2015) (Phipps et al., 2007). Eosinophils might also be able to use their reactive oxygen species (ROS) producing ability as well as promotion of T-cell responses to participate in host anti-viral defense (Samarasinghe et al., 2017).

Fungi

Several fungal diseases have been associated with eosinophilia and mouse models suggests that eosinophils are beneficial in combatting fungal infections. These are not to be confused with fungal allergic disorders which will be discussed later and in which the fungal colonization is very minor to absent but the eosinophil response is major and deleterious (Gaur et al., 2022)(Klion et al., 2020) . Finally, EETs could also be involved in fungal destruction as alongside eosinophil secretion of antimicrobial peptides alpha defensins.

2.3 Eosinophil-associated diseases

As exposed above, eosinophils have an extensive arsenal of antimicrobial, cytotoxic and inflammatory mediators at their disposal as well as mechanism such as EETosis and cytolysis which have the potential to deal a lot of damage to host tissues if the response is not adequately controlled. Deregulation of eosinophil abundance and behaviors have been associated with a vast array of diseases grouped under the umbrella of eosinophil-associated diseases(EADs)(fig6). However, those diseases can be heterogeneous and complex harboring multiple endotypes and in some instances it is still unclear if eosinophils are active drivers of the pathology or mere bystanders.

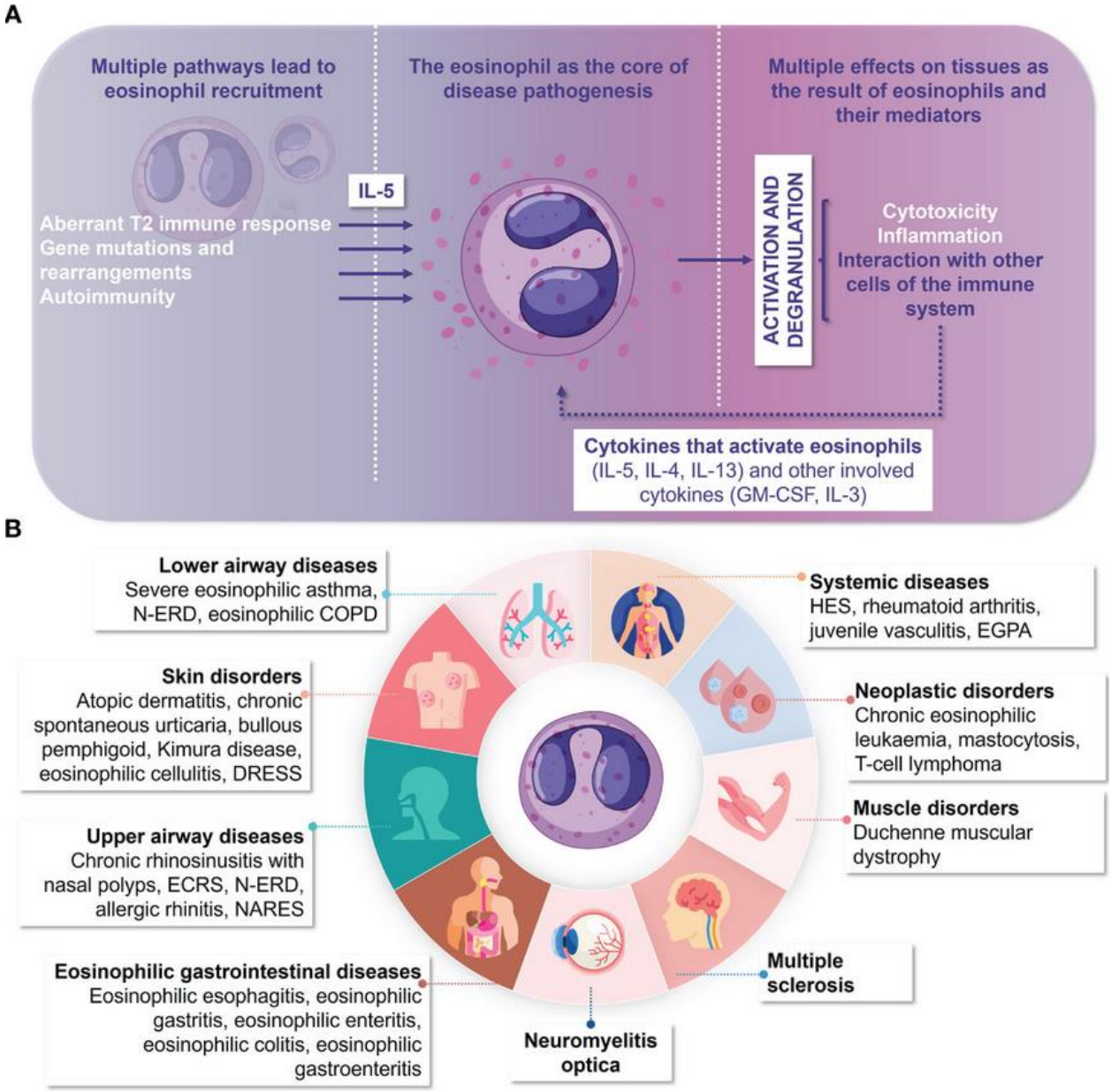


Figure 6: The eosinophil in eosinophil associated diseases (EADs).

Schematic of the cycle leading to eosinophilic inflammation (A) and spectrum of diseases in which eosinophils are involved or in which eosinophilia is observed (B)

From (Quirce et al., 2023)

EADs are often detected through one of their main common characteristics, namely blood or tissue eosinophilia. At steady state eosinophils have been described to be located in the bone marrow and spleen, circulating in the blood, marginated along blood vessels of certain tissues such as the lung and residing within certain tissues such as in the intestines (Gigon et al., 2023). In normal conditions eosinophils are not found in the esophagal mucosa, airway lumen or skin. The invasion of these tissues by eosinophils or an abnormal recruitment of eosinophils in tissues where they are already present at steady state is known as tissue eosinophilia. A comprehensive study on blood eosinophil numbers involving slightly more than 11000 subjects in Austria reported an average peripheral eosinophil blood eosinophil count (BEC) of 128 cells/µl with a median of 130 cells/µl (Hartl et al., 2020). The authors discussed that median blood eosinophil counts in adults are considerably lower than those currently regarded as normal. They also reported that eosinophil numbers are higher in children and that asthma, smoking, a positive skin prick test and metabolic syndrome were associated with increased blood eosinophil counts.

Peripheral blood eosinophilia is currently diagnosed by an absolute blood eosinophil count above 500/µl, while the threshold for hypereosinophilia is considered to be 1500 /µl (Kuang, 2020). EADs encompass a plethora of respiratory, cutaneous, digestive diseases and other conditions such as drug hypersensitivity (fig6B). In this introduction we will focus on and provide a short overview of eosinophilic asthma, since it is the most prevalent and well-known EAD. We will also briefly consider hypereosinophilic syndrome (HES) since it is in this syndrome that eosinophil lineage expansion is at its maximum. More details on these and other EADs are available in comprehensive reviews (Gigon et al., 2023; E. A. Jacobsen et al., 2012; Klion et al., 2020; Rothenberg & Hogan, 2006; Valent et al., 2021; Wechsler et al., 2021).

Hyper Eosinophilic syndrome (HES)

HES is characterized by end organ damaged resulting from persistent blood hypereosinophilia above 1500/µl. In this syndrome, eosinophil expansion can have several origins leading to a classification of HES in several categories: neoplastic, reactive, or idiopathic (Klion et al., 2020). The best known example of neoplastic HES, representing the vast majority of these cases, is a myelodysplastic syndrome involving a fusion protein in eosinophils. This fusion protein gene, FIP1L1-PDGFRA , is due to a deletion in chromosome 4 and is composed of FIP1L1 and the tyrosine kinase PDGFRA yielding a constitutively active tyrosine kinase that triggers unrestricted eosinophil proliferation (Pardanani et al., 2004). Thanks to the better understanding of its underlying mechanisms, this variant of HES, in which eosinophils were causing significant mortality due to endomyocardial fibrosis and thromboembolism, can now be treated with the tyrosine kinase inhibitor imatinib (Helbig, 2018). Reactive HES can have several origins but an interesting representative is the lymphocytic variant (Shi & Wang, 2022). This form of reactive HES is due to the clonal expansion of T cells producing massive amounts of Th2 cytokines, notably the main eosinophilopoietin IL-5, which in turns increase bone marrow eosinophil output. Among the diverse other causes of reactive hypereosinophilia, we can notably mention parasitic infections and adverse drug reactions such as Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS) (Calle et al., 2023). Finally, when the hypereosinophilia does not have an identified cause it is qualified as idiopathic but, fortunately, as our understanding of the mechanisms and the diagnostic methods progress, less and less patients are falling in this category.

Severe eosinophilic asthma

EADs are responsible for a big economic burden as well as high symptoms burden for the patients. Asthma alone is affecting more than 300 million people worldwide with an ever increasing prevalence (Braman, 2006; Loftus & Wise, 2015). Asthma is a heterogeneous chronic inflammatory airway disease characterized by several key features: airway inflammation and hyper reactivity, bronchoconstriction, mucus overproduction and increased thickness of the

airway wall (Hammad & Lambrecht, 2021). Although neutrophilic or non-eosinophilic phenotypes of asthma do exist, T2 high asthma involving deregulated type 2 immune responses and eosinophils represents 89% of the severe asthma cases (Oishi et al., 2016). Eosinophils are hence involved in a majority of asthma phenotypes with blood and tissue eosinophilia orienting the diagnosis. The potential of eosinophils to cause damage to the bronchi and their increased abundance in most of the cases has led to the hypothesis that unregulated eosinophil responses were the main driver of the disease (fig6A). Eosinophilic asthma is commonly related with atopy and elevated IgE levels and can be modelled in laboratory mice via exposition to allergens such as ovalbumin, dust mites or mold extracts (Haspeslagh et al., 2017; Snelgrove et al., 2014). Allergen sensitization and challenge induces aberrant Th2 responses. These are characterized by a production of alarmins, notably the potent eosinophil activator IL-33 (Snelgrove et al., 2014). Furthermore, these alarmins stimulate and induce the expansion of type 2 innate lymphoid cells and Th2 lymphocytes that produce large quantities of IL-5 and other cytokines which in turn increase bone marrow eosinophil output as well as eosinophil recruitment (Petrova et al., 2020; Sun et al., 2023). Once they infiltrate the airways and even the lumen, as eosinophil are also found in the sputum of asthmatic patient, eosinophil degranulation can sustain local airway inflammation and their secretion of cytokine and mediators such as TGF-β and IL-13 can contribute to airway remodeling and mucous hyper secretion (Hammad & Lambrecht, 2021; Schleich et al., 2013). In addition to their role in mucus hypersecretion, human eosinophils, via their degranulation of CLC protein which aggregates in crystals, contribute to airway plugging and Th2 inflammation hotspots (Persson et al., 2019). Because of their involvement in this prevalent disease eosinophil, several therapies targeting eosinophils and aiming at their reduction or complete depletion have been developed.

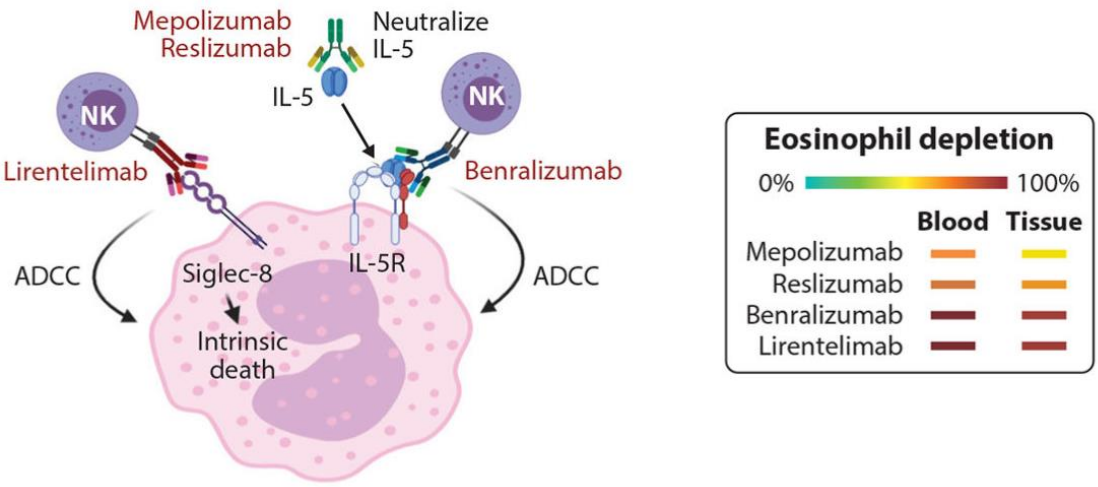


Figure 7: Anti-eosinophil monoclonal antibodies.

From (E. A. Jacobsen et al., 2021)

1.3.1 Anti eosinophil therapies

In the treatment of many EADs and especially in asthma, glucocorticoids play an important role in controlling the inflammation and reducing eosinophil numbers (Wechsler et al., 2021). Still, it is now common knowledge that prolonged steroid therapy does not go without significant side effects. This toxicity, added to the existence of corticoresistant forms of severe eosinophilic asthma underlined the need for new therapies (E. A. Jacobsen et al., 2021). Precision therapies were hence designed that would target eosinophils more specifically in eosinophilic asthma (fig7). These therapies consist in engineered monoclonal antibodies targeting IL-5, such as mepolizumab and reslizumab, or the IL-5Rα receptor subunit in the case of benralizumab. As we will discuss in the "IL-5, the do it all cytokine" section, IL-5 has been described to be involved in about every process related to eosinophils, including their maturation, proliferation, recruitment, activation, survival, etc (Pelaia et al., 2019). Mepolizumab and reslizumab repress IL-5 signaling by preventing the binding to IL-5Rα while benralizumab directly depletes eosinophil via the induction of Antibody Dependent Cellular Cytotoxicity (ADCC) (fig7). These therapies have first been designed and tested for the treatment of severe eosinophilic asthma and were shown, in most cases, to normalize eosinophil numbers, to decrease the number of exacerbations and to allow corticoid sparing (Busse et al., 2019; Castro et al., 2015; Henriksen et al., 2018). Considering this success monoclonal antibodies targeting eosinophils are also being tested in various clinical trials for other EADs (table1). While the exact mechanisms of action mepolizumab and reslizumab are yet to be explained, it has been hypothesized that the depletion of IL-5 via mepolizumab is interfering with eosinophil maturation, causing a maturational arrest in the eosinophil lineage (table1) (Menzies-Gow et al., 2003). Recently, an anti-Siglec8 monoclonal antibody, also inducing ADCC, was added to the anti-eosinophil arsenal and might be of interest in gastro-intestinal EADs thanks to its potential cumulative impact on mast cells that are also bearing this receptor (E. S. Dellon et al., 2020). Besides biologics, a promising small molecule dexpramipexole that was tested in clinical trial for amyotrophic lateral sclerosis, was serendipitously discovered to have potent eosinophil depleting effects (Panch et al., 2018). While the mechanism is yet to be discovered, there is ongoing interest regarding the treatment of HES (table1).

	Mepolizumab	Reslizumab	Benralizumab	AK002	Dexpramipexole
Target	IL-5	IL-5	IL-5R α	Siglec-8	unknown
Antibody (parent)	Humanized IgG1κ (murine 2B6)	Humanized IgG4κ (rat 39D10)	Humanized afucosylated IgG1κ	Humanized non-fucosylated IgG1	-
Max Dose in Clinical Trials	10 mg/kg iv 300 mg sc	3 mg/kg iv	3 mg/kg iv 200 mg sc	3 mg/kg iv	300 mg orally/day
Approved Indications	Severe Eosinophilic Asthma (100 mg sc monthly) EGPA (300 mg sc monthly)	Severe Eosinophilic Asthma (3 mg/kg iv monthly)	Severe Eosinophilic Asthma (30 mg sc monthly × 3 months and then every 2 months)	None	None
Pediatric Approval	>12 years of age	No	>12 years of age	No	No
Studies in multisystem HES	Phase 2 completed, phase 3 ongoing	Phase 2 completed	Phase 2 completed, Phase 3 planned	None	Phase 2 completed, Phase 3 planned
Studies in EGID	Phase 2 in EoE completed	Phase 2 in EoE completed	Phase 2 in eosinophilic gastritis ongoing	Phase 2 in eosinophilic gastritis and gastroenteritis ongoing	None
Studies in EGPA	Phase 3 completed	Phase 2 ongoing	Phase 2 ongoing	None	None
In vivo Effects on Target Cells					
Peripheral eosinophils	Profound reduction	Profound reduction	Complete depletion	Complete depletion (published abstract in JACI 2018)	Complete depletion
Tissue eosinophils	Partial depletion	Partial depletion	Complete depletion	NA	Complete depletion
Eosinophil precursors	Maturational arrest	NA	Complete depletion	NA	Maturational arrest
Basophils	NA	NA	Reduction	NA	Reduction
Mast cells	No effect	No effect	No effect	NA	No effect

Note: therapies that target mutations associated with eosinophilic myeloid neoplasms, including the tyrosine kinase inhibitor imatinib, are not included in this table

NA = published data not available

Table 1: Eosinophil-targated therapies approved or in clinical development.

As in (Klion et al., 2020)

On some occasions, the use of anti-eosinophil therapies and other anti-cytokine monoclonal antibodies led to seemingly contradictory observations. Despite a profound diminution of eosinophil numbers, some asthmatic patients are classified as partial responders or even non responders and one or several switch between different biotherapies are frequent (Caruso et al., 2022; Couillard et al., 2024; Evan S. Dellon & Spergel, 2023; E. A. Jacobsen et al., 2021; Scioscia et al., 2023). Moreover, it is hence common that patients be switched from "anti-eosinophil" to other precision therapies such as dupilumab, which targets the receptors to IL-4 and IL-13. A non-negligible proportion of patients switching from anti-eosinophil treatment to dupilumab will develop transient eosinophilia while still experiencing improvement in their symptoms (Ryser et al., 2023). This could be explained by a decrease in eosinophil aggressiveness or would simply depict them as bystanders in these cases. However, it is to be mentioned that these patients need to be carefully monitored because, although the transient eosinophilia is generally not a cause of concern, the therapy switch, especially if accompanied by corticoid dose decrease, can unmask an eosinophilic vasculitis or other eosinophilic manifestations in some rare cases (Olaguibel et al., 2022).

Similarly, a significant proportions of eosinophil esophagitis patients treated with benralizumab saw a decrease in eosinophil numbers and eosinophil biomarkers without improvement of clinical symptoms (Evan S. Dellon & Spergel, 2023; L., 2024). This observation led to the Local Immunity And/or Remodeling/Repair (LIAR) hypothesis (Kevin Range, 2012). The LIAR model hypothesizes that although eosinophils are mostly portrayed as destructive cells, they are likely also involved in tissue repair in certain diseases.

These discussions highlight the heterogeneity of EADs. Further research on biomarkers and therapeutic mechanisms is required to provide each patients with the most suited therapy depending on the specific case.

2.4 Eosinophil ontogeny

2.4.1 Original identification of eosinophil progenitors in mice and humans

Eosinophils originate from bone marrow progenitors. They develop and mature within the bone marrow before transiting via the blood to their different organs or residency (Gigon et al., 2023; Rothenberg & Hogan, 2006). Initially, to decipher the process of hematopoiesis, hematopoietic bone marrow progenitors were compartmentalized based on cell surface markers and sorted via flow cytometry (Challen et al., 2009). Different progenitor populations were then cultured in the presence of different hematopoietic growth factors and were given names according to their potential to generate certain lineages. This type of approaches was instrumental in delineating the first "hematopoietic trees". At the root of the classical hematopoietic tree is the compartment of hematopoietic stem cells (HSCs). HSCs can self-renew and are able to give rise to the whole spectrum of blood cells. When grafted to irradiated hosts, they can reconstitute all blood lineages. Depending on whether this reconstitution can last for months or not, HSCs have been divided into long-term (LT-HSCs) and short-term (ST-HSCs) HSCs, respectively (Kumar & Geiger, 2017; Zhong et al., 2005). Downstream of HSCs is the compartment of multipotent progenitors (MPPs), which can differentiate into multiple lineages but without the long term grafting potential. ST-HSCs are sometimes considered as MPPs. Further downstream in the classical tree, progenitors start acquiring more specialized differentiation potential, and only generate a few lineages. These progenitors were initially divided into two main populations; Common Lymphoid Progenitors (CLPs) giving rise to lymphoid cells and Common Myeloid Progenitors (CMPs) giving rise to all myeloid lineages including red blood cells and megakaryocytes. This myeloid potential is further narrowing in 2 different progenitor populations: Granulocytes Monocyte Progenitors (GMPs) giving rise to all the granulocytes (neutrophils, basophils and eosinophils) and monocytes and Megakaryocyte Erythrocyte Progenitors (MEPs) (Adolfsson et al., 2005; Böiers et al., 2010; Forsberg et al., 2006; Pietras et al., 2015)

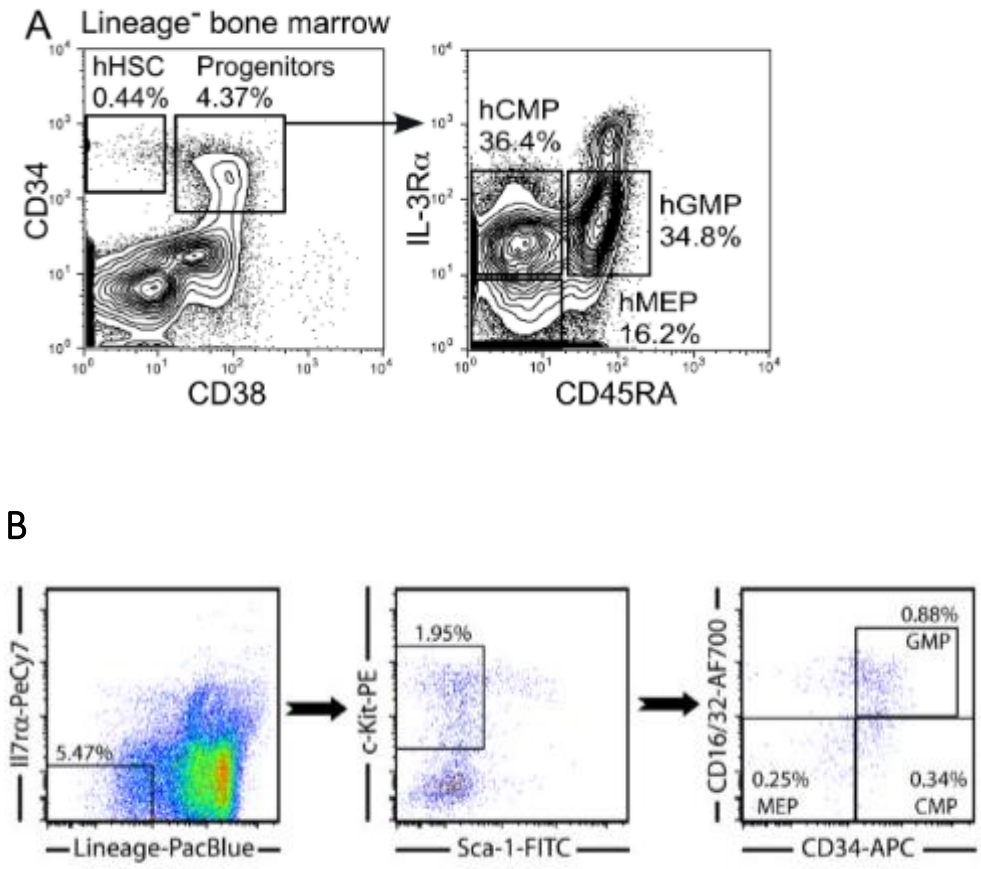
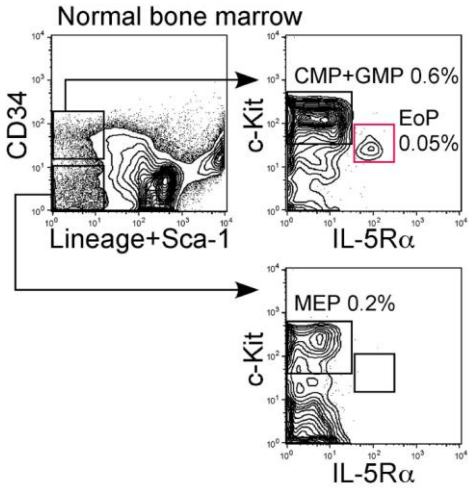


Figure 8: Gating strategies of CMP, GMP and MEP in humans and mice.

A: In humans as in (Mori et al., 2009)

B: In mice as in (Challen et al., 2009)

A



B

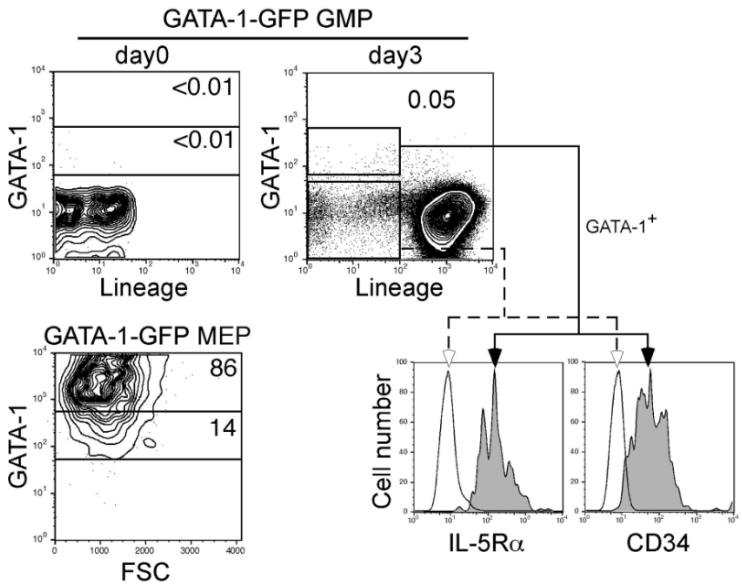


Figure 9: First identification of the EoP in murine bone marrow.

A: Original gating strategy for murine EoP

B: GMPs are GATA1$^-$ while MEPs are GATA1$^+$. After 3 days in culture in vitro, GATA1$^-$ GMPs presented a fraction of 0.05% of GATA1$^+$ cells which resembles EoPs.

As in (Iwasaki et al., 2005)

Eosinophil progenitors were first identified at a time where this dichotomic view of hematopoiesis prevailed. Seminal work by Koichi Iwasaki's group reported on bone marrow progenitors in human and murine bone marrow that were able to generate eosinophils in culture, which they called "EoPs" (Iwasaki et al., 2005). In mice, Iwasaki's group postulated that the EoPs arose from the GMP, whereas in humans, they would diverge earlier from other granulocytic lineages and arise from the CMP.

The gating strategies of CMPs and GMPs are displayed in figure 8 (fig8). These gating strategies remain to this day the most widely used method for identifying myeloid progenitors in flow cytometry. There are however a number of notable issues with this approach.

The general pool of HSCs in mice is characterized by a clear lack of expression of lineage markers and the expression of two highly expressed markers, namely cKit and Sca1, which provide more than appropriate resolution in flow cytometry. In contrast, the GMP and CMP populations are gated on intermediate levels of lowly expressed markers, offering only poor resolution (Challen et al., 2009).

To continue towards EoPs identification and since IL-5 was suggested to promote the commitment of bone marrow progenitors toward the eosinophil lineage, Kiyoshi Takatsu's group, developed several anti-IL-5Rα antibodies in the 1990's (Foster et al., 1996; Mita et al., 1991; Yamaguchi et al., 1990). They did so using whole cell lysates of the T88-M cell line, a murine IL-5-dependent early B-cell line, as immunogen. Two clones, H7 and T21 were selected due to their ability to block IL-5 binding to its receptor and reduce to IL-5Rα-bearing cell line proliferation. However, only one antibody, T21, brightly labelled a large myeloid population, which was considered intriguing or unexpected already at that point (Hitoshi et al., 1990). Unfortunately, as we will see in this work, T21 became later the most widely used antibody for the detection of IL-5Rα in mice Having previously discussed a potential issue with T21, Hiromi Iwasaki, Kiyoshi Takatsu and Koichi Akashi's group made use of the H7 antibody in 2005 in order to first identify the murine eosinophil progenitor via their expression of IL-5Rα (fig9). However the expression of IL-5rα is low and the resolution is poor which causes poor reproducibility.

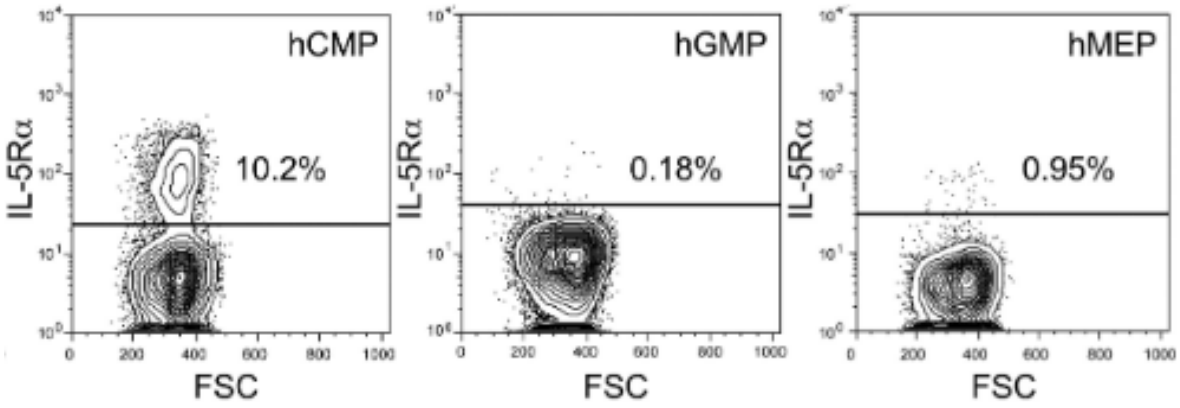


Figure 10: First identification of the EoP in human bone marrow.

Gating of the EoP as the IL5Ra expressing fraction of the human CMP

As in (Mori et al., 2009)

Knowing that granulocytes but especially eosinophils are autofluorescent cells and without being presented with the proper controls, one could argue that no actual stain is visible in their original flow cytometry plots figure (Weil & Chused, 1981).

We ourselves attempted to identify EoPs using anti-IL-5Rα antibodies in mice but we made some surprising discoveries and struggled with this method.

Let's go back to the first murine EoP identification article where the GMP origin of the EoP is a bit confusing (Iwasaki et al., 2005). Murine EoPs were first selected by gating $Sca1^-$ progenitors against Cd34 displaying low resolution and then $cKit^{int}$ IL-5Rα. This last gate does not actually exactly fall within the GMP gate (fig9A). To demonstrate the eosinophil potential within the GMP population, the authors performed in vitro limiting dilution assays, single progenitor cell culture and made use of a GATA1+ reporter mouse strain. The dilution assays showed eosinophil potential in 1/4 HSCs, 1/22 CMPs, and 1/72 GMPs. The higher eosinophil potential of HSCs and CMPs was hypothesized to be due to their capacity to generate several GMPs within the culture. They reported that single GMP culture with an eosinophil promoting medium showed less than 1.5% eosinophil potential. Some of those wells contained both eosinophils and neutrophils, suggesting the GMP displayed bipotency for those 2 lineages. Finally, the authors showed that the GMP, in contrast to the Megakaryocyte Erythrocyte Progenitor (MEP), was $GATA1^-$ although after 3 days of in vitro culture a very small fraction of $GATA1^+$ expressing IL-5rα and CD34 cells with eosinophil potential were obtained from this $GATA1^-$ GMP pool (fig9 B). In this study, the authors discussed the importance of GATA-1 and provided the community with an excellent tool; the GATA-1 reporter strain would be instrumental in later revising the models of hematopoiesis. Unfortunately, the authors modelled eosinophil ontogeny according to the CMP/GMP theory, in spite of their own evidence that GMPs failed to generate significant numbers of GATA-1^+ EoPs.

In humans, the first identification of EoPs was done by gating IL5rα positive cells directly within the CMP compartment (Mori et al., 2009). Although weak, the expression of the receptor seems to provide sufficient resolution (fig10). In vitro culture showed consistent results with the eosinophil potential residing in the CMP compartment and IL-5Rα$^+$ CMPs virtually yielding only eosinophils.

These seminal studies led to the elaboration of the models of eosinophilopoiesis presented in figure 11 (fig11). Intriguingly, this model would suggest that the developmental path of eosinophils differs in mice and humans, in spite of the eosinophil lineage being conserved in all jawed vertebrates also named gnathostomes (Stacy & Ackerman, 2021). In the model derived from Iwasaki's works, eosinophils would be a sister lineage of all other granulocytes, including neutrophils, and of monocytes. To this day, this view remains dominant but is incorrect.

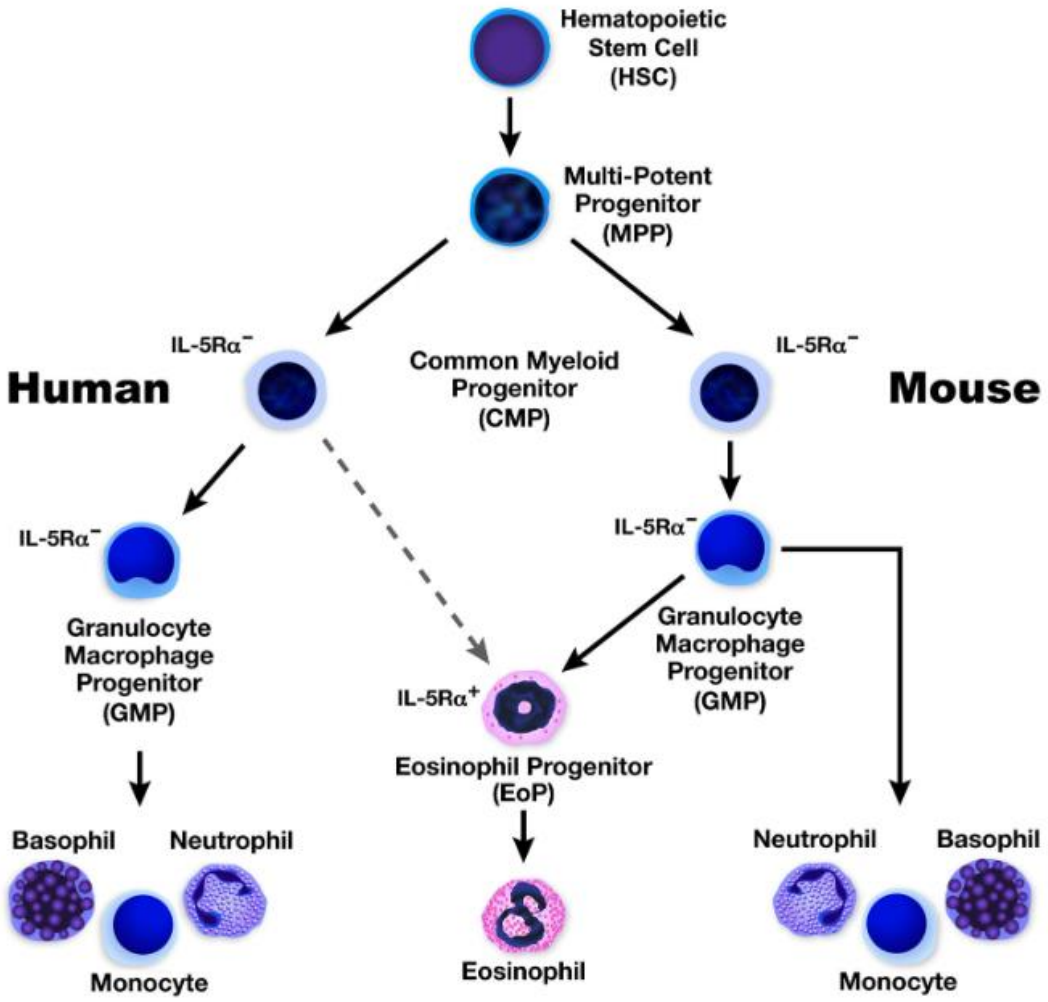


Figure 11: Hematopoeitic differentiation pathways leading to eosinophils in humans and mice.

As in (J. J. Lee et al., 2012)

2.4.2 Evolution of the hematopoietic tree model in light of new technologies.

The concepts of GMP and CMP are ancient, and are not aging very well. As outlined above, GMPs and CMPs are gated on a very limited set of markers displaying poor resolution. Although they are granulocytes, eosinophils do not seem to originate from GMPs in humans and attempts to bring murine EoPs under the GMP umbrella are technically weak. Recent advances in flow cytometry, lineage tracing, single-cell RNA-sequencing and reporter and genetically deficient mice led to a profound revision of the hematopoietic tree model (S. E. W. Jacobsen & Nerlov, 2019). The previous textbook hematopoietic tree is now challenged by 2 other proposed models of hematopoiesis. Three competing models are shown in figure 12 (fig12). Panel A shows the classical hematopoietic tree. Model B proposes a limited adaptation of the textbook model in A, in which the GMP is still proposed to give rise to all the granulocytes. Finally, model C proposes a radically different structure of hematopoiesis, and is arguably the best-supported model to date. Model C is supported by scRNAseq data, lineage tracing data, and data from reporter mouse strains in which transgenic expression of GFP is driven by the promoter of GATA1 (Drissen et al., 2016; L. Tang, 2020; Weinreb et al., 2020). GATA1 is a transcription factor and a master regulator of hematopoiesis. Indeed, GATA1 is an essential transcription factors in the development of erythrocytes, megakaryocytes and eosinophils. GATA1 deficient mice are not viable due to impaired myelopoiesis and anemia, but a deletion of a palindromic GATA DNA motif in front of the GATA1 promoter itself leads to total eosinophil deficiency (Yu et al., 2002). Accordingly, eosinophils in GATA1 reporter mice arise from a pool of GATA1-expressing "pre-GMP" progenitors, which also give rise to megakaryocytes, erythrocytes and basophils, but not to neutrophils and monocytes (Drissen et al., 2016). Eosinophils in model C are the sister lineage of basophil and mast cells, close cousins of erythrocytes and megakaryocytes but only distant relatives of neutrophils. Model C also makes the existence of CMPs and GMPs as a distinct progenitor cell state superfluous. ScRNAseq analyses indeed have revealed the highly heterogeneous nature of these progenitor pools (Kwok et al., 2020) . It should become widely accepted that GMPs and CMPs are only artificial pools of progenitors conflated together by rudimentary gating strategies in flow cytometry.

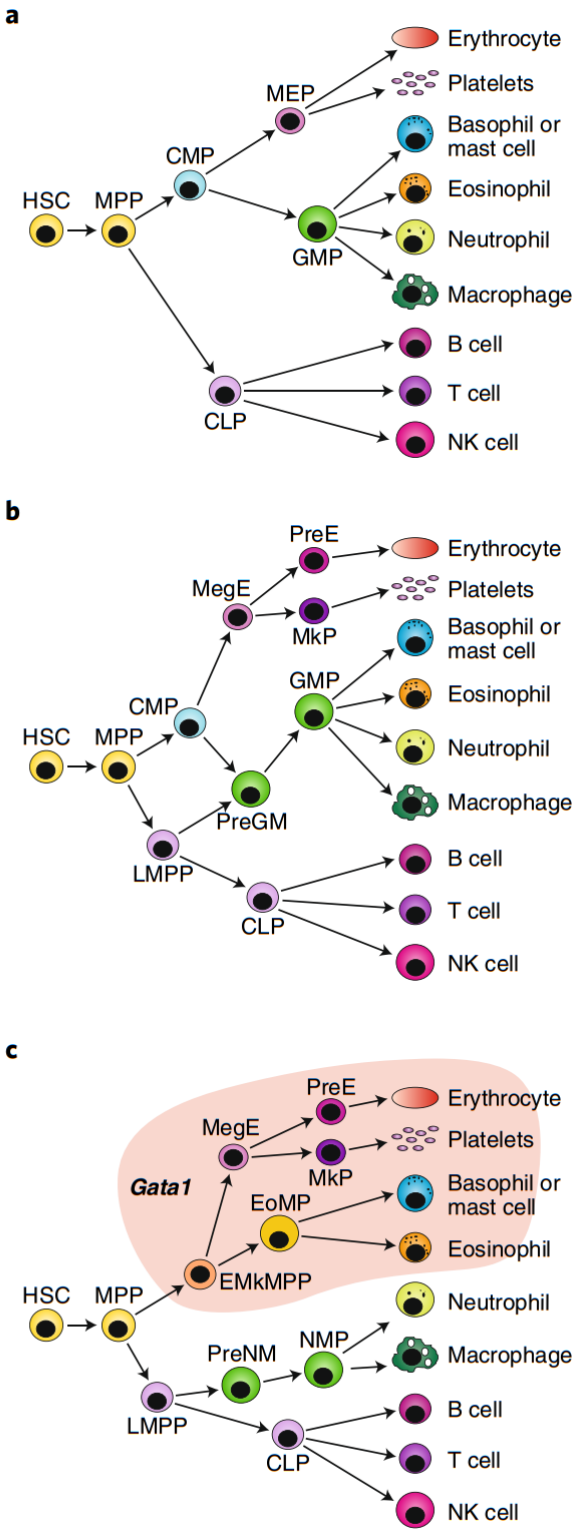
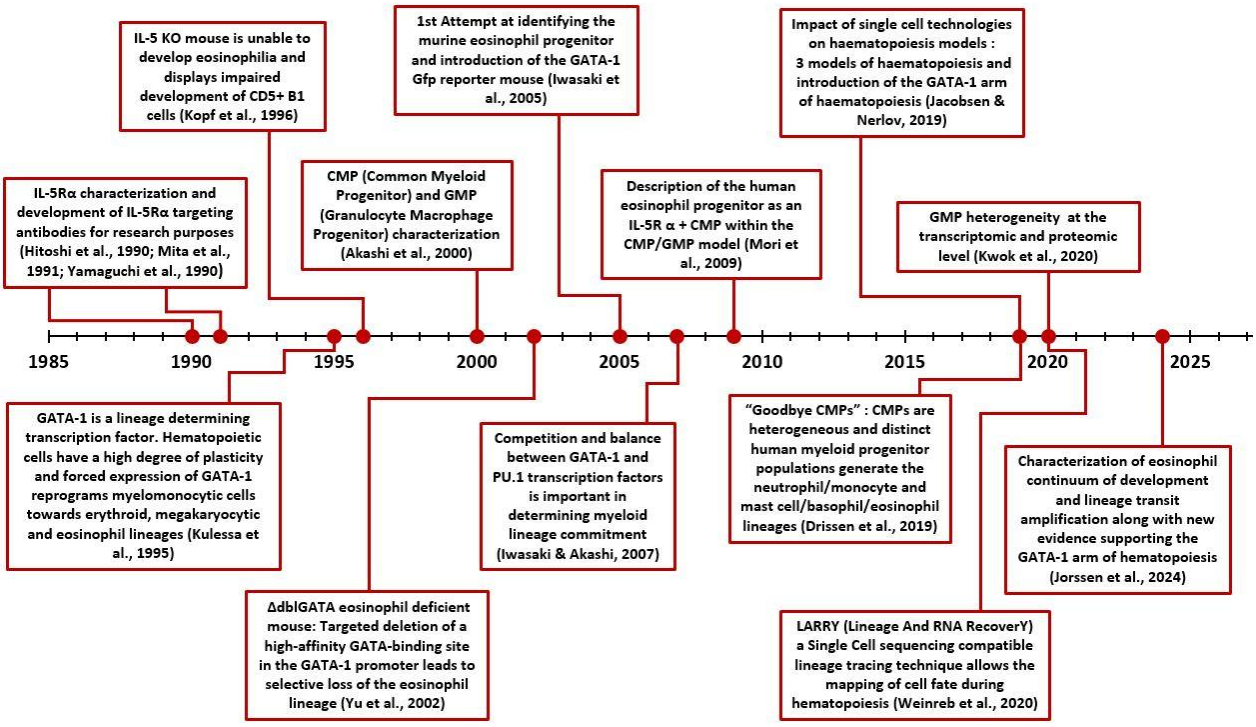


Figure 12: Comparison of the 3 different models of the hematopoietic tree.

As in (S. E. W. Jacobsen & Nerlov, 2019)

2.4.3 Incremental advances in eosinophil ontogeny

To provide an overview of the different steps leading to the recent models of eosinophil ontogeny we propose the following timeline.



Timeline 1: Milestones in research on eosinophil ontogeny and development (non-exhaustive)

This timeline is non-exhaustive and was built upon the following references (Akashi et al., 2000; Drissen et al., 2019; Hitoshi et al., 1990; Iwasaki et al., 2005; Iwasaki & Akashi, 2007; S. E. W. Jacobsen & Nerlov, 2019; Jorssen et al., 2024; Kopf et al., 1996; Kulessa et al., 1995; Kwok et al., 2020; Mita et al., 1991; Mori et al., 2009; Weinreb et al., 2020; Yamaguchi et al., 1990; Yu et al., 2002)

2.4.4 Molecular mechanisms of eosinophil development

The commitment of hematopoietic progenitors toward the eosinophil lineage and their subsequent development toward mature eosinophils depends on a complex interplay between several transcription factors (fig13). This field is still under investigation and more evidence remains required to pinpoint the exact role of each transcription factor in the process. Most of the current knowledge was obtained through the observation of the phenotype of mice deficient for said transcription factors, at least when they were viable, or by the analysis of the transcriptome of "GMPs", "CMPs" and "EoPs". We already discussed the master regulator GATA1, which is a master regulator of eosinophilopoiesis. The other transcription factors proposed to be involved in eosinophil development are GATA2, Friend Of GATA 1 (FOG1), PU.1 (also known as SPI-1), Interferon response factor 8 (IRF8), XBP1, ID2 and two members of the CEBP family: CEBPα and CEBPε (Fulkerson, 2017). The sequential activation and de-activation of these transcription factors orchestrates the transcription of specific sets of genes required at different moment of eosinophil maturation. FOG1 is described to be highly expressed in MPPs and to antagonize GATA1 (Du Roure et al., 2014; Querfurth et al., 2000). FOG1 expression must then be downregulated for eosinophil lineage commitment. IRF8 expression has also been described to be critical to eosinophil lineage commitment (Fulkerson, 2017) and we tested this proposition in one of our studies by studying eosinophil development in IRF8 deficient mice. C/EBPα-deficient mice are deficient in both eosinophils and neutrophils. In line with the old "GMP" concept, C/EBPα was proposed to regulate the balance between eosinophil and neutrophil commitment; an increase in C/EBPα expression would lead to increased neutropoiesis, while a decrease would favor an eosinophil fate (Satoh et al., 2013; Zhang et al., 1997). Deficiency in C/EBPε also leads to the absence of eosinophils and neutrophils and induced ectopic expression of C/EBPε in cord blood progenitors increases commitment toward the eosinophil lineage (Bedi et al., 2009; Yamanaka et al., 1997). It was hence postulated that eosinophil commitment requires a temporally regulated decrease in expression of C/EBPα combined with an increase in expression of C/EBPε to occur. Finally, XBP1 is a transcription factor involved in the unfolded protein response triggered by endoplasmic reticulum stress. XBP1 is expressed in EoPs, which need to produce large amounts of cytotoxic granules proteins

that understandably put a lot of stress on the protein machinery. In line with this notion, deficiency in XBP1 impairs eosinophil development in XBP1-deficient mice(Bettigole et al., 2015).

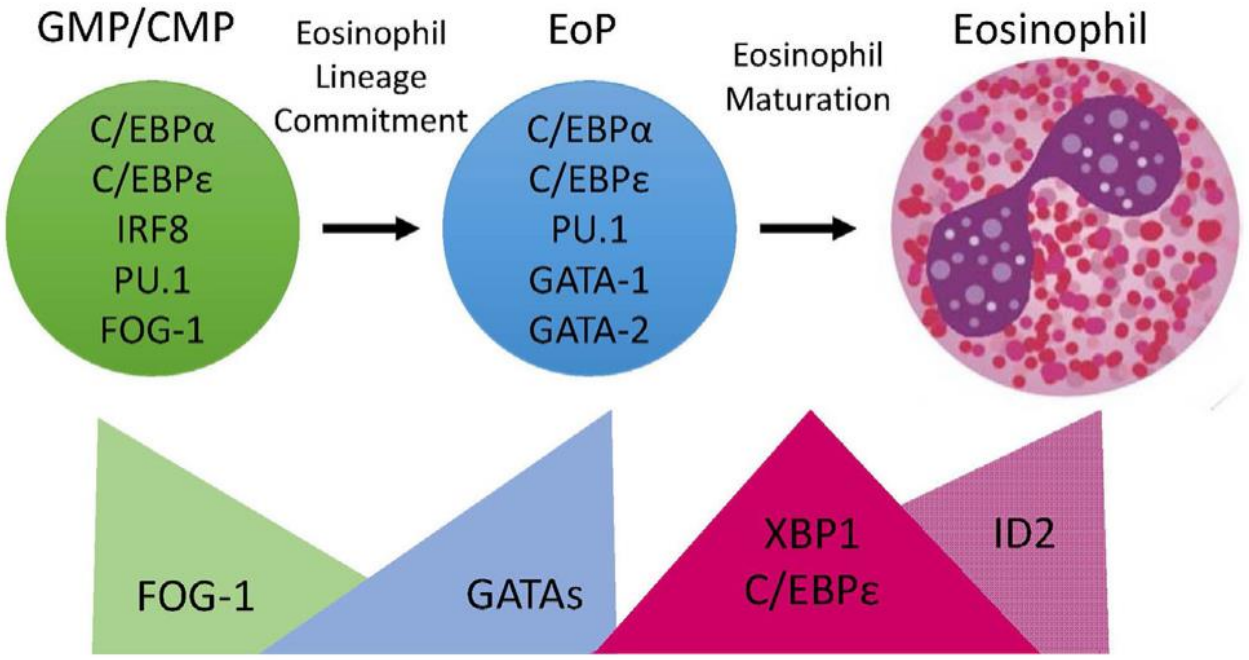


Figure 13: Transcription factors involved in eosinophil development.

Figure and following associated text as in (Fulkerson, 2017)

"Eosinophils differentiate in the bone marrow from an eosinophil lineage-committed progenitor (EoP) that is derived from the granulocyte/macrophage progenitor (GMP) in mice and the common myeloid progenitor (CMP) in humans. For eosinophil lineage commitment to occur, the myeloid progenitor (GMP orCMP) must express C/EBPα, C/EBPε, interferon regulatory factor 8 (IRF8), and PU.1. Expression of friend of GATA-1 (FOG-1) declines, allowing for increasing expression and activity of GATA TFs, which is necessary for EoP production. Following lineage commitment, eosinophil granule protein gene expression is markedly increased with the collaborative interaction between C/EBPε, PU.1, and GATA-1. To assist with the elevated granule protein synthesis in the EoP and eosinophil precursors, XBP1 expression is increased and promotes survival during the demanding maturation process. Expression of activator isoforms of C/EBPε peaks during eosinophil maturation and then declines during the final stages. Expression of ID2 increases during eosinophil maturation and enhances the rate of maturation."

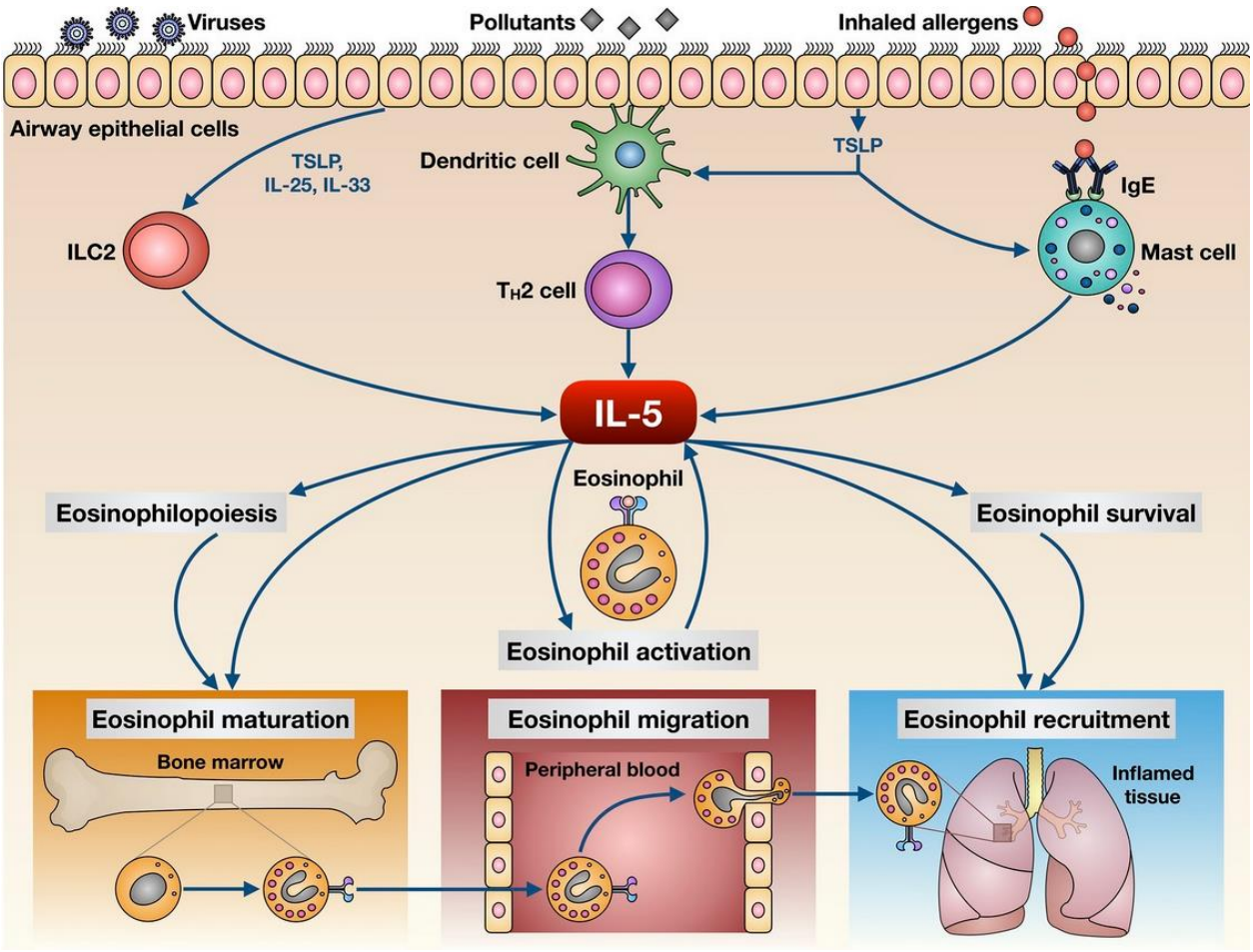


Figure 14: Production and roles of IL-5 in respiratory eosinophil associated diseases.

As in (Pelaia et al., 2019)

2.5 Eosinophilia and lineage expansion.

2.5.1 Inducers of eosinophilia

Eosinophilia, when not of neoplastic origin, is generally initiated by the production of alarmins also called “alarmin-cytokines”(Gauvreau et al., 2023). Alarmins are released by different cell types in response to damage to epithelia or other tissues, for example as happens during parasitic infections, or following exposure to allergens (Gauvreau et al., 2023). Three important alarmins have a significant potential to induce eosinophilia, namely thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 (fig14). By far the most potent alarmin in inducing eosinophilia is IL-33. However, IL-33 does not induce eosinophilia when IL-5 is depleted (Johnston et al., 2016). It is hence usually accepted that IL-33 promotes eosinophilia indirectly. IL-5 remains to this date the only direct major inducer of eosinophilia. IL-5 was first identified as a growth factor of B cells but is now considered mainly an essential growth factor for eosinophils (Kopf et al., 1996; Takatsu & Nakajima, 2008). Indeed, IL-5-deficient mice harbor very significantly decreased numbers of eosinophils (Van Hulst et al., 2022). Yet, they are not completely devoid of eosinophils like ΔdblGATA mice which lack a palindromic high-affinity GATA binding site in front of the GATA-1 promoter itself (Yu et al., 2002). Conversely, several IL-5 transgenic mouse strains (IL-5Tg), in which the IL-5 gene was inserted several times (a few to several tens of copies) under the dominant control region from promoter of the gene encoding human CD2, display constitutive expression and elevated production of IL-5 accompanied by hypereosinophilia (Dent et al., 1990). The link between IL-33 and IL-5 production was made via the discovery of ILC2s. ILC2s bear the IL-33 receptor ST2 and, upon stimulation with IL-33, amplify and secrete massive amounts of IL-5 (Sun et al., 2023). Th2 cells have also been shown to contribute to the production of IL-5 albeit in smaller quantities. On figure 14, you can also appreciate the pleotropic effects attributed to IL-5 which we will discuss later within this work. As a way to induce eosinophilia in our studies, we used IL-33 intraperitoneal injections. In order to corroborate our data with more physiological models, we also used repeated allergen exposure in the form of *Alternaria alternata* or house dust mice intranasal injections as well as helminth infection models with *Nippostrongylus Brasiliensis*.

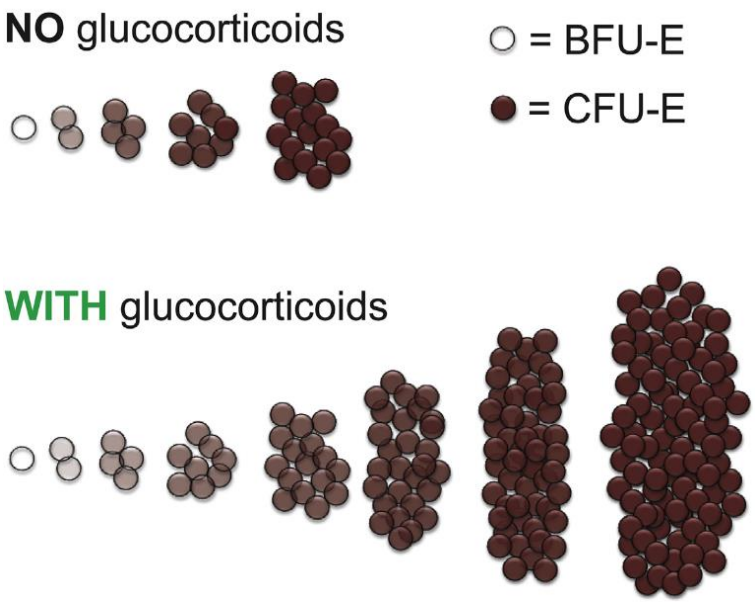


Figure 15: Model of erythropoiesis amplification during glucocorticoids treatment.

This model proposes a decoupling of maturation and proliferation within the erythrocyte lineage during treatment with glucocorticoids. A decrease in the rate of maturation is allowing an increased number of divisions along the maturation process leading to improved erythrocyte output from the bone marrow.

CFU-E: Colony Forming Unit – Erythroid BFU-E: Burst Forming Unit – Erythroid.

As in (Li et al., 2019a)

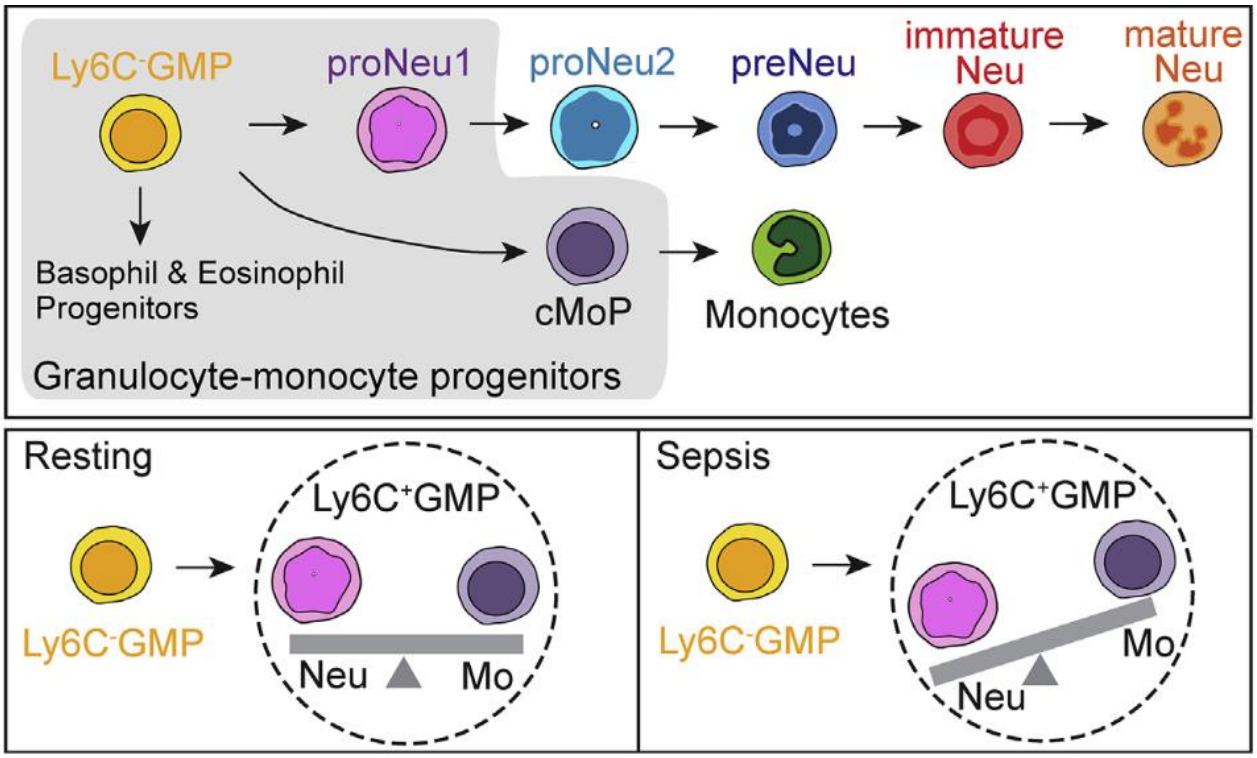


Figure 16: Refined model of neutrophil ontogeny and development and sepsis induced shift in lineage commitment.

This model proposes that bipotent progenitors possessing neutrophil and monocyte potential see an increased commitment towards the neutrophil lineage at the expense of the monocyte lineage during sepsis. This bipotent progenitor is likely to correspond to the neutrophil monocyte/macrophage progenitor (NMP) modelled in figure 12 C.

As in (Kwok et al., 2020)

2.5.2 - Mechanisms of lineage amplification

Two different mechanisms of lineage amplification have been described in the literature. We will briefly discuss these before starting the investigation of the proposed mechanisms of expansion of the eosinophil lineage.

Erythropoiesis and its continuum of transit amplifying progenitors

Li and colleagues were interested in erythrocyte expansion at steady state and during its amplification via exposure to glucocorticoids (Li et al., 2019b). They used flow cytometry and ScRNAseq to show that erythrocytes develop along a continuum of maturating progenitors and that their divisions were symmetrical (fig15). By comparing the transcriptomes of erythroid progenitors exposed or not to glucocorticoids they observed that glucocorticoids slow down the induction of genes involved in erythrocyte maturation. The decrease in rates of maturation allows erythrocyte progenitors to undergo more rounds of cell division before their terminal maturation, leading to a greatly enhance total cell output. This process of delayed maturation combined with extended proliferative capacity is called transit amplification.

Emergency neutropoiesis and its shift in lineage commitment

Kwok, Becht and colleagues used ScRNAseq analyses as well as high dimension flow cytometry in order to identify an early committed neutrophil progenitor within the heterogeneous GMP and observe neutrophil developmental stages (fig16) (Kwok et al., 2020). They also discuss the heterogeneity of the GMP which is resolved in several committed progenitors by increasing the resolution via the use of hundreds of other markers in flow cytometry (Becht et al., 2021). They showed that GMPs could be better subdivided by their differential expression of 81 markers for which at least some of them stained positive. In order to further study the mechanism of neutrophil expansion, they used a model in which mice undergo cecal ligation and puncture (CLP) which exposes the immune system to bacterial products demanding an increase of neutrophil output from the bone marrow. They observed an expansion of early neutrophil

progenitors mediated by Granulocyte-Colony Stimulating Factor G-(CSF) and they describe a skewing effect in the commitment of upstream progenitors towards the neutrophil lineage at the expense of the monocyte lineage. The mechanism of expansion proposed in this case was thus a shift in commitment of multi potential bone marrow progenitors towards a particular lineage.

3. Objectives

In this thesis, we aimed to better resolve the ontogeny of eosinophils and the mechanisms regulating the expansion of their lineage. We aimed at providing the community with reliable methods for the identification of eosinophils progenitors and for following their full developmental path toward mature eosinophils. In order to circumvent the refractory nature of eosinophils towards ScRNAseq we combined this method with the use of a high dimensional flow cytometry technique called “Infinity Flow”. We leveraged the obtained datasets to investigate the mechanisms involved in eosinophil expansion during eosinophilia. We hope that this work will allow to broaden the understanding of the impact of anti-IL-5 biotherapies on eosinophils. To this end, we also aimed at comparing mature eosinophils from severe asthmatic patients treated with control anti-IgE or anti-IL-5 biotherapies as well as mature murine eosinophils from IL-5-deficient versus wild-type mice. Finally, facing difficulties in staining the murine IL-5Rα led us to generate an IL5ra reporter mouse strain in order to evaluate the potential role of IL-5 in eosinophil lineage commitment.

4. Results

4.1 Participation

This works is the result of a collaborative project and was made possible by a joint effort between Glenn Van Hulst and myself. This project led to 2 peer- reviewed publications within the eosinophil field in which Glenn Van Hulst and I shared first authorship. The first publication describes the development of murine and human eosinophils and their mechanism of amplification in the bone marrow. The second publication compared mature eosinophils in IL-5 depleted or -replete conditions in human and mice. Contributions to the body of work leading to these publications can be described as follows: I oversaw the animal handling, establishment of animal models, and took the lead in the RNA and single-cell RNA sequencing analyses, and the bioinformatics necessary to create the Infinity Flow datasets. Glenn Van Hulst was responsible for investigating the role of IL-5 and took the lead in aspects related to flow cytometry, including panel design, conducting flow cytometry experiments, cell sorting, conventional & spectral cytometry data analyses, analyses of the Infinity Flow datasets, as well as creating figure artwork and layouts. Both Glenn Van Hulst and I contributed equally to sample preparation for all experiments and to data interpretations.

Both studies will now be presented in this result section in a format similar to their publication. Additional datasets or tables can be found following the doi of the studies or on array express database and the Gene Expression Omnibus database as mentioned in each method section.

4.2 Study 1: Single-cell proteomics and transcriptomics capture the development of eosinophils and the role of IL-5 in their lineage transit amplification

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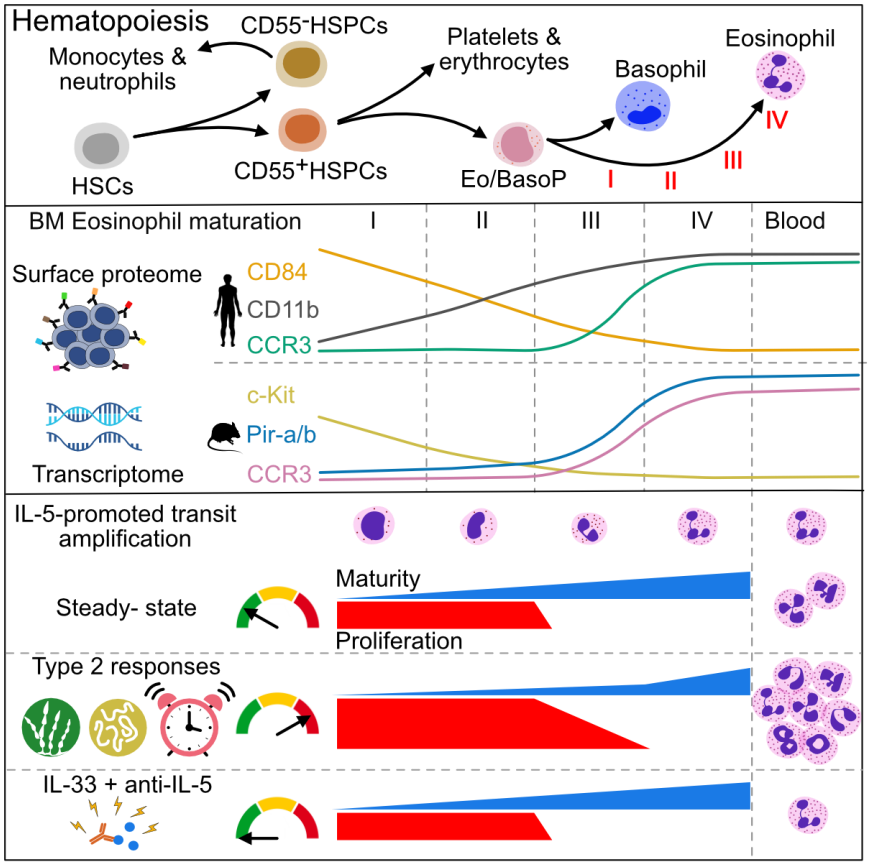
Note that figures in this section are numbered as in the original publication.

4.2.1 Abstract

The activities, ontogeny, and mechanisms of lineage expansion of eosinophils are less well resolved than those of other immune cells, despite the use of biological therapies targeting the eosinophilia-promoting cytokine interleukin (IL)-5 or its receptor, IL5RA. We combined single-cell proteomics and transcriptomics and generated transgenic IL-5ra reporter mice to revisit eosinophilopoiesis. We reconciled human and murine eosinophilopoiesis and provided extensive cell-surface immunophenotyping and transcriptomes at different stages along the continuum of eosinophil maturation. We used these resources to show that IL-5 promoted eosinophil-lineage expansion via transit amplification, while its deletion or neutralization did not compromise eosinophil maturation. Informed from our resources, we also showed that interferon response factor-8, considered an essential promoter of myelopoiesis, was not intrinsically required for eosinophilopoiesis. This work hence provides resources, methods, and insights for understanding eosinophil ontogeny, the effects of current precision therapeutics, and the regulation of eosinophil development and numbers in health and disease.



4.2.2 Introduction

Eosinophils are specialized granulocytes whose exact physiologic and pathophysiological activities are still not fully understood [1,2]. Initially recognized to accumulate in response to helminth infection [3], eosinophils are also increasingly acknowledged for their role in immune homeostasis [4,5], microbial defense [6–8], metabolism [9] or anticancer protection [10]. Despite their proposed beneficial functions, eosinophils are predominantly considered for their diagnostic value and implication in a broad spectrum of non-communicable diseases termed eosinophil-associated diseases (EADs) [1,2]. EADs are most often driven by type 2 immune processes, and are characterized by an accumulation of eosinophils in blood and diseased tissues. EADs include highly prevalent mucosal diseases such as eosinophilic asthma and eosinophilic chronic rhinosinusitis with nasal polyps but also less common eosinophilic vasculitis and idiopathic hypereosinophilic syndromes.

In EADs, the increased presence of circulating eosinophils, known as eosinophilia, serves as a valuable diagnostic marker and is used for treatment allocation. It is commonly assumed that blood eosinophilia primarily results from increased eosinophil production by the bone marrow (BM) [1]. The first identified eosinophil-committed hematopoietic progenitors, often called "EoPs", appeared as a subset of the heterogeneous granulocyte/monocyte progenitor pool (GMP) in mice [11], or of the common myeloid progenitor pool (CMP) in human [12]. However, the traditional perspective proposing that eosinophils share developmental proximity to all other granulocytes including neutrophils, arising from the hypothesis that the GMP and CMP represent defined oligopotent developmental stages of hematopoietic progenitors, has been invalidated. Recent studies concur in showing that myelopoiesis proceeds along 2 distinct arms; one consisting of lineages expressing the *GATA1* transcription factor, which give rise to eosinophils, basophils, erythrocytes and megakaryocytes, and a separate arm that leads to the development of neutrophils and monocytes [13–17]. Therefore, previous assumptions on eosinophilopoiesis built within the framework of the GMP and CMP concepts need revision.

Due to the currently limited resolution of the eosinophil lineage, the mechanisms leading to increased eosinophil production from hematopoietic progenitors are also not well understood [18]. It is known that the cytokine interleukin IL-5 [19] is essential to eosinophilia, as was established first in *IL-5*-deficient mice [20,21]. Alarmin cytokines such as IL-33 can also trigger eosinophilia but appear to do so indirectly by stimulating type 2 innate lymphoid cells (ILC2s) and helper T (Th2) cells, thereby increasing IL-5 production [22].

The dependency of eosinophilia on IL-5 led to the development of neutralizing anti-IL-5 monoclonal antibodies for the treatment of EADs [23,24]. These precision therapies alleviate blood eosinophilia and are used in the treatment of severe forms of EADs including severe eosinophilic asthma [25]. Cytotoxic antibodies to IL5RA were also developed, which deplete virtually all tissue and circulating eosinophils [26]. Other precision therapies for the treatment of EADs impact on blood eosinophil counts as well. For instance, the IL4RA-targeting antibody dupilumab elicits transient blood eosinophilia in a fraction of patients [27]. Alternatively, the anti-TSLP tezepelumab [28] and the anti-IL-33 itepekimab [29] both reduce blood eosinophil counts in asthmatic patients. With the current shift toward precision therapies for EADs comes the need for a refined understanding of eosinophil development and lineage expansion.

Here, we aimed to better resolve eosinophil development in human and mice. We obtained single-cell resolution of eosinophilopoiesis by combining single-cell proteomic screening by flow cytometry, generating a transgenic Il5ra reporter mouse strain and (single-cell) transcriptomic analyses. Our comparative analyses highlighted the evolutionarily conserved ontogeny of eosinophils, along a continuum of immunophenotypic stages of maturation in human and mice. We illustrate how these transcriptomic and immunophenotypic resources may be leveraged to investigate the molecular requirements and cellular dynamics of eosinophil progenitor maturation and expansion in eosinophilia, and to elucidate how IL-5 depletion impacts eosinophil development.

4.2.3 STAR Methods

Mice

C57BL/6J and Il5$^{-/-}$ (C57BL/6-Il5$^{tm1Kopf/J}$) mice were purchased from The Jackson Laboratory. Irf8-/- mice were described previously (Sichien et al., 2016). Il5ra reporter mice were generated by PolyGene AG (Rümlang, Switzerland) using homologous gene targeting in C57BL/6 embryonic stem (ES) cells. The *Il5ra* gene is stretched roughly over 38kb on mouse chromosome 6. The gene codes for 3 known primary transcripts, translated into two different peptides: the Il5ra protein (415 amino acid residues, coded by transcripts 201 and 202) and a 75-amino acid peptide (coded by transcript 203). We exchanged the first coding exon of all transcripts (corresponding to exon 4 in transcript 202) with the CDS of eGFP-T2A-Cre in a way that the original initiating ATG becomes the initiating ATG of the eGFP-T2A-Cre construct, which is supplemented with a SV40 poly(A) signal. An FRT-flanked neomycin selection cassette is inserted immediately downstream and a fragment of 2.2 kbp is deleted, eliminating the region of exon 5. This targeting strategy disrupts the *Il5ra* gene, leading to its knock-out. The targeting construct, K128.8a, was assembled by conventional cloning using homology arms straddling exons 4 and 5 of the *Il5ra* gene retrieved from BAC RP23-238B21, some synthesized elements, and selection and expression cassettes available at PolyGene. The flanking homology arms were short (2.6 /2.5 kbp) due to DNA elements that are refractory to cloning in bacteria, and the linearized vector yielded poor targeting frequency upon electroporation. Consequently, CRISPR targeting was used to enhance the frequency of homologous targeting. CRISPR guides were designed to induce cuts as close as possible to homology arms, cutting the genome but not affecting the targeting vector or the recombined target. ES clones were generated via colipofection (Invitrogen Lipofectamin LTX) using 1µg of the guide plasmids and 1µg of the unmodified circular homology donor vector followed by selection with 0.8µg /ml puromycin and 200µg/ml G418. Out of 288 tested clones, six clones with correct integration at both homology sites were identified by PCR and injected into C57Bl/6Ng blastocysts. Five chimeric males obtained from the blastocyst injections were mated to Flp-deleter mice on

C57Bl/6Ng background to assess transmission to the germ line, and cause elimination of the neo cassette via Flp/FRT excision. Chimeric males derived from 2 separate clones displayed germline transmission within their first litter. The heterozygous genotype was physio pathologically unsuspicious.

All mice were housed and bred in institutional specific pathogen-free facilities. Age- and sex-matched (female or male) mice were used at 8–16 weeks of age. All animal experiments were approved by the animal ethics committee of the University of Liege and complied with the Animal Research: Reporting of In Vivo Experiments guidelines, the European Union directive 2010/63/EU and the Declaration of Helsinki for the use and care of animals.

Human subjects

Bone marrow aspirates were obtained from the femur of 5 healthy donors (age 48-60 years) undergoing total hip replacement. Bone marrow was collected by aspiration before removal of the femoral head. A bone biopsy needle was inserted at the greater trochanter. The surgeon aspirated bone marrow using a heparinised 10 mL syringe and transferred it to heparin tubes. The first 2 samples were used to setup a backbone panel and run a flow cytometric screening, respectively. The 3 other samples were used to sort and perform RNA sequencing on the 4 stages of eosinophil maturation. The study was approved by the local ethics committee (IACUC, University of Liège) and written informed consent was obtained from all study participants. This research was undertaken in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and followed the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals of the International Committee of Medical Journal Editors.

10X single-cell RNA sequencing

Myeloid progenitors were sorted from the bone marrow of steady-state C57Bl/6 mice using a BD FACSAria III (BD Biosciences) cell sorter as described in Rosu et al.(Rosu et

al., 2021). Siglec-F + bone marrow leukocytes were sorted using the same instrument from C57Bl/6 mice injected for 7 days with IL-33. Cells were resuspended in Ca and Mg free PBS containing 0.4mg/ml of UltraPure™ BSA (Thermofisher, AM2616). In total, 16,000 sorted cells were submitted to the 10X Genomics pipeline for encapsulation aiming for a recovery of ~10,000 sequenced single cells as described in the manufacturer's instructions. Sequencing libraries were prepared using Chromium Single Cell 3' Reagent Kit V3 (10X Genomics) as per manufacturer's instructions. CDNA quality and quantity were controlled using Agilent High Sensitivity DNA Kit (Agilent) on a 2100 Bioanalyser (Agilent). Sequencing was performed on an Illumina NovaSeq 6000 sequencer using the following read lengths: 28 bp for Read1, 8bp for sample index and 80bp for Read2. Reads were mapped to the GRCm38 reference genome using Cell Ranger (v3.0.2).

10X Flex single-cell RNA sequencing combined with CITE-Seq

Single-cell suspension of mouse bone marrow from steady state CB57BL/6 mice was first incubated with TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody (BioLegend, 156603) for 10 mins at 4°C,washed with PBS 1 per cent BSA, spun at 300g for 5 mins and then stained for Ly6G-FITC (BioLegend, 127606), CD90.2-FITC (BioLegend, 140204), NK1.1-FITC (eBioscience, 11-5941-85), CD45R-FITC (eBioscience, 11-0452-85), CD55-Biotin (R&D systems, BAF5376), CD45-AF700 (BioLegend, 103128) CD115-BV786 (BD Biosciences, 750888) along with the following TotalSeq-B antibodies: TotalSeq™-B0014 antimouse/human CD11b (BioLegend, 101273), TotalSeq™-B0130 anti-mouse Ly-6A/E (Sca-1) (BioLegend, 108149), TotalSeq™-B0114 anti-mouse F4/80 (BioLegend, 123155), TotalSeq™-B0012 anti-mouse CD117 (c-Kit) (BioLegend, 105849), TotalSeq™-B0203 antimouse CD150 (SLAM) (BioLegend, 115951), TotalSeq™-B0115 anti-mouse FcεRIα (BioLegend, 134341), TotalSeq™-B0808 anti-mouse CD193 (CCR3) (BioLegend, 144529), TotalSeq™ B0570 anti-mouse/rat CD29 (BioLegend, 102239), TotalSeq™-B0431 anti-mouse CD170 (Siglec-F) (BioLegend, 155517) for 30 minutes on ice. Cells were washed and stained with TotalSeq™-B0952 PE Streptavidin (BioLegend, 405287) for 30 minutes on ice and washed again. Next,

cells were fixed for 1 hour at room temperature using the Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (10X Genomics, 1000414) according to manufacturer's instructions and CD45+CD55+ dump- CD115- cells were sorted using a BD FACSAria III (BD Biosciences) cell sorter and collected into PBS containing 1 per cent ultrapure BSA (Fisher Scientific, 10743447) and 0.4Unit/µl Protector RNase Inhibitor (Merck, 3335399001). 800,000 sorted cells were subsequently submitted to probe hybridization using the Chromium Fixed RNA Kit, Mouse Transcriptome (10X Genomics, 1000495) according to manufacturer's instructions and 16000 probed cells were submitted to GEM generation, barcoding and construction of libraries were performed using the Chromium Next GEM Chip Q Single Cell Kit (10X Genomics, 1000422) and the Fixed RNA Feature Barcode Kit (10X Genomics, 1000419) following the CG00047 user guide. cDNA quality and quantity were controlled using Agilent High Sensitivity DNA Kit (Agilent) on a 2100 Bioanalyser (Agilent). Sequencing was performed on an Illumina NovaSeq 6000 and reads were mapped to the probe set and antibody barcode sequences using Cell Ranger (v3.0.2).

Single-cell RNA sequencing data processing and analysis

Raw feature matrices obtained from Cell Ranger (v3.0.2) were converted into a Seurat object and analysed using Seurat V4 (v4.3.0)(Hao et al., 2021) in R (v4.0.3). Genes contained in at least 3 cells and cells containing at more than 725 (Myeloid progenitors) or 200 (Siglec-F + Leukocytes) but less than 7000 RNA features and with a percentage of mitochondrial genes comprised between 1 and 10 (Myeloid progenitors) or below 20 (Siglec-F + Leukocytes) were selected for subsequent analysis. Clustering was performed using the Leiden algorithm (v0.4.3)(Traag et al., 2019). Slingshot (v1.8.0)(Street et al., 2018) was used for lineage trajectory inference and pseudotime calculation. Regulon activities were computed with SCENIC (v1.3.1) (Aibar et al., 2017) and AUCell(v1.21.2) as in: http://htmlpreview.github.io/?https://github.com/aertslab/SCENIC/blob/master/inst/doc/SCE NIC_Running.html.

For fixed single-cell RNA sequencing combined CITE-seq, data was also analysed using Seurat V4 following the WNN pipeline (https://satijalab.org/seurat/articles/weighted_nearest_neighbor_analysis) and cells with at least 10 RNA features and 30 RNA counts were selected for analysis. Cells with low RNA content but at least 500 counts of any protein feature were also integrated to the analysis. Erythroid , basophil and eosinophil clusters were subselected in order to rerun the analysis focusing only on these lineages.

Single-cell RNA sequencing data from magnetically enriched Siglec-F^{+} cells from the bone marrow of steady state IL-5 transgenic mice was downloaded from: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182001 (Gurtner et al., 2022). Cells with at least 200 but no more than 6000 RNA features, at least 500 RNA counts and a percentage of mitochondrial genes between 0 and 10 were used for subsequent analysis with SeuratV4. The 20 first principal components were used to compute Nearest Neighbours and UMAP and a resolution of 0.5 was used for Louvain Clustering.

Mouse and human single-cell suspensions

Mouse blood was collected from the orbital sinus of terminally anesthetized mice and incubated with in-house prepared ammonium chloride lysis buffer (UltraPure distilled water (Invitrogen) supplemented with 150mM NH4Cl, 10mM KHCO, and 0.1mM EDTA) at room temperature for 5 minutes to lyse red blood cells. Cell suspension was passed through a 70μM cell strainer to remove cell clumps and washed twice prior to surface marker staining. Mouse bone marrow was recovered from hind leg bones (pelvis, tibia, and femur). Bones were flushed with 10mL ice-cold PBS and passed through a 70μM cell strainer to remove cell clumps. Recovered single-cell suspensions were incubated with ammonium chloride lysis buffer for red blood cell lysis. Single-cell suspensions were washed twice with PBS prior to surface marker staining. Human bone marrow aspirates were washed twice by diluting 1:50 in PBS. Red blood cells were depleted using EasySep™ RBC Depletion Reagent for a total of three cycles (Stemcell, 18170) following the manufacturer's instructions. The recovered single-cell suspensions were washed twice with PBS prior to surface marker staining.

High dimensional flow cytometric screening

Single-cell suspensions of mouse bone marrow from CB57BL/6 mice were first stained for Ly6G-FITC (BioLegend, 127606), CD90.2-FITC (BioLegend, 140204), NK1.1-FITC (eBioscience, 11-5941-85), CD45R-FITC (eBioscience, 11-0452-85) for 30 minutes on ice. Samples were immunomagnetically enriched for cells of interests using EasySep™ FITC positive selection kit II (Stemcell, 17682) as described in the manufacturer's instructions. The negative fractions were subsequently stained on ice for 30 minutes using a 13-color backbone panel with the following antibodies: SCA-1-BUV395 (BD Biosciences, 563990), CD11bBUV737 (BD Biosciences, 621800), CD115-BV421 (BD Biosciences, 743638), CD55-biotinstreptavidin-BV510 (R&D, BAF5376, BD Biosciences, 563261), CXCR2-BV605 (BD Biosciences, 747814), Siglec-F-BV711 (BD Biosciences, 740764), CD16/32-BV786 (BD Biosciences, 740851), c-Kit-BB700 (BD Biosciences, 566414), CD200R3-PE-cy7 (eBioscience, 25-2001-82), CCR3-APC (BioLegend, 144512), CD45-Alexa fluor 700 (BD Biosciences, 560510), and FcεR1α-APC-eFluor780 (eBioscience, 47-5898-82) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes on ice (Figure S2A). Cells were washed twice and aliquoted into individual wells (3.105 cells/well) all containing a different PE-conjugated antibody (LEGENDScreen™, BioLegend, 700003) and incubated for 30 minutes on ice.

Single cell suspensions of human bone marrow were stained on ice for 30 minutes with a 12-color backbone panel containing the following antibodies: CD38-BUV395 (BD Biosciences, 563811), CD34-BUV737 (BD Biosciences, 748739), CD125-biotin-streptavidinBV421 (Miltenyi, 130-110-543, BD Biosciences, 563259), FcεR1α-BV510 (BD Biosciences, 747786), CD11b-BV711 (BioLegend, 101242), CD45-BV786 (BD Biosciences, 563716), lineage-FITC (BioLegend, 348801), Siglec-8-BB700 (BD Biosciences, 747867), CD200R-PEcy7 (BioLegend, 329312), CCR3-APC (Miltenyi, 130-116-507), c-Kit-APC-R700 (BD Biosciences, 565195), and CD66b-APC-cy7 (BD Biosciences, 305126) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes at room temperature (Figure S3A). Cells were washed twice and aliquoted into individual wells all containing a specific PEconjugated marker

(LEGENDScreen™, BioLegend, 700007) and incubated for 30 minutes at room temperature.

All mouse and human samples were washed twice and the final cell pellet was resuspended in FACS buffer supplemented with 5nM BD Via-Probe™ Green (BD Biosciences, 565802). The 96-wells plates were acquired on a 5-laser BD LSRFortessa cell analyser (BD biosciences).

All data were pre-processed using FlowJo™ software (BD Biosciences, version 10.8, Supplementary Figures 2B & 3B). Each FCS file was assessed for quality control and single viable CD45+ dumpcells were selected for further analysis. Only markers with expression higher than background on any of the events within the pool of target cells were provided to the InfinityFlow pipeline totalling 163 markers for the human analysis and 142 for the mouse (Figure S2B and Figure S3B). The InfinityFlow pipeline was run as described in Becht et al.(Becht et al., 2023). with 4.104 target cells as input for human data and 5.104 cells for mouse data. Output FCS files were concatenated into a single file for downstream analysis using FlowJo™ software.

Cell sorting of stages of eosinophil maturation from mouse and human bone marrow

Suspensions of mouse bone marrow cells from CB57BL/6 at steady state and following 7 daily IL-33 intraperitoneal injections were first stained with anti-Ly6G-FITC (BioLegend, 127606), CD90.2-FITC (BioLegend, 140204), NK1.1-FITC (eBioscience, 11-5941-85), CD45R-FITC (eBioscience, 11-0452-85) for 30 minutes on ice. Samples were immunomagnetically enriched for cells of interest using EasySep™ FITC positive selection kit II (Stemcell, 17682) as described in the manufacturer's instructions. The negative fractions were subsequently stained for CD29-pacblue (BioLegend, 102224), F4/80-BV510 (BioLegend, 123125), SiglecFBV711(BD Biosciences,740764), CD55-biotin-streptavidin-BV786 (R&D, BAF5376, BD Biosciences, 563858), c-Kit-BB700 (BD Biosciences, 566414), Pir-A/B-PE (BioLegend, 144104) and CD200R3-PE-cy7

(BioLegend, 142212) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes on ice.

Human bone marrow single cell suspensions were first stained with Lineage-cocktail (BioLegend, 348801) for 30 minutes on ice. Samples were immunomagnetically enriched for cells of interests using EasySep™ FITC positive selection kit II (Stemcell, 17682) as described in the manufacturer's instructions. The negative fractions were next stained for CD84-BV421 (BD Biosciences, 566904), FcεR1α-BV510 (BD Biosciences, 747786), CD11b-BV711 (BioLegend, 101242), CD45-BV786 (BD Biosciences, 563204), Siglec-8-BB700 (BD Biosciences, 747867), CD38-PE (BioLegend, 356604), CD200R-PE-cy7 (BioLegend, 329312), CCR3-APC (Miltenyi, 130-123-300), and CD66b-APC-cy7 (BioLegend, 305126) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes at room temperature.

All mouse and human cells suspensions were washed twice and resuspended in PBS supplemented with 5nM BD Via-Probe™ Green (BD Biosciences, 565802). Mouse and human eosinophil stages were sorted directly into TRIzol (ThermoFisher, 15596026) or into FACS buffer for bright-field microscopy using a BD FACSAria III (BD Biosciences) cell sorter with a 100µm nozzle. Sort purity was at 95 per cent or higher and samples were stored at -80°C for downstream RNA applications or processed immediately for bright-field microscopy.

Bright-field microscopy

Sorted human- and mouse bone marrow cells were sedimented at 300xg for 5 minutes and resuspended in Freeflex Geloplasma 3 per cent (Fresenius Kabi, RVG 20107), loaded into a cytofunnel and spun at 800rpm for 4 minutes on a 12 samples rotor in a Tharmac Cellspin I machine. Slides were left to dry 30 minutes and cells were stained using Hemacolor Rapid staining kit (Sigma-Aldrich, 1116610001). Bright-field cell images were taken at 1500x total magnification using an oil immersion objective.

RNA extraction

Human blood and mouse bone marrow eosinophils were sorted as described above directly into TRIzol (ThermoFisher). For every mL of TRIzol, 200μL of chloroform was added, and the samples were vigorously mixed and incubated for 2 minutes at room temperature. Samples were centrifuged at 10,000xg for 15 minutes at 4°C to separate the phases. The RNAcontaining upper aqueous phase was transferred to a new microcentrifuge tube containing 475μL of isopropanol and 2μL of glycoblue (ThermoFisher, AM9515). Samples were centrifuged at 10,000xg for 15 minutes and supernatant was discarded. One volume of 75 per cent ethanol was added to wash the RNA pellet and samples were centrifuged at 10,000xg for 1 minute and supernatant was discarded. RNA pellet was resuspended in 40μL of DNase/RNasefree water for a 15-minute DNase treatment (Zymo Research, E1010). DNase treatment was followed by column-based RNA purification with the RNA Clean & concentrator-5 kit (Zymo Research, R1016). Briefly, 100μL of RNA binding buffer was added to every 50μL sample and mixed thoroughly. One volume of 100 per cent ethanol was added and the sample was transferred into a Zymo-Spin™ IC column in a collection tube. Columns were centrifuged at 10,000xg for 30 seconds and flow-through was discarded. The column was washed once with RNA prep buffer and twice with RNA wash buffer, following the manufacturer's instructions. RNA was eluted in 10μL of DNase/RNase-free water and stored at -80°C. Integrity and quantity of purified RNA were assessed using the RNA 6000 Pico kit (Agilent) for the presence of 18s and 28s rRNA peaks.

RNA sequencing & data processing

Full length cDNA was prepared from isolated RNA using SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio, 634889) following the manufacturer's instructions, with 17 cycles of cDNA amplification. Final cDNA quality was assessed using Agilent High Sensitivity DNA kit (Agilent, 5067-4626). cDNA libraries were prepared for sequencing using Nextera XT

DNA library preparation kit (Illumina, FC-131-1024) using the manufacturer's instructions and samples were sequenced on a NovaSeq™ 6000 sequencing system (Illumina).

Differential gene expression analyses

Sequenced reads were aligned to the mouse genome (UCSC mm10) or the human genome (HG19) with RNA-seq Alignment (v2.0.2) using STAR aligner (version 2.6.1a) on BaseSpace (https://basespace.illumina.com). Uniquely mapped reads were used to calculate gene expression. Differential gene expression was calculated using DESeq2 (v1.26.0) in R (v4.1.2) (Love et al., 2014). For analysis of differential gene expression along steady-state human and murine eosinophilopoiesis, we performed a paired likelihood ratio test (LRT) comparing the full model (gene count~donor+stage) with a reduced model (gene count~donor) on wellexpressed expressed genes (baseMean>100). For comparison of eosinophil maturation stages in steady-state and IL33-stimulated mice, we used a likelihood ratio test (LRT) comparing the full model (gene count~stage*condition) with a reduced model (gene count~stage) on wellexpressed expressed genes (baseMean>100). Genes with FDR100 or >50 genes.

Gene set enrichment analyses

Enrichment of specified gene sets in GeneOntology biological process gene signatures was calculated using ShinyGO (v0.77, http://bioinformatics.sdstate.edu/go/) with default parameters. Regulon activities were computed with SCENIC (v1.3.1) and AUCell(v1.21.2). Genes not represented by at least 1 UMI in each sample on average were discarded. The subsequent first quartile of lowly expressed genes was also filtered out. In bulk RNAseq analyses, only regulons with at least 0.3 activity score in any of the samples were considered for subsequent analysis.

Models of eosinophilia using IL-33, Nippostrongylus brasiliensis, and Alternaria alternata

For the IL-33 model, CB57BL/6 mice were injected intraperitoneally with 400ng of recombinant murine IL-33 in sterile PBS (Biolegend, 580506) daily for 7 days. Single cell bone marrow suspensions were harvested at steady state and at days 2, 4, 7, 9, and 12 (Figure 6A). Single cell bone marrow suspensions of Il5-/- mice were harvested at steady state and at day 7. CB57BL/6 mice also receiving anti-IL-5 treatment in addition to IL-33 were injected intraperitoneally with 400µg of anti-IL-5 (BioLegend, 504318) in sterile PBS at day 6, 7, and 8.

Nippostrongylus brasiliensis parasites were maintained in male Wistar rats as described previously (Bouchery et al., 2017; Rolot et al., 2018). L3 larvae were subsequently isolated from 9 to 16 day fecal cultures using a Baermann apparatus. CB57BL/6 mice were subcutaneously infected with 500xL3 larvae in sterile PBS. Single cell bone marrow suspensions were harvested at steady state and at days 5, 8, and 15 (Figure 6E).

In the *Alternaria alternata* model, we intranasally administered 200µg of *Alternaria alternata* extract (Citeq biologics, 09.01.26) in sterile PBS daily for 22 days to CB57BL/6 mice. Single cell bone marrow suspensions were harvested at steady state and at days 4, 8, 15, 22, and 30 (Figure 6F).

All recovered single cell bone marrow suspensions were subsequently immunophenotyped using the following 9-color conventional flow cytometry panel: CD29- Pacific Blue (BioLegend, 102224), F4/80-BV510 (BioLegend, 123125), Siglec-F-BV711(BD Biosciences,740764), CD55-biotin-streptavidin-BV786 (R&D, BAF5376, BD Biosciences, 563858), Ly6G-FITC (BioLegend, 127606), CD90.2-FITC (BioLegend, 140204), NK1.1- FITC (eBioscience, 11-5941-85), CD45R-FITC (eBioscience, 11-0452-85), c-Kit-BB700 (BD Biosciences, 566414), Pir-A/B-PE (BioLegend, 144104) and CD200R3-PE-cy7 (BioLegend, 142212), and CCR3-APC (BioLegend, 144512) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes on ice. Stained samples were washed twice and resuspended in FACS buffer supplemented with 5nM BD Via-Probe™ Green (BD Biosciences, 565802). All samples were acquired on a 5-

laser BD LSRFortessa cell analyser (BD biosciences) and recorded data were analysed using FlowJo™ software (BD Biosciences, version 10.8). Instrument daily laser variations were corrected using BD FACSDiva™ CS&T research beads (BD Biosciences, 655050) and linked custom application settings system. Viable single cells were normalised across the different samples and eosinophil stages were quantified as cells per million bone marrow cells.

DNA replication assays

CB57BL/6 mice at steady-state and in models of eosinophilia were injected intraperitoneally with 1mg of EdU (5-ethynyl-2'-deoxyuridine) in PBS exactly 1 hour before sacrifice. Bone marrow cells were submitted to Click-iT™ EDU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Thermofisher, C10424) following the manufacturer's instructions. Single cell bone marrow suspensions were stained with anti-CD29-pacblue (BioLegend, 102224), F4/80- BV510 (BioLegend, 123125), Siglec-F-BV711 (BD Biosciences,740764), CD55-biotinstreptavidin-BV786 (R&D, BAF5376, BD Biosciences, 563858), Ly6G-FITC (BioLegend, 127606), CD90.2-FITC (BioLegend, 140204), NK1.1-FITC (eBioscience, 11-5941-85), CD45R-FITC (eBioscience, 11-0452-85), c-Kit-BB700 (BD Biosciences, 566414), Pir-A/BPE (BioLegend, 144104) and CD200R3- PE-cy7 (BioLegend, 142212) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes on ice. All stained suspensions were washed twice and acquired on a 5-laser BD LSRFortessa cell analyser (BD biosciences) and recorded data were analysed using FlowJo™ software (BD Biosciences, version 10.8).

Characterization of Il5ra expression in murine bone marrow progenitor cells

Bone marrow cells were harvested from Il5ra reporter heterozygote ($Il5ra^{KI/+}$) and CB57BL/6 ($Il5ra^{+/+}$) mice. Cells were first stained for Ly6G-biotin (BioLegend, 127604), CD90.2-biotin (BioLegend, 140314), NK1.1-biotin (BioLegend, 108704), CD45R-biotin (BioLegend, 103204) for 30 minutes on ice. Samples were then immunomagnetically enriched for cells of interest using EasySep™ biotin positive selection kit II (Stemcell,

17683) as described in the manufacturer's instructions. The negative fractions were subsequently stained for anti-SCA1-BV421 (BD Biosciences, 553108), F4/80-BV510 (BioLegend, 123125), CD150-BV711 (BioLegend, 115941), c-Kit- BB700 (BD Biosciences, 566414), CD55-PE (BioLegend, 131804), CD200R3- PE-cy7 (BioLegend, 142212), CD45-Alexa Fluor 700 (BioLegend, 110724), CD16/32-APC-Cy-7 (BioLegend, 101328), CD11b-BUV737 (BD Biosciences, 612800), and streptavidin-BUV395 (BD Biosciences, 564176) in 1X BD Horizon™ Brilliant stain buffer (BD Biosciences, 563794) for 30 minutes on ice. Samples were washed twice and acquired on a 5-laser BD LSRFortessa cell analyser (BD biosciences) and recorded data were analysed using FlowJo™ software (BD Biosciences, version 10.8).

Characterization of Il5ra-expressing bone marrow leukocytes

We built a 19-color antibody panel allowing the characterization of a wide variety of white blood cell lineages within the bone marrow. Bone marrow cells were harvested from $Il5ra^{KI/+}$ and $Il5ra^{+/+}$ mice and were stained with SiglecH-BV480 (BD Biosciences, 752585), CD125-BV421 (BD Biosciences, 565015), FcεR1α-Pacific Blue (Sony biotechnology, 1271570), CD138-BV605 (Sony biotechnology, 1312580), c-Kit-BV650 (Sony biotechnology, 1275625), CD64-BV711 (Sony biotechnology, 1296555), CD19-BV750 (Sony biotechnology, 1177805), CD14-BV785 (Sony biotechnology, 1216685), CD3-Spark Blue 574 (Sony biotechnology, 1101380), Ly6G-PerCP (Sony biotechnology, 1238270), Siglec-F-PE (BD Biosciences, 552126), F4/80-PE-Dazzle 594 (Sony biotechnology, 1215730), NK1.1-Pe-Cy5 (Sony biotechnology, 138620), CD200R3-Pe-Cy7 (BioLegend, 329312), CD8a-PE-Fire700 (Sony biotechnology, 1103960), CCR3-APC (BioLegend, 144512), CD4-APC-Fire480 (Sony biotechnology, 1102400), and CD45-Alexa Fluor™ 700 (BioLegend, 110724) on ice for 30 minutes. Cells were washed twice and acquired on a 5 laser ID7000™ spectral cell analyser (Sony biotechnology). All recorded data were analysed using FlowJo™ software (BD Biosciences, version 10.8).

Assessment of staining and specificity of anti-murine CD125 antibodies

Bone marrow cells were harvested from homozygote Il5ra reporter ($Il5ra^{KI/KI}$), which are knock-out for Il5ra) and $Il5ra^{+/+}$ mice. Cell suspensions were stained with SiglecF-BV421 (BD Biosciences, 562681), Ly6G-PerCP-Cy5.5 (BioLegend, 127615), and CCR3-APC (BioLegend, 144512) on ice for 30 minutes. Samples were subsequently aliquoted and stained with one of the following PE-conjugated anti-CD125 clones: T21 (BD Biosciences, 558488), REA343 (Miltenyi, 130-125-513), or DIH37 (BioLegend, 153403). All samples were washed twice and acquired on a BD FACSAria III (BD Biosciences) cell sorter. CD125+Ly6G+ and CD125+Ly6G- were sorted using a 100µm nozzle into FACS buffer for bright field microscopy and recorded data were analysed using FlowJo™ software (BD Biosciences, version 10.8).

Statistical analyses

All statistical analyses were performed in R (v4.1.2). All experiments followed a randomized design. Sample sizes were determined by power analysis. Respect of tests assumptions and model fit were evaluated using diagnostic plots. Raw data were transformed when needed and back-transformed for graphical presentation. Statistical tests applied to each dataset are indicated in figure legends. For clarity of presentation, only results of intergroup comparisons of interest are displayed in figures.

Data availability

Bulk and single-cell RNA-sequencing data is available from the Gene Expression Omnibus database at the National Centre for Biotechnology Information under accession number GSE249011 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE249011). Other data and scripts are available upon reasonable request.

4.2.4 Results

Droplet-based single-cell RNA sequencing captures the first stages of eosinophilopoiesis

We first aimed to resolve the development of eosinophils starting from their first identifiable progenitors in the BM of mice, with the aim of finding tractable cell surface markers for further identification. We resorted to single-cell RNA sequencing (scRNAseq) of lineage (lin)-negative c-Kit^{+} Il7ra^{-} progenitors sorted from the BM of steady-state mice using a 10X droplet-based approach to generate a "snapshot" of the ontogenic relationships of early eosinophil progenitors [13,30]. Uniform Manifold Approximation and Projection (UMAP [31]) displayed the 2 major branches of myelopoiesis (Figure 1A), with one arm giving rise to monocytes and neutrophils and another arm of *Gata1*-expressing progenitors leading to the erythroid, megakaryocyte, basophil and eosinophil lineages (Figure 1B). Early eosinophil-committed progenitors were identified based on their expression of eosinophil marker genes (*Epx*, *Prg3*, …) and displayed quality of data comparable to that of other progenitors (Figure 1C, Table S1 and Figure S1A).

To zoom in on eosinophil ontogeny, we sub-selected hematopoietic stem and progenitor cells (HSPCs) clusters containing hematopoietic stem cells (HSCs, marked by the expression of the *Hlf* transcription factor [32]) and those belonging to the *Gata1*-expressing subbranch leading to the basophil/mast cell and eosinophil lineages (Figure 1B-C). UMAP of this sub-selection and Slingshot trajectory inference displayed a continuum from HSPCs toward eosinophil/basophil/mast cell (EBM) progenitors, which in turn separated into basophil -committed and eosinophil-committed progenitors identifiable by lineage marker genes such as *Cd200r3* and *Epx*, respectively (Figure 1D-F). We did not detect progenitors with specific mast cell markers different from markers also shared with basophils, possibly owing to the rarity of mast cells in normal murine BM. Previous scRNAseq analyses on larger number of progenitors identified putative mast cell progenitors in murine BM, which were proposed to share a common progenitor pool with basophils [33]. Based on this current hypothesis, we postulate that EBM clusters identified here likely encompassed progenitors of both basophils and BM-derived mast

cells, but we will not refer to mast cells in subsequent analyses of our scRNAseq dataset.

We then used single-cell regulatory network inference and clustering (SCENIC) [34] to position the activity of key transcription factors involved in eosinophilopoiesis in this actualized developmental path. We observed 4 major patterns among regulons. The first cluster consisted in regulons of transcription factors associated with HSCs such as *Hlf* (Figure S1B). A second cluster displayed upregulated activity of transcription factors in EBM progenitors, which included *Gata1* and *Gata2*, consistent with their belonging to the "*Gata1*" arm of myelopoiesis. EBM progenitors also upregulated the activity of transcription factors involved in endoplasmic reticulum homeostasis including *Xbp1*, which is particularly important for eosinophil maturation [35]. A third cluster of transcription factors involved in terminal myeloid cell maturation and function was upregulated in basophil progenitors. This cluster also contained *Cebpa,* which was already upregulated in EBM progenitors, in line with its role in basophil and eosinophil differentiation [18]. Finally, eosinophil commitment was characterized by a small cluster of regulons mostly displaying upregulation of *Cebpe*, a known promoter of eosinophil differentiation [36]. Eosinophil differentiation also involved the downregulation of *Cebpa* activity, consistent with the required balance between *Cebpa* and *Cebpe* for normal eosinophil development [37]. One unexpected observation was the low activity of Interferon response factor-8 (*Irf8*) along eosinophil ontogeny (Figure S1B), since *Irf8* has been proposed to play an important role in eosinophil differentiation, maturation and expansion [18,38].

Within this actualized transcriptomic landscape of eosinophilopoiesis, we finally aimed to find cell surface markers of early eosinophil progenitors. Noticeably, *Il5ra* gene expression was detectable in the first identifiable eosinophil progenitors, but not earlier (Figure S1C). Aside from *Il5ra*, we did not identify detectable or discriminating expression of other cell surface markers, including *Siglec-f*, *Adgre1* (encoding F4/80), or *Ccr3* (Table S1 and Figure S1C). The earliest identifiable eosinophil progenitors in mice hence might be best defined by exclusion of other lineages.

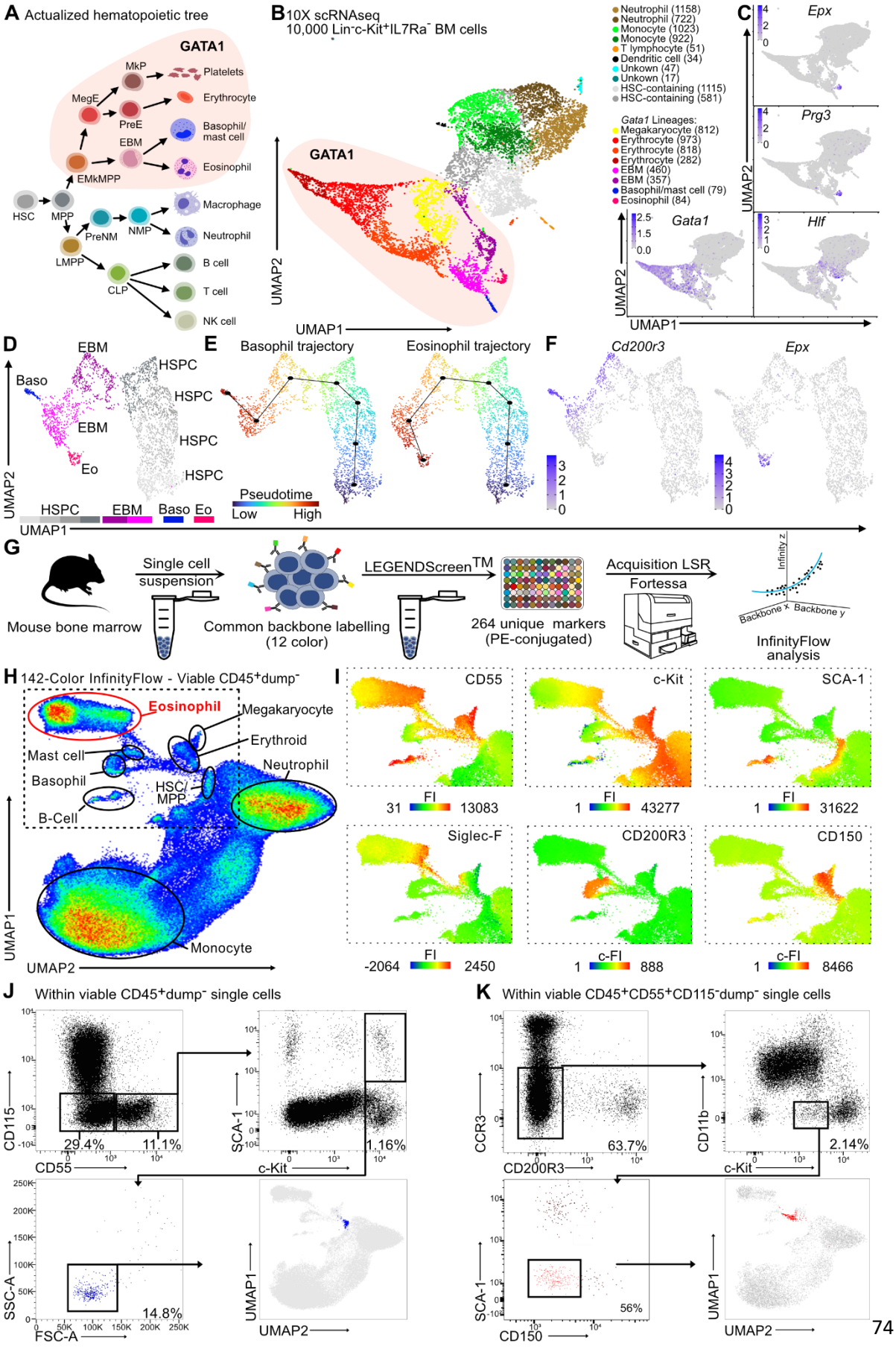

A Actualized hematopoietic tree
GATA1
MkP
Platelets
MegE
Erythrocyte
PreE
Basophil/ mast cell
EBM
Eosinophil
EMkMPP
HSC
MPP
Macrophage
PreNM
NMP
Neutrophil
LMPP
B cell
CLP
T cell
NK cell
B 10X scRNAseq
10,000 Lin-c-Kit+IL7Ra- BM cells
GATA1
UMAP2
UMAP1
Neutrophil (1158)
Neutrophil (722)
Monocyte (1023)
Monocyte (922)
T lymphocyte (51)
Dendritic cell (34)
Unkown (47)
Unkown (17)
HSC-containing (1115)
HSC-containing (581)
Gata1 Lineages:
Megakaryocyte (812)
Erythrocyte (973)
Erythrocyte (818)
Erythrocyte (282)
EBM (460)
EBM (357)
Basophil/mast cell (79)
Eosinophil (84)
C
Epx
Prg3
Gata1
Hlf
UMAP2
UMAP1
D
EBM
HSPC
Baso
HSPC
EBM
HSPC
Eo
HSPC
UMAP2
HSPC EBM Baso Eo
UMAP1
E
Basophil trajectory
Eosinophil trajectory
Pseudotime
Low High
F
Cd200r3
Epx
G
Mouse bone marrow
Single cell suspension
Common backbone labelling (12 color)
LEGENDScreen™
264 unique markers (PE-conjugated)
Acquisition LSR Fortessa
Infinity z
Backbone x Backbone y
InfinityFlow analysis
H 142-Color InfinityFlow - Viable CD45+dump-
Eosinophil
Megakaryocyte
Erythroid
Mast cell
Neutrophil
Basophil
HSC/ MPP
B-Cell
Monocyte
UMAP1
UMAP2
I
CD55
c-Kit
SCA-1
31 FI 13083
1 FI 43277
1 FI 31622
Siglec-F
CD200R3
CD150
-2064 FI 2450
1 c-FI 888
1 c-FI 8466
J Within viable CD45+dump- single cells
CD115
CD55
29.4%
11.1%
SCA-1
c-Kit
1.16%
SSC-A
FSC-A
14.8%
UMAP1
UMAP2
K Within viable CD45+CD55+CD115-dump- single cells
CCR3
CD200R3
63.7%
CD11b
c-Kit
2.14%
SCA-1
CD150
56%
UMAP1
UMAP2

Figure 1. Single-cell RNA sequencing combined with high dimension flow cytometric screening resolves murine eosinophilopoiesis.

A. Revised hematopoietic tree adapted from (Jacobsen and Nerlov, 2019). **B.** UMAP and lineage annotation, based on lineage marker genes in Table S1, of steady-state murine BM progenitors in droplet-based scRNAseq. Insert shows overlayed expression of *Gata1* defining Gata1 lineages. **C.** Overlayed expression of HSC and eosinophil marker genes in A. **D-E.** Slingshot trajectory inference in HSC-containing clusters (HSPC) and eosinophil and basophil/mast cell lineage clusters subselected from B (D), overlayed with pseudotime on basophil and eosinophil cell trajectories (E). **F.** Overlayed expression of eosinophil marker gene Epx and of basophil marker gene Cd200r3 in E. **G.** Experimental outline of a flow cytometric screening focused on the murine eosinophil lineage (more detail in Figure S3A-B). **H.** UMAP of an InfinityFlow-integrated 142-marker staining of dump-negative CD45+ murine BM cells with major lineages annotated based on cell surface markers in Figure S3C and Table S3. **I.** Overlayed staining intensity of select markers in indicated insert in H. **J.** Gating strategy of $CD55^{+}$ HSPCs and projection on UMAP in H. **K.** Gating strategy of the eosinophil/basophil/mast cell progenitor pool and projection on UMAP in H. (Baso: basophil, c-FI: background-corrected fluorescence intensity, EBM: eosinophil/basophil/mast cell progenitors, Eo: eosinophil, Ery: erythroid, FI: fluorescence intensity, HSC: hematopoietic stem cell)

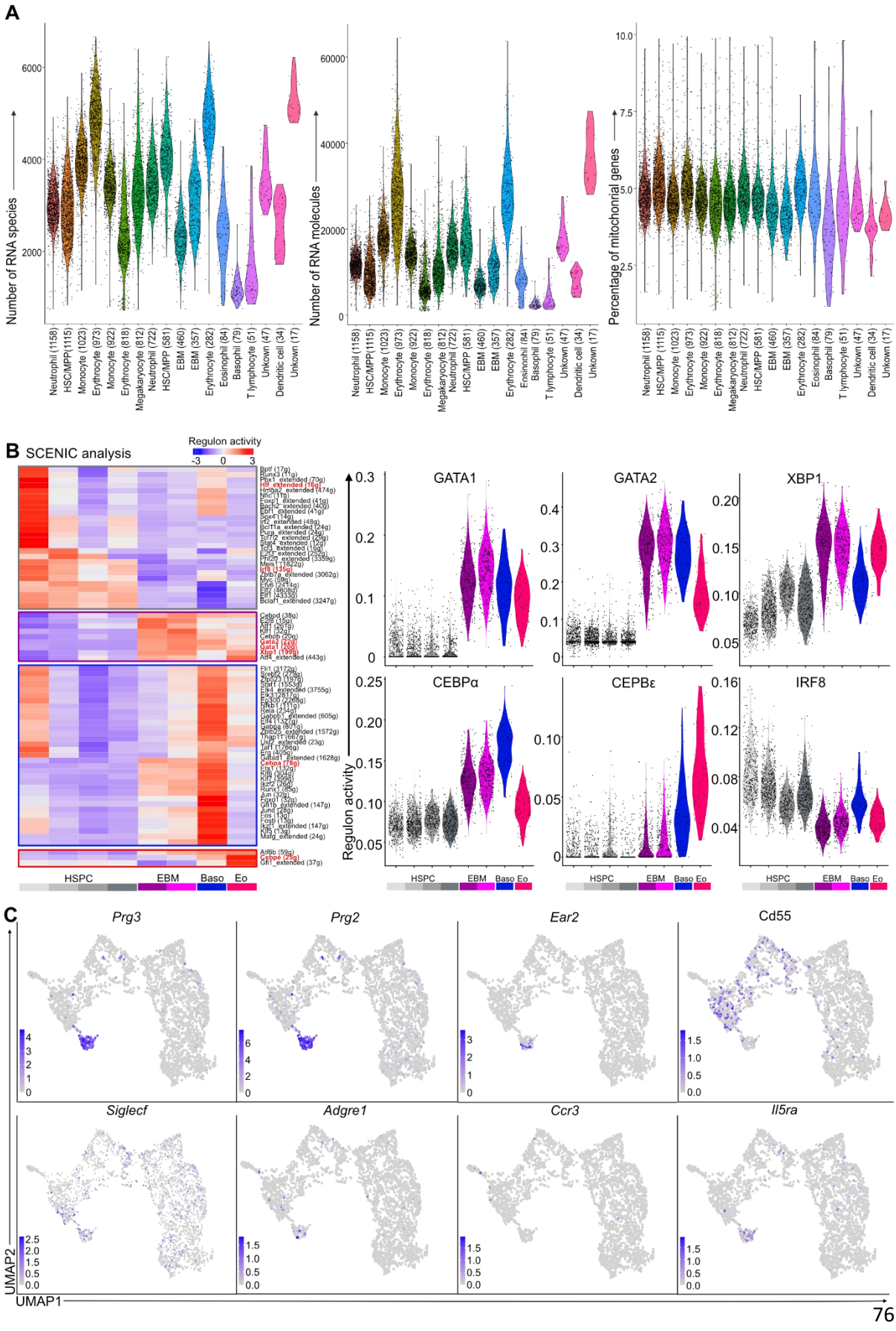

A
Number of RNA species
Number of RNA molecules
Percentage of mitochonrial genes
Neutrophil (1158)
HSC/MPP (1115)
Monocyte (1023)
Erythrocyte (973)
Monocyte (922)
Erythrocyte (818)
Megakaryocyte (812)
Neutrophil (722)
HSC/MPP (581)
EBM (460)
EBM (357)
Erythrocyte (282)
Eosinophil (84)
Basophil (79)
T lymphocyte (51)
Unkown (47)
Dendritic cell (34)
Unkown (17)
B SCENIC analysis
Regulon activity
HSPC
EBM
Baso
Eo
GATA1
GATA2
XBP1
CEBPα
CEPBε
IRF8
Regulon activity
C
Prg3
Prg2
Ear2
Cd55
Siglecf
Adgre1
Ccr3
Il5ra
UMAP2
UMAP1

Figure S1 (related to Figure 1). Single-cell RNA sequencing analysis and SCENIC analysis of murine bone marrow progenitors.

A. Violin plots of number of RNA species, number of RNA molecules, and percentage of mitochondrial genes in cell clusters of murine BM progenitors in Figure 1B. **B.** SCENIC analysis of cell clusters in Figure 1D presented as row-scaled heatmaps (left) or absolute activity of select regulons (right). **C.** Overlayed expression of eosinophil marker and cell surface marker genes on UMAP in Figure 1D. (Baso/mast: basophil/mast cell progenitors, EBM: eosinophil/basophil/mast cell progenitors, Eo: eosinophil, Ery: erythroid)

Single-cell RNA sequencing is limited for resolving eosinophil maturation

We next evaluated whether different automated methods of scRNAseq could be implemented to identify cell surface markers resolving eosinophil maturation downstream of lineage commitment. We reanalyzed a recently published dataset of well-based scRNAseq on Siglec-F^{+} BM cells from highly eosinophilic IL-5 transgenic mice [8]. Using a recommended resolution in Seurat returned 4 clusters of eosinophil lineage cells in the BM of these mice, which differed by their expression of genes associated with eosinophil function (*Epx, Ccr3,* …) and the cell cycle (*Mki67, Top2a,* …) (Figure S2A). Complicating the analysis, no differential expression of cell surface marker genes could be detected that discriminated the different clusters of eosinophils from each other, except for a putatively more mature *Ccr3*-expressing subset (Table S2). We likewise performed a 10X droplet-based analysis on Siglec-F^{+} BM cells from mice rendered highly eosinophilic by repeated injections of IL-33. This method too recovered analyzable eosinophils, this time in 3 predicted clusters differing again by their expression of genes associated with eosinophil function (*Epx, Ccr3, Prg3*, …) and the cell cycle (*Pcna*, …) (Figure S2B). Yet again, only the presumably more mature eosinophil subset differentially expressed cell surface marker genes such as *Ccr3* (Table S2). Finally, we tested whether fixing cells in droplet-based 10X Flex scRNAseq could provide more RNA data in eosinophils among CD55^{+} Dump (Ly6G, B220, NK1.1, CD90.2)$^{-}$ cells of Gata1 lineages from steady-state wild-type mice. Additionally, cells were labeled with oligo-tagged antibodies to improve lineage identification and the recovery of cells with low RNA content. Combining RNA and protein information allowed the identification of the major Gata1 lineages and captured a continuum of eosinophil maturation from c-Kit^{+} CCR3^{-} to c-Kit^{-} CCR3^{hi} eosinophils along 4 putative clusters (Figure S2C-E). Nonetheless, the cellular RNA information dropped along eosinophil maturation, leading to a resolution that relied mostly on protein data. Altogether, while different platforms were able to capture eosinophils, depth of analysis and cell recovery in scRNAseq were suboptimal, especially for mature eosinophils. Reasons for this issue remain to be fully resolved but are often assumed to relate to high amounts of inhibitor-resistant RNAses in eosinophils [39].

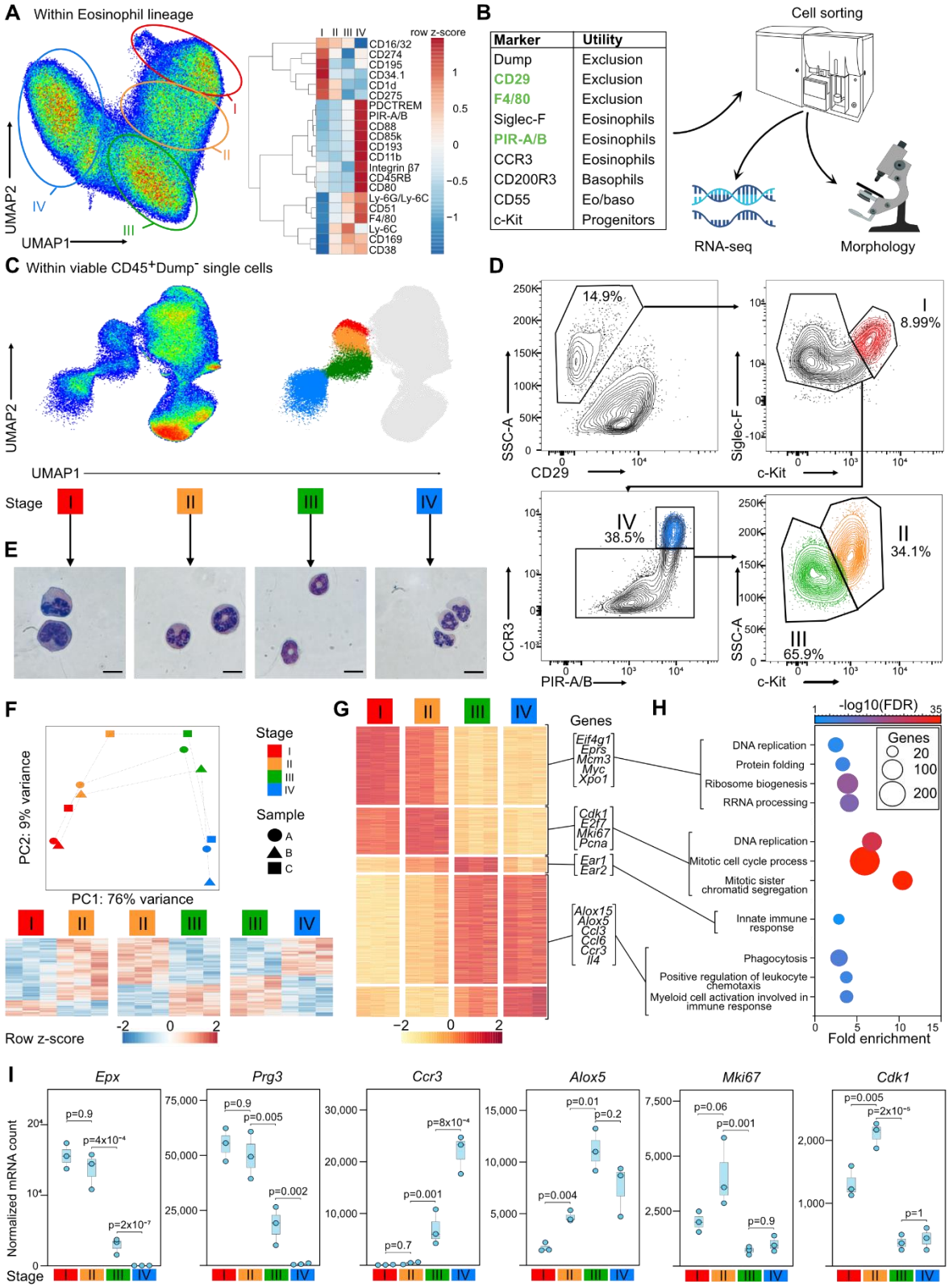

A
Within Eosinophil lineage
UMAP2
UMAP1
I
II
III
IV
I II III IV
row z-score
CD16/32
CD274
CD195
CD34.1
CD1d
CD275
PDCTREM
PIR-A/B
CD88
CD85k
CD193
CD11b
Integrin β7
CD45RB
CD80
Ly-6G/Ly-6C
CD51
F4/80
Ly-6C
CD169
CD38
B
Marker
Utility
Dump
Exclusion
CD29
Exclusion
F4/80
Exclusion
Siglec-F
Eosinophils
PIR-A/B
Eosinophils
CCR3
Eosinophils
CD200R3
Basophils
CD55
Eo/baso
c-Kit
Progenitors
Cell sorting
RNA-seq
Morphology
C
Within viable CD45+Dump- single cells
UMAP2
UMAP1
Stage
I
II
III
IV
D
14.9%
SSC-A
CD29
I
8.99%
Siglec-F
c-Kit
IV
38.5%
CCR3
PIR-A/B
II
34.1%
III
65.9%
SSC-A
c-Kit
E
F
PC2: 9% variance
PC1: 76% variance
Stage
Sample
A
B
C
Row z-score
G
Genes
Eif4g1
Eprs
Mcm3
Myc
Xpo1
Cdk1
E2f7
Mki67
Pcna
Ear1
Ear2
Alox15
Alox5
Ccl3
Ccl6
Ccr3
Il4
H
-log10(FDR)
Genes
20
100
200
DNA replication
Protein folding
Ribosome biogenesis
RRNA processing
DNA replication
Mitotic cell cycle process
Mitotic sister chromatid segregation
Innate immune response
Phagocytosis
Positive regulation of leukocyte chemotaxis
Myeloid cell activation involved in immune response
Fold enrichment
I
Epx
Prg3
Ccr3
Alox5
Mki67
Cdk1
Normalized mRNA count
Stage

Figure 2. Murine eosinophilopoiesis progresses along a continuum of immunophenotypes.

A. InfinityFlow-derived UMAP of eosinophil lineage cells in murine BM displaying 4 main immunophenotypes used to partition their maturation continuum into 4 stages (I to IV) with pseudodensity overlayed (left) and heatmap of their relative expression of highly expressed markers (right, signal intensity of all markers staining eosinophil-lineage cells is in Table S4). **B.** 9-color flow cytometric panel allowing the separation of murine eosinophil maturation into 4 (I-IV) immunophenotypic stages (InfinityFlow-inferred markers in green). **C.** UMAP of murine viable dump-negative (Ly6G- B220- NK1.1- CD90.2-) CD45+ bone marrow cells stained with marker panel in B as pseudodensity plot (left) or with maturation stages I to IV overlayed (right). **D.** Gating strategy, downstream of gating strategy in Figure S3D, used for partitioning eosinophil maturation into stages I to IV. **E.** Light imaging photographs of stage I-IV eosinophils (scale bar: 15 μm). **F-G.** Bulk RNA sequencing comparisons of stage I-IV BM eosinophils from 3 donors presented in a principal component analysis (F, upper, dashed lines connect samples from the same donor), as heatmaps of differentially expressed genes (DEG) between each stage (F, lower) and as major co-regulated modules (G and Table S5). **H.** Gene ontology (GO) enrichment analysis on co-expressed gene modules in G (FDR: false discovery rate). **I.** Comparison of normalized gene expression of select genes in F (one-way ANOVA followed by TukeyHSD tests).

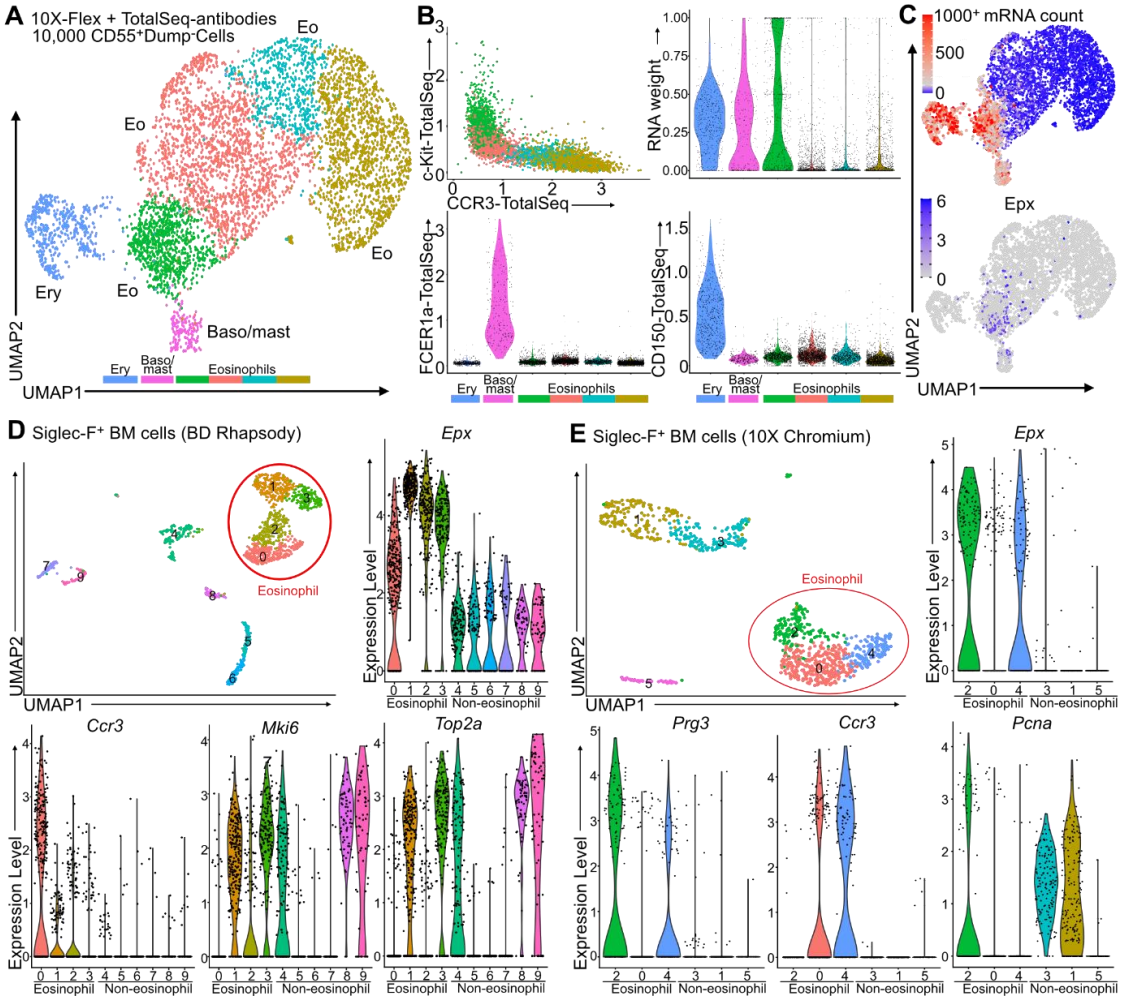

A
10X-Flex + TotalSeq-antibodies
10,000 CD55+Dump-Cells
Eo
Ery
Baso/mast
Eosinophils
UMAP1
UMAP2
B
c-Kit-TotalSeq
CCR3-TotalSeq
FCER1a-TotalSeq
RNA weight
CD150-TotalSeq
C
1000+ mRNA count
Epx
D
Siglec-F+ BM cells (BD Rhapsody)
Eosinophil
Non-eosinophil
Expression Level
Ccr3
Mki6
Top2a
E
Siglec-F+ BM cells (10X Chromium)
Prg3
Pcna

Figure S2. Single-cell RNA sequencing analysis of murine bone marrow progenitors using different platforms.

A. UMAP with eosinophil lineage clusters highlighted in a reanalysis of SiglecF+ BM cells from IL-5 transgenic mice in (Gurtner et al., 2022) using a resolution of 0.5 in Seurat, with violin plots of the indicated eosinophil marker and cell cycle-associated genes. **B.** UMAP with lineage annotation of a droplet-based scRNAseq analysis of SiglecF+ BM cells from mice injected for 7 days with IL-33, with violin plots of expression probability distribution of the indicated eosinophil marker and cell cycle-associated genes. **C.** UMAP and lineage annotation of Gata1 lineage progenitors of steady-state murine BM in fxed droplet-based scRNAseq with oligo-antibody tags. **D.** Plot of c-Kit versus Ccr3 oligo-tag antibody signal (upper left) and violin plots of RNA weight (upper right) and oligo-antibody tag signal (bottom) in cell clusters in C. **E.** Overlay of mRNA count (upper) and overlay of *Epx* mRNA signal (lower) in C. (Baso/mast: basophil/mast cell progenitor, Eo: eosinophil, Ery: erythroid)

Single-cell proteomic screening in flow cytometry resolves the maturation of murine eosinophils

Due to limitations of scRNAseq in resolving eosinophil maturation, we turned to single-cell surface proteome screening by flow cytometry, which has been previously used to resolve neutropoiesis [40]. We used our scRNAseq analyses to inform an initial backbone marker panel aimed at capturing the continuum between the earliest identifiable eosinophil progenitors among c-Kit^{+} CD55^{+} "Gata1" progenitors, and mature eosinophils known to express Ccr3 and Siglec-F, while excluding other lineages (Figure S3A). We used Infinity Flow [41] to combine this panel with a large-scale flow cytometric screening for 264 surface markers (LEGENDScreen, Biolegend, Figure 1G, Figure S3B). Major cell populations were classified using lineage markers based on prior knowledge (Figure S3C and Table S3). UMAP of the Infinity Flow output provided a consistent snapshot of the ontogenic relationships of eosinophils (Figure 1H, I). The eosinophil lineage emerged from a pool of CD55^{+} c-Kit^{+} Sca-1^{+} HSPCs, from which also diverged the erythroid, megakaryocytic, mast cell and basophil lineages, while monocytes and neutrophils were excluded from this branch of myelopoiesis (Figure 1H, J). Downstream of the early divergence of CD55^{+} Gata1 lineages, eosinophils arose together with basophils and mast cells from a pool of CD45^{+} CD55^{+} CD115^{-} Ccr3^{-} dump^{-} CD200R3^{-} CD11b^{-} c-Kit^{int} Sca-1^{-} CD150^{-} progenitors (Figure 1H, K).

We next inspected the 132 antibodies that labelled cells of the eosinophil lineage and improved the resolution of eosinophil maturation (Table S4). UMAP of the Infinity Flow output identified a continuum of eosinophil maturation comprising 4 main immunophenotypes appearing as distinguishable density nodes. To facilitate the further characterization of eosinophil progenitors at different stages of their maturation process, we chose to partition the continuum of eosinophil maturation based on these 4 immunophenotypes, which we refer to here as stages I to IV (Figure 2A). Among the 21 antibodies delivering strong signal (background-corrected median fluorescence intensity (MFI) > 103) at any stage of eosinophil maturation were markers previously associated with murine eosinophil progenitors such as CD34, which became downregulated along eosinophil maturation, as well as markers of mature eosinophils and granulocytes such

as Ccr3/CD193 and CD11b, which were progressively upregulated (Figure 2A). We optimized our initial panel by incorporating 3 additional markers: Pir-a/b, a marker highly upregulated along eosinophil maturation (Figure 2A-D), as well as F4/80 and CD29 (also known as integrin-b1, Itgb1) as discriminating markers that help exclude contaminant cells (Figure 2B-D, Figure S3D). The resulting 9-color conventional flow cytometric panel allowed to identify and sort 4 stages of murine eosinophil maturation (Figure 2C-E). Progenitors in stages I and II displayed eosinophilic granules and large, often contorted nuclei, with central hollowing of the nucleus visible in a majority of stage II cells (Figure 2E). Cells in stage III cells were distinctively smaller than in stages I-II, and exhibited ring-shaped nuclei, while stage IV cells displayed the classical morphology of mature murine eosinophils.

Next, we aimed to characterize the relationship between these 4 stages of eosinophil development using bulk RNA-sequencing. Principal component analysis (PCA) positioned the 4 stages on a continuum along the first principal component (PC1) that captured 76 per cent of the variance (Figure 2F). This analysis highlighted a major transcriptional transition happening between stages II and III, even though pairwise comparisons returned hundreds of differentially expressed genes (FDR<0.05) at transitions between stages I-II and III-IV (Figure 2F). We used hierarchical clustering to identify prominent patterns of gene regulation (>100 genes/pattern) in a likelihood ratio test (LRT) identifying well-expressed genes (baseMean>100) that were differentially expressed along eosinophilopoiesis (FDR<1.10-4), and tested their enrichment in Gene Ontology (GO) biological pathways. Stage I progenitors expressed on average the highest expression of genes associated with protein synthesis and ribosome biogenesis, while eosinophil granule protein-coding genes and genes associated with the cell cycle were highly expressed in both stages I and II (Figure 2G-I, Table S5). Expression of these genes decreased in stages III and IV. Stage III cells displayed higher expression of a small set of genes associated with innate immunity including eosinophil-associated RNAses. Finally, genes associated with mature myeloid cell function were progressively upregulated along eosinophil maturation, reaching their peak expression in stage IV eosinophils. The downregulation of eosinophil granule- and cell cycle-associated genes in the transition between stages II and III, along with the upregulation of myeloid

function-associated genes were the most prominent transcriptomic changes along steady-state eosinophilopoiesis. The above data were consistent with cells in stage I-II being myelocytes primarily involved in eosinophil granule production and lineage expansion in the steady-state, before transitioning toward non-proliferating stage III metamyelocytes and stage IV mature eosinophils.

Single-cell proteomic screening in flow cytometry shows conservation of human and murine eosinophilopoiesis

We performed a similar surface proteome screening in healthy human BM (Figure 3A, Figure S4A). We used UMAP visualization of the Infinity Flow output and annotated major cell populations using lineage markers (Figure 3B-C, Figure S4B-C and Table S3). Confirming the robustness of the approach, a sub-analysis of neutrophil lineage cells captured 4 previously proposed stages of neutrophil maturation 40 (Figure S4D). As in mice, human eosinophils, basophils and mast cells shared a common pool of progenitors (Figure 3B, D). Of note, CD125 surface expression was upregulated in committed eosinophil progenitors compared to cells engaging toward the basophil and mast cell lineages (Figure 3D).

We then focused on eosinophil maturation and observed that the continuum of maturation of human eosinophils encompassed 4 main immunophenotypes in our analysis, similarly to mice (Figure 4A). Hence, we opted to partition the continuum of human eosinophil maturation into 4 stages as well (I to IV). To generate a conventional flow cytometric antibody panel for human eosinophil maturation, we inspected the 153 screening antibodies that stained eosinophil lineage cells (Table S6) and focused on the 43 antibodies generating strong signal (background-corrected MFI>2.103) in at least one of the 4 stages of eosinophil maturation (Figure 4B). This selection contained known surface markers of human eosinophils in addition to markers not previously reported on human eosinophils such as TNFRSF12A (CD266/TWEAKR/FN14) (Figure 4B). Because of its dynamic regulation along human eosinophilopoiesis, we included CD84 into an optimized 10-color flow cytometric panel. We used this panel to isolate and study consecutive immunophenotypic stages of human eosinophil maturation (Figure 4C-E).

Based on their distinctive eosinophilic granule content and nuclear shape, cells in stage I-II were eosinophilic myelocytes, whereas stage III cells were metamyelocytes and stage IV cells were mature eosinophils (Figure 4E).

Next, we sorted the above 4 stages of human eosinophil maturation from 3 healthy donors and compared their transcriptome by RNAseq. A PCA positioned these 4 stages on a continuum along PC1 that captured 71 per cent of the variance (Figure 4F, upper). As in mice, the most prominent transcriptomic changes happened between stages II and III, even though transitions between stages I-II and III-IV involved changes in the expression of hundreds of genes (Figure 4F, lower). Three main patterns of changes of gene expression were observed along human eosinophilopoiesis (Figure 4G, Table S7). Like in mice, progenitors in stage I displayed the highest expression of genes involved in ribosome biogenesis and protein synthesis, while cells in stages I and II displayed the highest expression of eosinophil granule- and cell cycle-associated genes (Figure 4G-I). Transition to stage III metamyelocytes was accompanied by a downregulation of these genes, while the expression of myeloid function-associated genes steadily increased from stages I to IV. These analyses highlight a conserved developmental program of eosinophilopoiesis in both humans and mice, providing a basis for translational studies as well as opportunities to study shared mechanisms of eosinophil development.

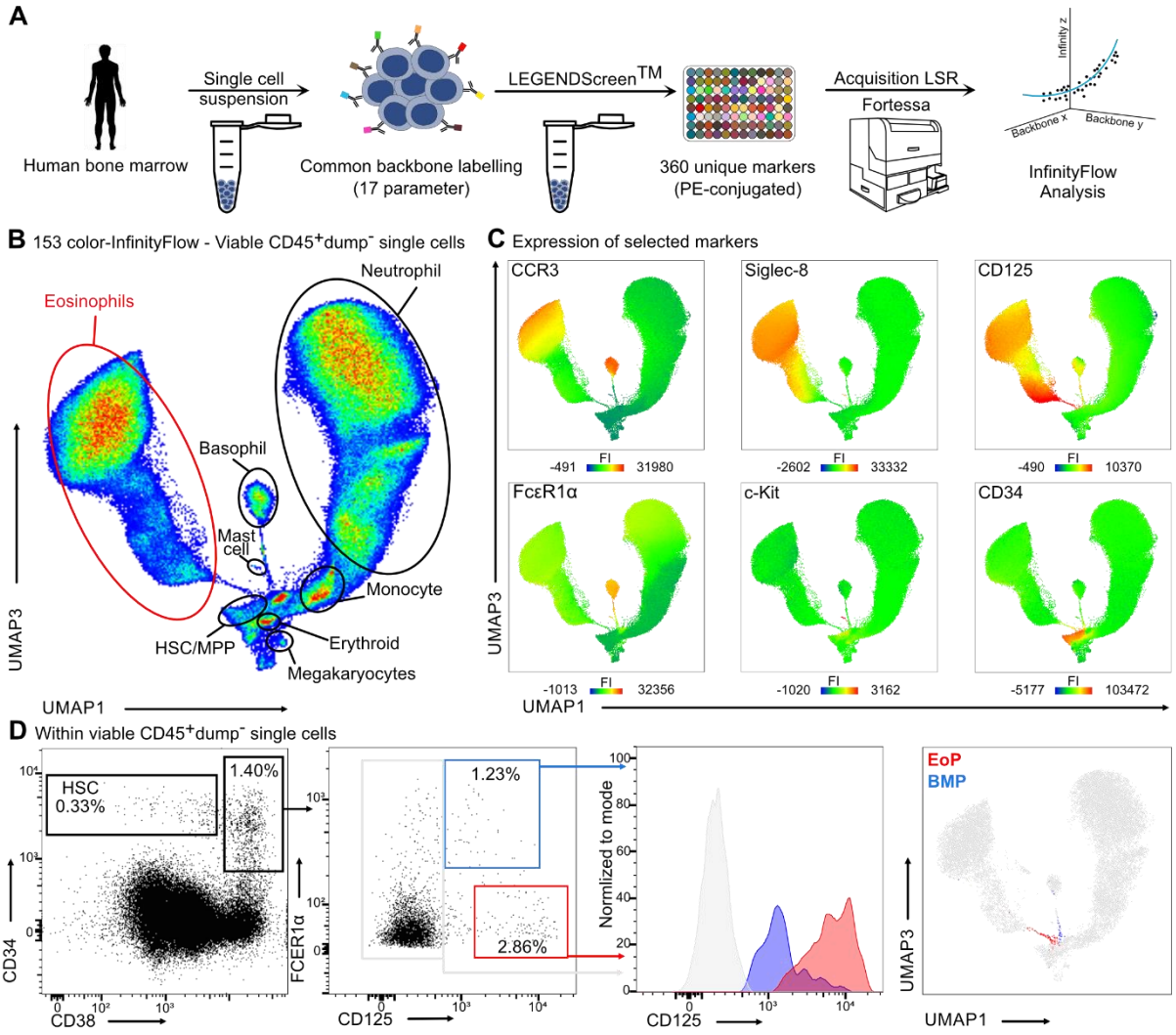


Figure 3. Resolution of human eosinophil ontogeny by high dimension flow cytometric screening.

A. Experimental outline of a flow cytometric screening focused on the human eosinophil lineage (more detail in Figure S4A-B). **B.** UMAP of an InfinityFlow-integrated 153-marker staining of viable lineage-negative CD45$^+$ human BM cells with major lineages annotated based on cell surface markers in Figure S4C and Table S6. **C.** Overlayed staining intensity of select markers in B. **D.** Gating strategy of early eosinophil progenitors (EoP, red) and basophil/mast cell progenitors (BMP, blue), relative staining intensity with anti-CD125 and projection on UMAP in B. (HSC: hematopoietic stem cells, MPP: multipotent progenitors)

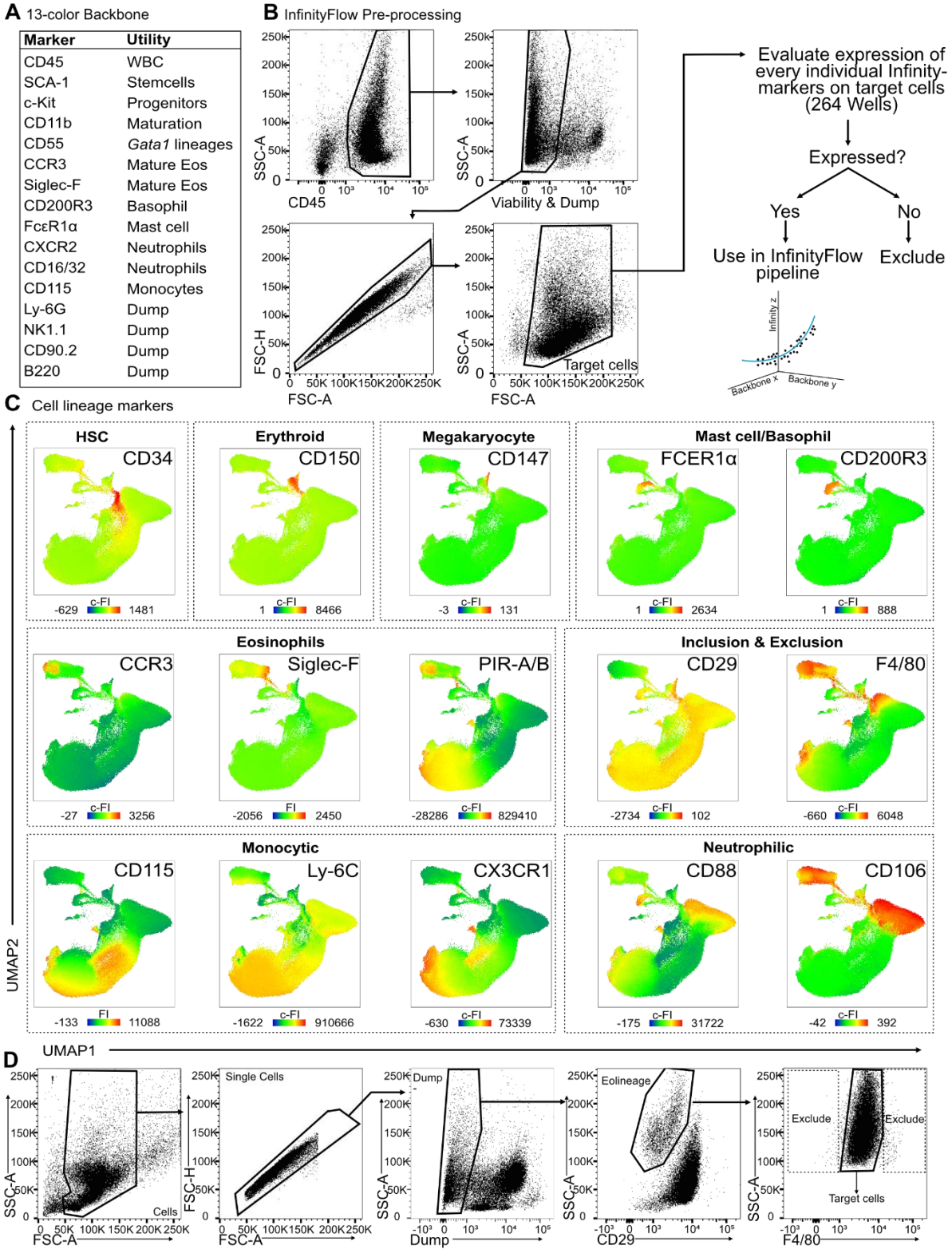

A 13-color Backbone
Marker Utility
CD45 WBC
SCA-1 Stemcells
c-Kit Progenitors
CD11b Maturation
CD55 Gata1 lineages
CCR3 Mature Eos
Siglec-F Mature Eos
CD200R3 Basophil
FcεR1α Mast cell
CXCR2 Neutrophils
CD16/32 Neutrophils
CD115 Monocytes
Ly-6G Dump
NK1.1 Dump
CD90.2 Dump
B220 Dump
B InfinityFlow Pre-processing
SSC-A
CD45
Viability & Dump
FSC-H
FSC-A
Target cells
Evaluate expression of every individual Infinity-markers on target cells (264 Wells)
Expressed?
Yes
No
Use in InfinityFlow pipeline
Exclude
Infinity z
Backbone x
Backbone y
C Cell lineage markers
HSC
CD34
-629 c-FI 1481
Erythroid
CD150
1 c-FI 8466
Megakaryocyte
CD147
-3 c-FI 131
Mast cell/Basophil
FCER1α
1 c-FI 2634
CD200R3
1 c-FI 888
Eosinophils
CCR3
-27 c-FI 3256
Siglec-F
-2056 FI 2450
PIR-A/B
-28286 c-FI 829410
Inclusion & Exclusion
CD29
-2734 c-FI 102
F4/80
-660 c-FI 6048
Monocytic
CD115
-133 FI 11088
Ly-6C
-1622 c-FI 910666
CX3CR1
-630 c-FI 73339
Neutrophilic
CD88
-175 c-FI 31722
CD106
-42 c-FI 392
UMAP2
UMAP1
D
Cells
Single Cells
Dump
Eolineage
Exclude
Exclude
Target cells
CD29
F4/80

Figure S3 (related to Figures 1 and 2). High dimension flow cytometric screening resolves murine eosinophilopoiesis.

A. Backbone panel for flow cytometric screening of the murine eosinophil lineage. **B.** Gating strategy of murine bone marrow cells of interest and post-processing strategy for the inclusion of detected markers in the InfinityFlow computation. **C.** Overlayed staining intensity of lineage markers on the UMAP in Figure 1H. **D.** Gating strategy upstream of Figure 2D. (c-FI: backgroundcorrected fluorescence intensity, Eos: eosinophil, FI: fluorescence intensity, HSC: hematopoietic stem cell).

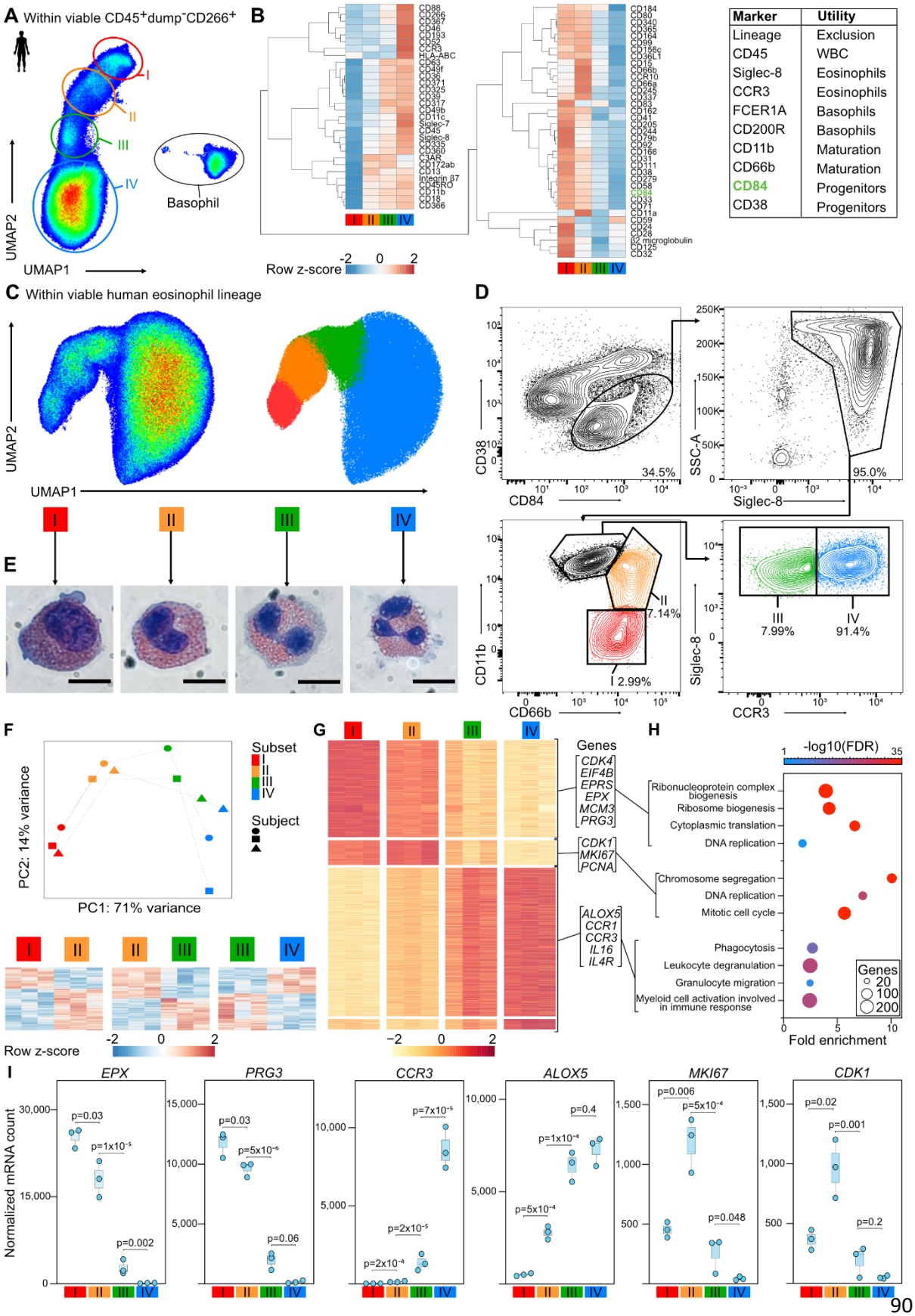

A Within viable CD45+dump-CD266+
I
II
III
IV
Basophil
UMAP2
UMAP1
B
CD88
CD266
CD367
CD46
CD193
CD52
CCR3
HLA-ABC
CD63
CD49f
CD36
CD371
CD325
CD39
CD317
CD49b
CD11c
Siglec-7
CD45
Siglec-8
CD335
CD360
CD45
CD172ab
CD13
Integrin β7
CD45RO
CD11b
CD18
CD366
CD184
CD80
CD340
CD365
CD164
CD99
CD156c
CD36L1
CD15
CD66b
CCR10
CD66e
CD245
CD337
CD83
CD162
CD41
CD205
CD244
CD79b
CD62
CD166
CD31
CD111
CD38
CD279
CD58
CD84
CD33
CD71
CD11a
CD59
CD24
CD28
β2 microglobulin
CD125
CD32
I II III IV
-2 0 2
Row z-score
Marker | Utility
Lineage | Exclusion
CD45 | WBC
Siglec-8 | Eosinophils
CCR3 | Eosinophils
FCER1A | Basophils
CD200R | Basophils
CD11b | Maturation
CD66b | Maturation
CD84 | Progenitors
CD38 | Progenitors
C Within viable human eosinophil lineage
UMAP2
UMAP1
D
CD38
CD84
34.5%
SSC-A
Siglec-8
95.0%
CD11b
CD66b
II 7.14%
I 2.99%
III 7.99%
IV 91.4%
CCR3
E
I
II
III
IV
F
PC2: 14% variance
PC1: 71% variance
Subset
I
II
III
IV
Subject
I II II III III IV
-2 0 2
Row z-score
G
I II III IV
Genes
CDK4
EIF4B
EPRS
EPX
MCM3
PRG3
CDK1
MKI67
PCNA
ALOX5
CCR1
CCR3
IL16
IL4R
-2 0 2
H
-log10(FDR)
1 35
Ribonucleoprotein complex biogenesis
Ribosome biogenesis
Cytoplasmic translation
DNA replication
Chromosome segregation
DNA replication
Mitotic cell cycle
Phagocytosis
Leukocyte degranulation
Granulocyte migration
Myeloid cell activation involved in immune response
Genes
20
100
200
0 5 10
Fold enrichment
I
EPX
PRG3
CCR3
ALOX5
MKI67
CDK1
Normalized mRNA count
p=0.03
p=1x10-5
p=0.002
p=0.03
p=5x10-6
p=0.06
p=7x10-5
p=2x10-5
p=2x10-4
p=0.4
p=1x10-4
p=5x10-4
p=0.006
p=5x10-4
p=0.048
p=0.02
p=0.001
p=0.2

Figure 4. Resolution of human eosinophil maturation by high dimension flow cytometric screening

A. InfinityFlow-derived UMAP of eosinophil lineage cells in human BM displaying 4 main immunophenotypes used to partition their maturation into 4 stages (I to IV) with pseudodensity overlayed. **B.** Heatmap of the relative expression of highly expressed markers in maturation stage I-IV eosinophils (signal intensity of all markers staining eosinophil-lineage cells is in Table S7) and 10-color flow cytometric panel for the partition of the maturation continuum of human eosinophils into 4 stages. **C.** UMAP of human eosinophil lineage cells stained with marker panel in C as pseudodensity plot (left) or with maturation stages overlayed (right). **D.** Gating strategy for partitioning human eosinophil maturation into stages I to IV. **E.** Light imaging photographs of stage I-IV eosinophils (scale bar: 10 μm). **F-G.** Bulk RNA sequencing comparisons of stage I-IV BM eosinophils from 3 donors presented in a principal component analysis (F, upper, dashed lines connect samples from the same donor), as heatmaps of differentially expressed genes (DEG) between each stage (F, lower) and as major coregulated modules (G and Table S8). **H.** Gene ontology (GO) enrichment analysis on co-expressed gene modules in G (FDR: false discovery rate). **I.** Comparison of normalized gene expression of select genes in F (one-way ANOVA followed by TukeyHSD tests).

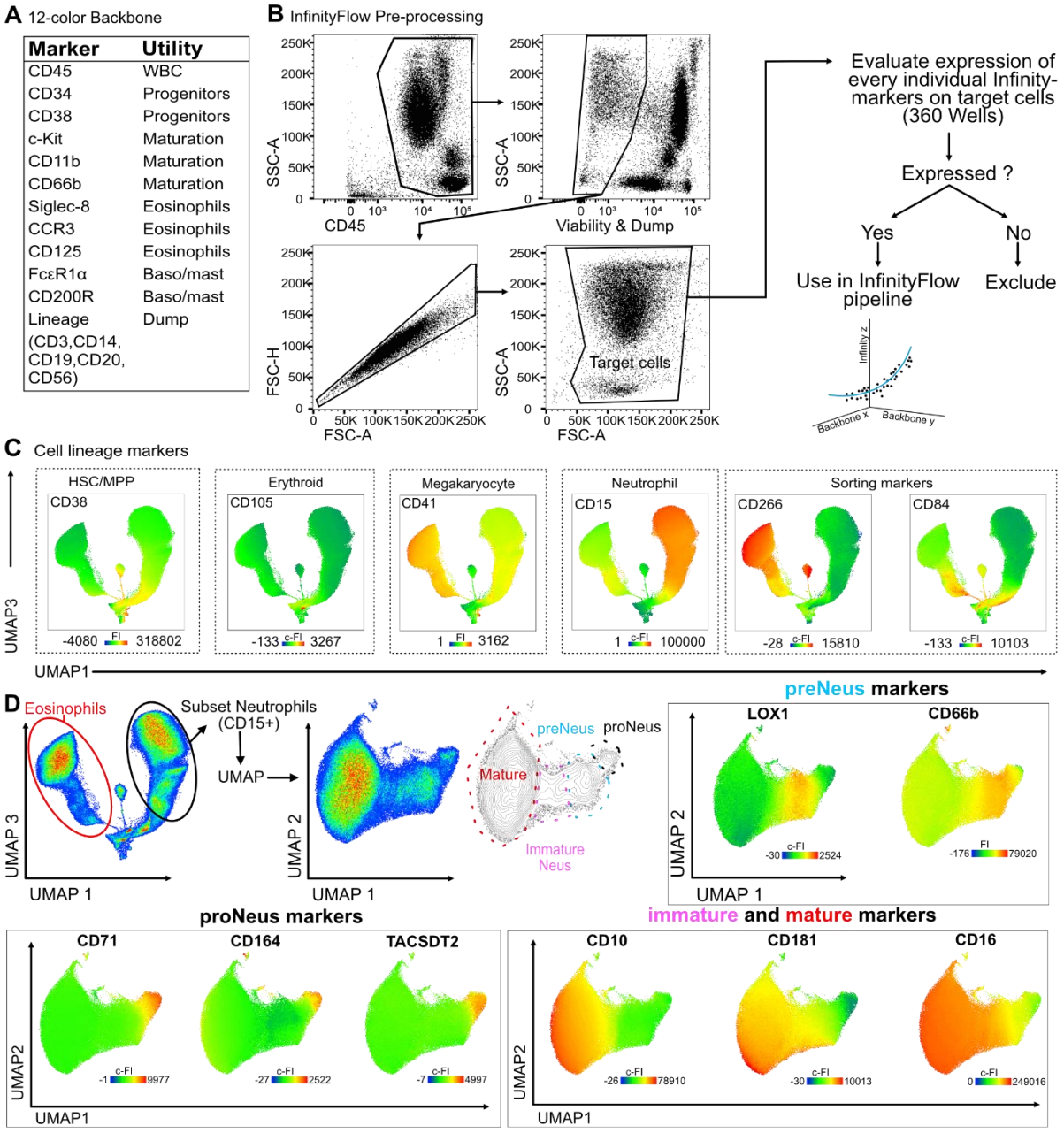

A 12-color Backbone
Marker Utility
CD45 WBC
CD34 Progenitors
CD38 Progenitors
c-Kit Maturation
CD11b Maturation
CD66b Maturation
Siglec-8 Eosinophils
CCR3 Eosinophils
CD125 Eosinophils
FcεR1α Baso/mast
CD200R Baso/mast
Lineage (CD3,CD14, CD19,CD20, CD56) Dump
B InfinityFlow Pre-processing
SSC-A
CD45
Viability & Dump
FSC-H
FSC-A
Target cells
Evaluate expression of every individual Infinity-markers on target cells (360 Wells)
Expressed ?
Yes
No
Use in InfinityFlow pipeline
Exclude
Infinity z
Backbone x
Backbone y
C Cell lineage markers
HSC/MPP
CD38
Erythroid
CD105
Megakaryocyte
CD41
Neutrophil
CD15
Sorting markers
CD266
CD84
UMAP3
UMAP1
D Eosinophils
Subset Neutrophils (CD15+)
UMAP
UMAP 1
UMAP 2
UMAP 3
preNeus
proNeus
Mature
Immature Neus
preNeus markers
LOX1
CD66b
proNeus markers
CD71
CD164
TACSDT2
immature and mature markers
CD10
CD181
CD16
UMAP2
UMAP1

Figure S4 (related to Figure 3). High dimension flow cytometric screening resolves human eosinophilopoiesis.

A. Backbone panel for flow cytometric screening of the human eosinophil lineage. **B.** Gating strategy of human BM cells of interest and post-processing strategy for the inclusion of detected markers in the InfnityFlow computation. **C.** Overlayed staining intensity of lineage markers on the UMAP in Figure 3B. **D.** UMAP of neutrophil lineage cells subselected from Figure 3B and highlighting 4 previously described stages of neutrophil maturation (Kwok et al., 2020) with overlayed expression of previously identified cell surface markers. (c-FI: background-corrected fluorescence intensity, FI: fluorescence intensity, HSC: hematopoietic stem cell, MPP: multipotent progenitor)

Eosinophil progenitor expansion is driven by increased transit amplification

Having resolved a conserved developmental trajectory of eosinophilopoiesis, we aimed to uncover the mechanisms underlying eosinophil progenitor expansion using models of eosinophilic disease in mice. We first used a very robust model of eosinophilia with features of eosinophilic granulomatosis polyangiitis 42 consisting of daily intraperitoneal injections of IL-33 and followed changes in the maturation stages of eosinophils through time by flow cytometry (Figure 5A-D). Importantly, the immunophenotyping panel established for steady-state eosinophilopoiesis continued to resolve 4 main immunophenotypic stages of eosinophil maturation in eosinophilic conditions (Figure S5A). Abundance of cells in maturation stages I, II and III increased over time, reaching a peak fold amplification at the end of the 7 days of IL-33 treatment (Figure 5B, C). Discontinuation of IL-33 stimulation led to a drop in the abundance of stage III progenitors, paralleled by a further increase of mature stage IV eosinophils in the BM and blood. Abundance of stage I-III progenitors returned close to baseline values within 5 days, while blood and BM mature eosinophils started to decrease.

A similar dynamic expansion of the eosinophil lineage was observed in response to subcutaneous infection with *Nippostrongylus brasiliensis* larvae. Eosinophil progenitors were expanded on day 8, the time around which the parasite is cleared in mice 43,44 (Figure 5E). Progenitor abundance returned toward a steady-state profile by day 15 post-infection, even though blood and BM mature eosinophils remained increased (Figure 5E). In a model of repeated intranasal administration of extracts of the allergenic mold *Alternaria alternata*, abundance of eosinophil lineage cells increased after 8 days of continued stimulation and returned to baseline values within 7 days of cessation of exposure, even though this model induced milder progenitor expansion and BM and blood eosinophilia than the other models (Figure 5F). In all 3 models, there was a noticeable correlation between the abundance of stage IV eosinophils in the BM and circulating blood eosinophils, which shared a similar $Ccr3^{+}$ Siglec-F^{+} phenotype (Figure 5B-F).

To identify the most prominent changes occurring in eosinophil lineage cells in response to eosinophilia-promoting stimuli, we compared the transcriptomes of stage I to IV eosinophils from IL-33-treated mice with steady-state counterparts. In a PCA, stage I-III progenitors of IL-33-treated mice were shifted leftward in PC1, which captured 67 per cent of the variance (Figure 6A). Genes with negative loadings in PC1 were enriched in cell-cycle related genes, whereas genes with positive loadings were enriched in genes associated with myeloid cell function (Figure 6B). Two major patterns of transcriptomic changes were noticeable in eosinophilia (Figure 6C-E and Table S8); first, 2 clusters enriched in genes related to leukocyte responses and maturation, such as *Ccr3*, displayed delayed upregulation along eosinophilopoiesis. Second, another large cluster of genes containing eosinophil granule-, cell cycle- and translation-associated genes retained elevated expression in stage III progenitors of IL-33-treated mice. In addition, bulk SCENIC analysis identified 2 major clusters of regulons differing between steady-state and eosinophilia that were congruent with the above mRNA expression patterns (Figure 6F); one cluster comprising transcription factors associated with immune responses such as *Ap-1* and *Nfkb*, whose upregulation was delayed in eosinophilia, and another cluster containing *E2fs* and *Myc* that remained upregulated in stage III. SCENIC also identified a third cluster comprising *Gata2* that was upregulated at all stages of maturation and a fourth cluster featuring *Irf8* that was downregulated throughout eosinophilopoiesis in IL-33-treated mice. Gene expression of the aforementioned transcription factors followed the same pattern as their regulons (Figure S5B).

The above transcriptomic observations suggested eosinophil progenitors from IL-33-treated mice acquired a fully mature phenotype slower, while retaining cell cycling activity for longer. *In vivo* 5-ethynyl-2'-deoxyuridine (EdU) nucleotide incorporation assays using a short 1-hour pulse confirmed this assumption. Not only was the percentage of EdU^+ stage I-II progenitors increased in the BM of IL-33-treated mice (Figure 6G, Figure S5C), the percentage of EdU^+ stage III eosinophils also increased up to ~20 per cent, compared to ~2 per cent in steady-state mice. Eosinophil progenitors developing during IL-33-promoted eosinophilia hence displayed increased cell cycling activity and retained the ability to divide for longer. We obtained similar results in models of *Nippostrongylus brasiliensis* infection as well as in the milder model of exposure to

Alternaria alternata (Figure 6H-I). Altogether, the above findings support the notion that eosinophil progenitor expansion in eosinophilia was sustained by a dynamic increase in transit amplification relying on the slower acquisition of a fully mature phenotype and increased and prolonged proliferation capacity.

Irf8 is not intrinsically essential to eosinophil maturation and expansion

To illustrate the tractability of our data in clarifying the molecular determinants of eosinophil development and expansion, we investigated the yet uncertain role of Irf8 in eosinophilopoiesis (Figure S5D-I). The pattern of mRNA expression of *Irf8* and its predicted activity in eosinophilia were inconsistent with an essential intrinsic role in eosinophil lineage development or expansion. Still, in the steady-state, the BM of *IR8*-deficient mice were eosinopenic (Figure S5F), as described previously 38. Nevertheless, all stages of maturation of eosinophils were present in *Irf8*-deficient mice and their relative abundance was comparable to that of wild-type counterparts. Furthermore, in response to IL-33, stage I-III eosinophil progenitors of *Irf8*-deficient mice expanded and incorporated EdU with a magnitude comparable to wild-type controls (Figure S5D-G, compare with Figure 6G). BM eosinopenia in *Irf8*-deficient mice was on the other hand accompanied by a general depression of Gata1 lineage cells up to the HSPC pool, reflecting the profound perturbations of hematopoiesis in these mice (Figure S5G-I). Hence, eosinopenia in *Irf8*-deficient mice was not primarily caused by an intrinsic defect of the eosinophil lineage, but mainly by perturbations of HSPCs upstream of eosinophil lineage commitment.

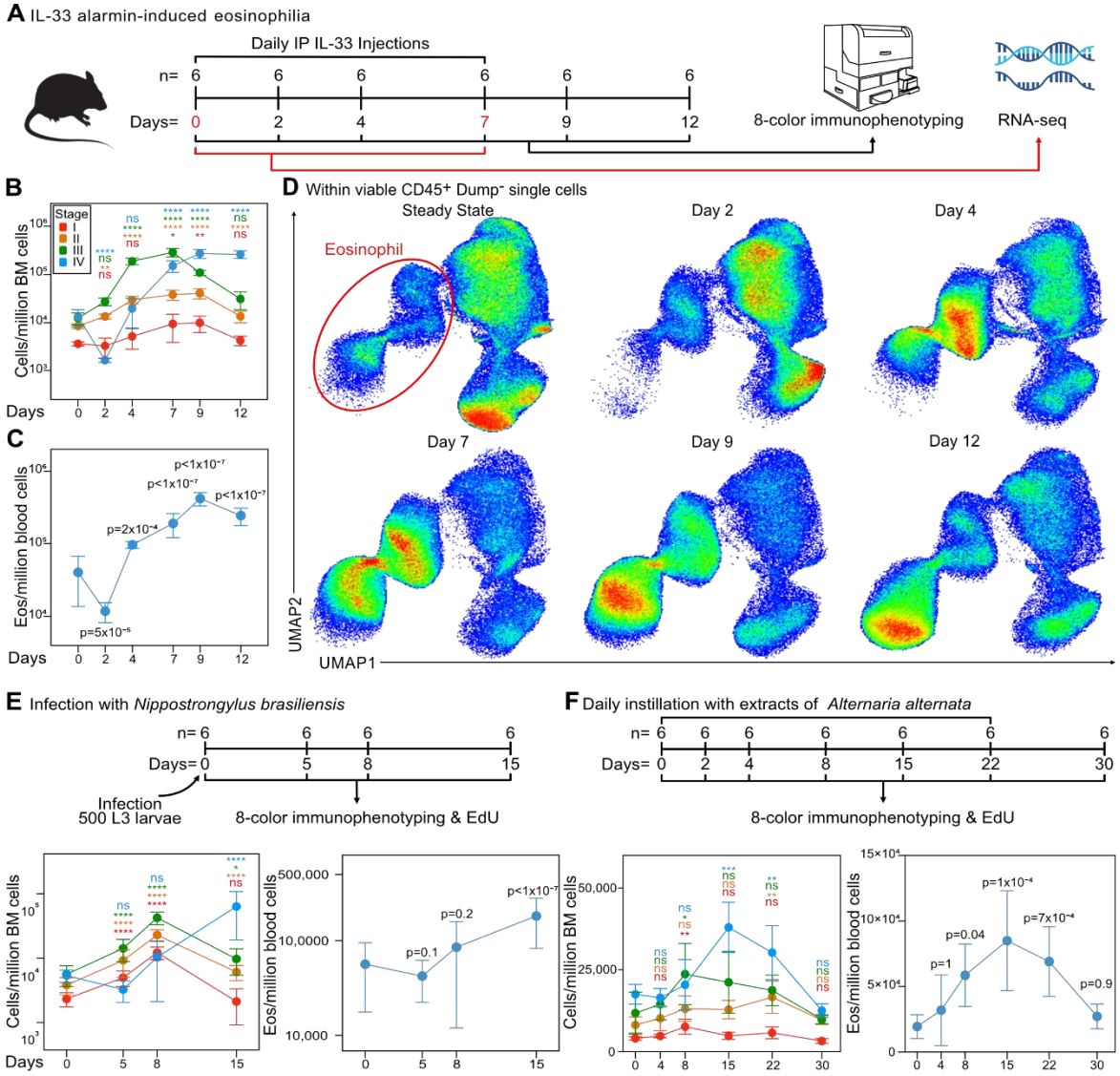

A IL-33 alarmin-induced eosinophilia
Daily IP IL-33 Injections
n= 6 6 6 6 6 6
Days= 0 2 4 7 9 12
8-color immunophenotyping
RNA-seq
B
Stage I II III IV
Cells/million BM cells
Days
C
Eos/million blood cells
p<1x10⁻⁷
p=2x10⁻⁴
p=5x10⁻⁵
D Within viable CD45⁺ Dump⁻ single cells
Steady State
Eosinophil
Day 2
Day 4
Day 7
Day 9
Day 12
UMAP1
UMAP2
E Infection with Nippostrongylus brasiliensis
n= 6 6 6 6
Days= 0 5 8 15
Infection
500 L3 larvae
8-color immunophenotyping & EdU
p=0.1
p=0.2
p<1x10⁻⁷
F Daily instillation with extracts of Alternaria alternata
n= 6 6 6 6 6 6 6
Days= 0 2 4 8 15 22 30
8-color immunophenotyping & EdU
p=1
p=0.04
p=1x10⁻⁴
p=7x10⁻⁴
p=0.9

Figure 5. Eosinophil lineage amplification in models of eosinophilic conditions.

A. Experimental outline of the induction of eosinophilia in mice by repeated treatment with recombinant IL-33. **B.** Abundance of stage I-IV eosinophils in the bone marrow of mice in A. **C.** Abundance of eosinophils in the blood of mice in A. **D.** UMAP of bone marrow cells in A with pseudodensity overlayed. **E.** Experimental outline (up) of the analysis of the abundance of stages I-IV BM eosinophils (lower left) and of blood eosinophils (lower right) in mice infected with Nippostrongylus brasiliensis. **F.** Experimental outline (up) of the analysis of the abundance of stages I-IV BM eosinophils (lower left) and of blood eosinophils (lower right) in mice intranasally-instilled with extracts of the mold Alternaria alternata. (Data pooled from 2 independent experiments with n=3/group presented as mean ± SD and analyzed by one-way ANOVA on each eosinophil subpopulation with TukeyHSD tests. Ns: not significant, *p

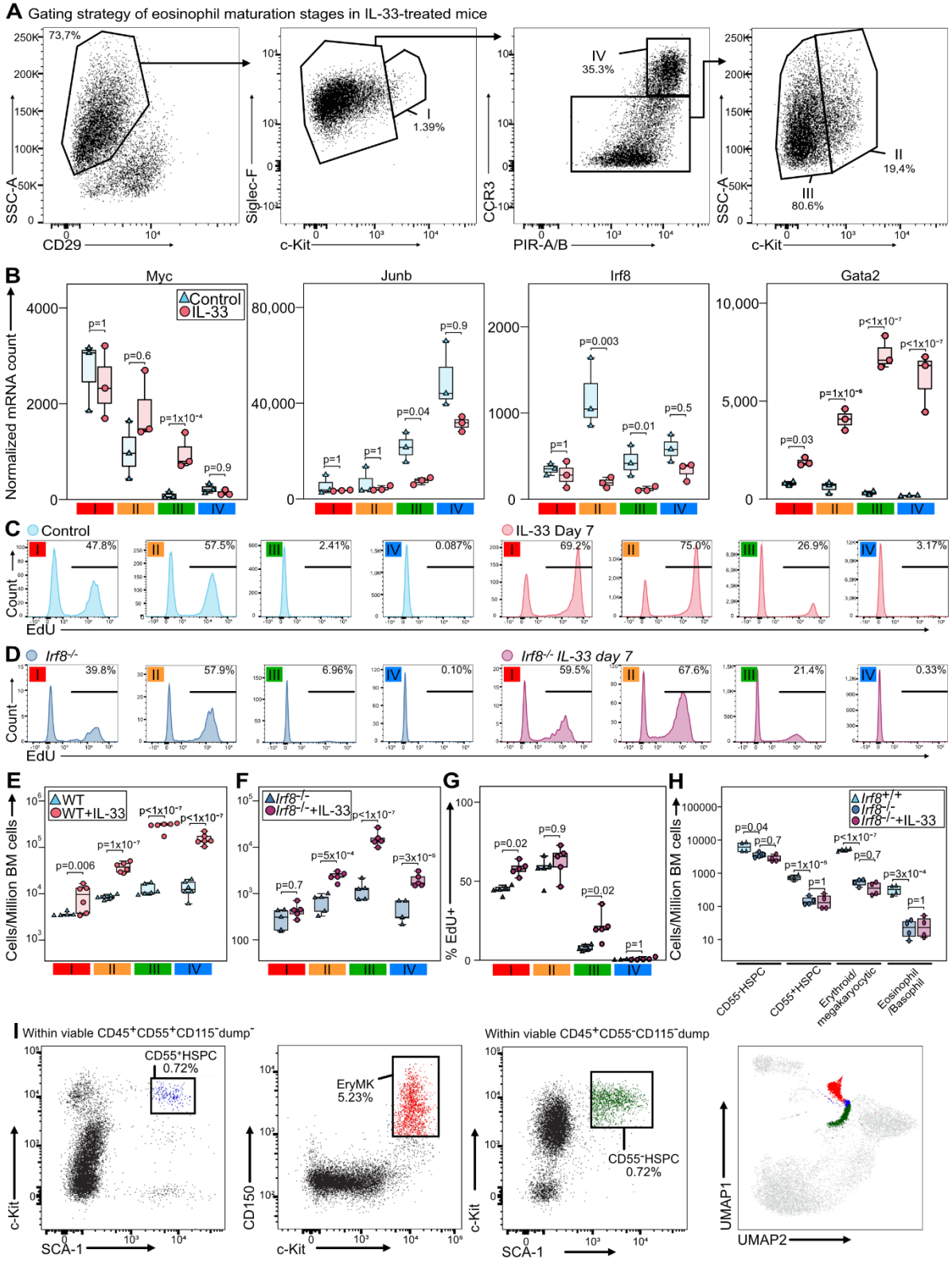

A Gating strategy of eosinophil maturation stages in IL-33-treated mice
73,7%
SSC-A
CD29
Siglec-F
c-Kit
I
1.39%
CCR3
PIR-A/B
IV
35.3%
II
19,4%
III
80.6%
B
Myc
Junb
Irf8
Gata2
Control
IL-33
Normalized mRNA count
C
Control
IL-33 Day 7
Count
EdU
47.8%
57.5%
2.41%
0.087%
69.2%
75.0%
26.9%
3.17%
D
Irf8-/-
Irf8-/- IL-33 day 7
39.8%
57.9%
6.96%
0.10%
59.5%
67.6%
21.4%
0.33%
E
WT
WT+IL-33
Cells/Million BM cells
F
Irf8-/-
Irf8-/-+IL-33
G
% EdU+
H
Irf8+/+
Irf8-/-
Irf8-/-+IL-33
CD55+HSPC
CD55+HSPC
Erythroid/ megakaryocytic
Eosinophil /Basophil
I Within viable CD45+CD55+CD115-dump-
CD55+HSPC
0.72%
c-Kit
SCA-1
EryMK
5.23%
CD150
Within viable CD45+CD55-CD115-dump-
CD55-HSPC
0.72%
UMAP1
UMAP2

Figure S5 (related to Figures 5 and 6). Cell cycling activity of stage I-IV eosinophils in eosinophilic conditions.

A. Gating strategy of the 4 maturation stages of eosinophils in the BM of mice treated with recombinant IL-33 i.p. for 7 days. Compare with Figure 2D. **B.** Comparison of normalized gene expression of select transcription factors in Figure 6F (n=3/group). **C-D.** Representative flow cytometric histograms of the incorporation of EdU after a 1h pulse in stage I-IV progenitors of control mice, mice treated with recombinant IL-33 for 7 days (C), or $Irf8^{-/-}$ mice treated or not with IL-33 for 7 days (D). **E-F.** Comparison of the abundance of stage I-IV eosinophils in the bone marrow of $Irf8^{-/-}$ mice (E) or wild-type (WT) mice (E, data from Figure 5B) treated or not with IL-33 for 7 days (n=5-6/group, pooled from 2 independent experiments). **G.** Comparison of 5-ethynyl-2'- deoxyuridine (EdU) incorporation after a 1h pulse in mice in (F). **H.** Comparison of the abundance of $CD55^{-}$ and $CD55^{+}$ HSPCs ($Dump^{-}$ $Sca1^{+}$ $c\text{-}Kit^{+}$), erythroid/megakaryocytic progenitors ($Dump^{-}$ $Sca1^{-}$ $c\text{-}Kit^{+}$ $CD150^{+}$) and eosinophil/basophil progenitors in mice in (G) (one-way ANOVA followed by TukeyHSD post-hoc tests for each subset). **I.** Gating strategy of cell populations in (H). (B, E-G: 2-way ANOVA followed by TukeyHSD post-hoc tests)

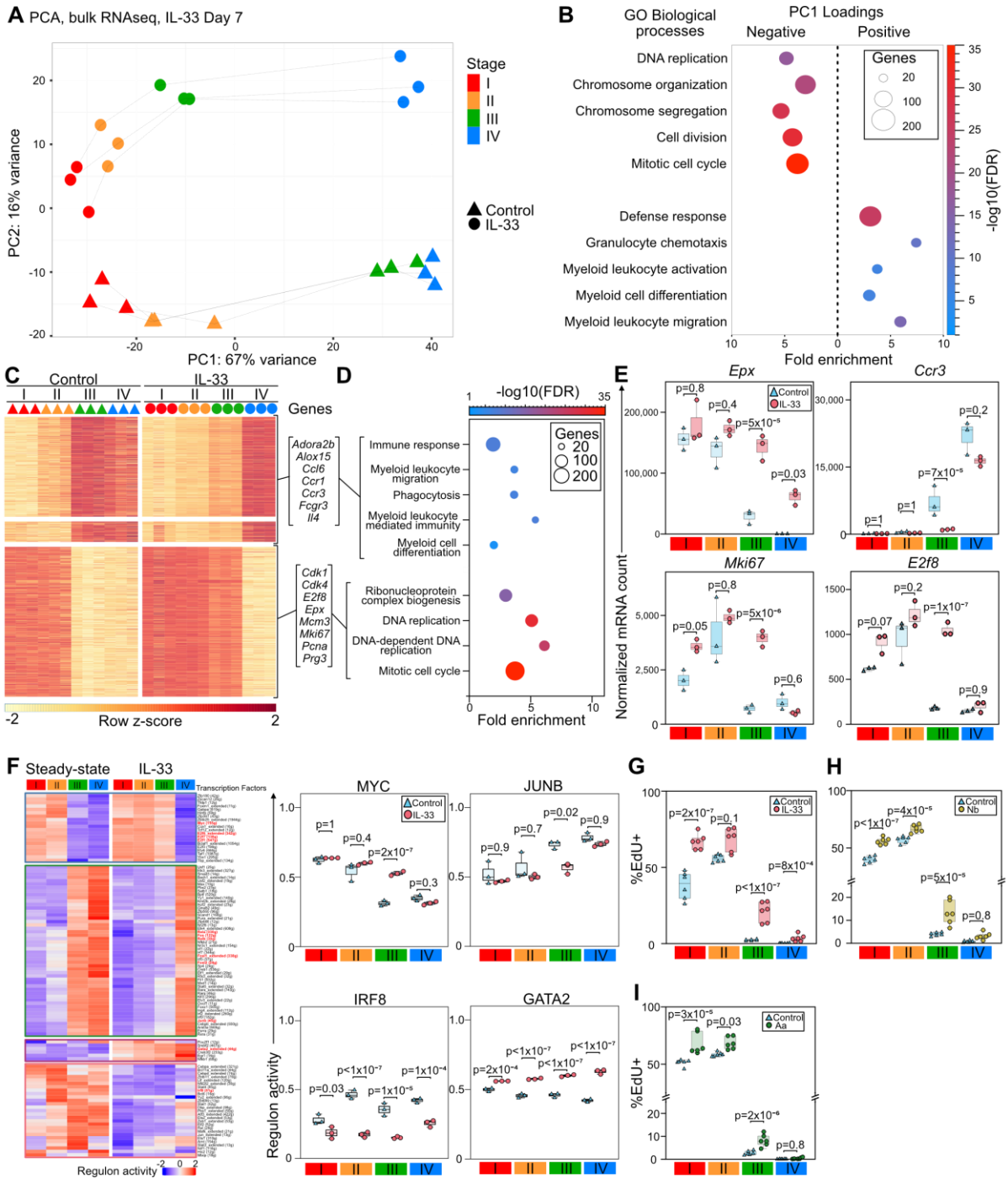

A PCA, bulk RNAseq, IL-33 Day 7
PC2: 16% variance
PC1: 67% variance
Stage
I
II
III
IV
Control
IL-33
B
GO Biological processes
PC1 Loadings
Negative
Positive
DNA replication
Chromosome organization
Chromosome segregation
Cell division
Mitotic cell cycle
Defense response
Granulocyte chemotaxis
Myeloid leukocyte activation
Myeloid cell differentiation
Myeloid leukocyte migration
Genes
20
100
200
-log10(FDR)
Fold enrichment
C
Control
IL-33
Genes
Adora2b
Alox15
Ccl6
Ccr1
Ccr3
Fcgr3
Il4
Cdk1
Cdk4
E2f8
Epx
Mcm3
Mki67
Pcna
Prg3
Row z-score
D
-log10(FDR)
Immune response
Myeloid leukocyte migration
Phagocytosis
Myeloid leukocyte mediated immunity
Myeloid cell differentiation
Ribonucleoprotein complex biogenesis
DNA replication
DNA-dependent DNA replication
Mitotic cell cycle
Fold enrichment
E
Epx
Ccr3
Mki67
E2f8
Control
IL-33
Normalized mRNA count
F
Steady-state
IL-33
Transcription Factors
Regulon activity
MYC
JUNB
IRF8
GATA2
Regulon activity
G
%EdU+
Control
IL-33
H
%EdU+
Control
Nb
I
%EdU+
Control
Aa

Figure 6. Increased transit amplification sustains eosinophil lineage expansion.

A. PCA of the transcriptomes of stage I-IV murine eosinophils from mice in the steady-state or made eosinophilic by the administration of IL-33 for 7 days as in Figure 5A (n=3/group, dashed lines connect stages in each donor). **B.** GO enrichment analysis on the genes with positive or negative loadings in PC1 in A. **C.** Bulk RNA sequencing comparison of stage I-IV eosinophils in A with major co-regulated modules (see Table S9) and select genes of interest. **D.** GO enrichment analysis on co-regulated gene modules in C. **E.** Comparison of normalized gene expression of select genes in C. **F.** Row-scaled heatmap of bulk SCENIC analysis of samples in A (left) and comparison of select regulon activity (right) **G-I.** Comparison of 5-ethynyl-2'- deoxyuridine (EdU) incorporation after a one-hour pulse in models of eosinophilia elicited by recombinant IL-33 (G), *Nippostrongylus brasiliensis* infection (H) or intranasal instillation of *Alternaria alternata* (I) as in Figure 5. (Data pooled from 2 independent experiments with n=3/group). (E-I: 2-way ANOVA followed by TukeyHSD tests. Aa: *Alternaria alternata*, FDR: false discovery rate, Nb: *Nippostrongylus brasiliensis*).

Depletion of IL-5 impairs expansion of eosinophil progenitors in steady-state and eosinophilia

Several mechanisms have been proposed to explain the reduction in blood eosinophilia following neutralization of IL-5, ranging from reduced commitment and expansion to maturational arrest of eosinophil progenitors 45,46. To help better characterize the activities of IL-5 in vivo, we generated Il5ra reporter (IL5RAporter) mice allowing straightforward identification of cells expressing Il5ra. IL5RAporter mice harbor an inactivating knock-in eGFP-T2A-Cre transgene in frame with the start codon of the native Il5ra locus (Figure 7A). The IL5RAporter allele labelled stages I to IV of the eosinophil lineage as well as a small subset of B cells (Figure 7B, Figure S6A), in line with previous studies that reported on *Il5ra* gene expression in eosinophils and in a subset of B cells 47,48. IL5RAporter mice may also be used to induce Cre recombination in the eosinophil lineage of floxed mice (Figure 7B). Of note, neutrophils have been reported to display high surface staining with T21 and REA343 anti-Il5ra antibodies in mice 5,49 (Figure S6B). Yet, neutrophils did not express the IL5RAporter transgene (Figure S6C) and neutrophils of Il5ra-deficient IL5RAporter$^{KI/KI}$ mice stained similarly to neutrophils from wild-type control mice with these anti-CD125 clones (Figure S6B), indicating they cross-react with an unidentified neutrophil antigen. A third clone, DIH37, did not display this unspecific staining of neutrophils, but generated only mildly higher median fluorescence intensity in control compared to Il5ra-deficient eosinophils (Figure S6B). IL5RAporter mice were therefore superior to currently available alternatives in identifying cell types expressing Il5ra in mice.

We used IL5RAporter$^{KI/+}$ mice to identify the earliest Il5ra-expressing hematopoietic progenitors in the murine BM by conventional flow cytometry. Consistent with our scRNAseq analysis, the IL5RAporter allele became expressed after the divergence point of the basophil/mast cell and eosinophil lineages, only in cells committed to the eosinophil fate (Figure 7C-D, and Figure S6D). These results establish that IL-5 can only be expected to directly influence eosinophilopoiesis after lineage commitment in mice.

Finally, we assessed the impact of the depletion of IL-5 on eosinophilopoiesis in the steady-state and in eosinophilia. We first inspected the consequences of genetic deletion of IL-5 using $Il5^{-/-}$ mice. All stages of eosinophil maturation were still present in $Il5^{-/-}$ mice (Figure 7E). The abundance of stage I progenitors in $Il5^{-/-}$ mice was comparable to that of reference control values (Figure 7F), but lineage expansion along stages II and III was reduced, resulting in reduced mature BM eosinophil abundance. In addition, stimulation by repeated administration of IL-33 for 7 days failed to expand stage I-III progenitors in $Il5^{-/-}$ mice (Figure 7F). We also assessed the consequences of depleting IL-5 on established eosinophilia using neutralizing monoclonal antibodies. Treatment with anti-IL-5 antibodies of wild-type mice rendered eosinophilic by injections of IL-33 accelerated the contraction of stage I-III eosinophil progenitors toward steady-state reference values (Figure 7G). Anti-IL-5 treatment led to earlier upregulation of genes associated with mature myelocyte function alongside earlier downregulation of genes associated with cell cycling and translation (Figure 7H-I), consistent with a decrease in transit-amplification. Altogether, these data indicate that IL-5 was an essential promoter of the post-commitment expansion of eosinophil progenitors through transit amplification in both the steady-state and in response to eosinophilia-promoting signals, but was not required for their maturation.

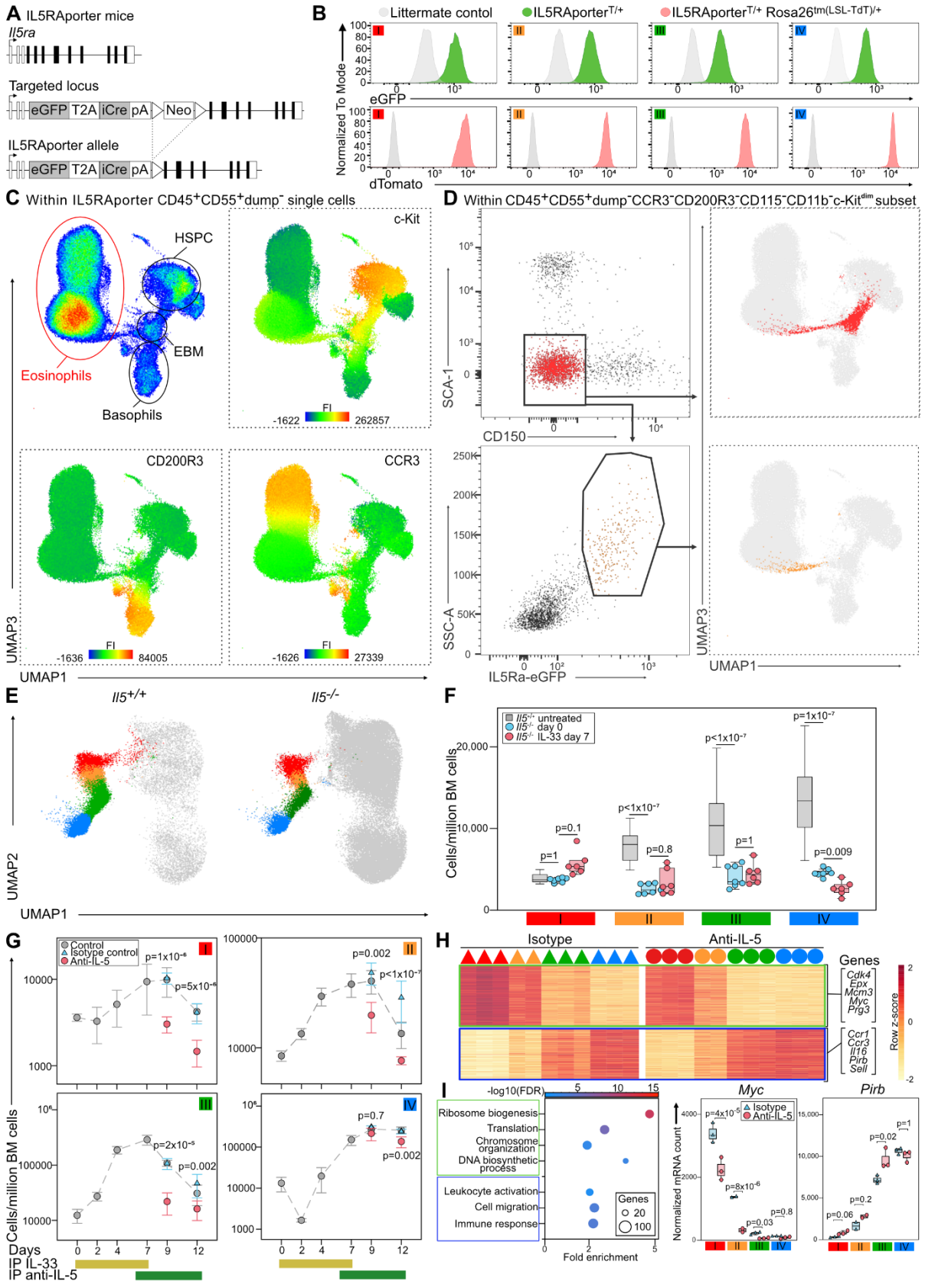

A IL5RAporter mice
Il5ra
Targeted locus
eGFP T2A iCre pA
Neo
IL5RAporter allele
B
Littermate contol
IL5RAporterT/+
IL5RAporterT/+ Rosa26tm(LSL-TdT)/+
Normalized To Mode
eGFP
dTomato
C Within IL5RAporter CD45+CD55+dump- single cells
HSPC
EBM
Eosinophils
Basophils
c-Kit
CD200R3
CCR3
FI
UMAP3
UMAP1
D Within CD45+CD55+dump-CCR3-CD200R3-CD115-CD11b-c-Kitdim subset
SCA-1
CD150
SSC-A
IL5Ra-eGFP
E
Il5+/+
Il5-/-
UMAP2
F
Il5-/- untreated
Il5-/- day 0
Il5-/- IL-33 day 7
Cells/million BM cells
G
Control
Isotype control
Anti-IL-5
Cells/million BM cells
Days
IP IL-33
IP anti-IL-5
H
Isotype
Anti-IL-5
Genes
Cdk4
Epx
Mcm3
Myc
Prg3
Ccr1
Ccr3
Il16
Pirb
Sell
Row z-score
I
-log10(FDR)
Ribosome biogenesis
Translation
Chromosome organization
DNA biosynthetic process
Leukocyte activation
Cell migration
Immune response
Fold enrichment
Myc
Pirb
Normalized mRNA count

Figure 7. IL-5 regulates transit amplification of committed eosinophil progenitors.

A. Schematic of the targeting of the Il5ra locus for the generation of IL5RAporter mice. **B.** Representative flow cytometric analysis of the expression of the eGFP reporter (upper) and TdTomato expression (lower) in stage I-IV eosinophils from the indicated transgenic mice with non-transgenic littermate as control. **C.** UMAP of HSPCs (marked by c-kit^{hi} expression) and eosinophil (marked by Ccr3) and basophil (marked by CD200R3) lineages in murine dump- CD45^{+} CD55^{+} BM cells (EBM: eosinophil/basophil/mast cell progenitors). **D.** Projection on the UMAP in C of gated eosinophil and basophil progenitors (red, gated as in Figure S6D) and of eosinophil-committed progenitors identified by expression of the eGFP IL5RAporter transgene within EBM (orange). **E-F.** UMAP and abundance of stage I-IV eosinophils in the BM of steady-state Il5$^{-/-}$ mice and Il5$^{-/-}$ mice stimulated for 7 days with IL-33 as in Figure 5. Reference wild-type values were from steady-state mice in Figure 5 (data pooled from 2 independent experiments with n=3/group and analysed by separate 2-way ANOVA followed by TukeyHSD tests comparing stage I-IV cell abundance in control versus IL-33-stimulated Il5$^{-/-}$ mice on the one hand, and in control Il5$^{-/-}$ mice versus reference wild-type values on the other hand). **G.** Abundance of stage I-IV eosinophils in the BM of mice treated for 7 days with IL-33 as in Figure 5 and receiving either an isotype control or anti-IL-5 neutralizing antibodies starting on the 6th day. For comparison, data is overlayed on data from Figure 5B (grey) (data pooled from 2 independent experiments with n=3/group presented as mean ± SD and analyzed by one-way ANOVA followed TukeyHSD tests). **H-I.** Heatmap of co-regulated modules (H) and GO enrichment analysis and comparison of the expression of the indicated genes in G (I, 2-way ANOVA followed by TukeyHSD tests).

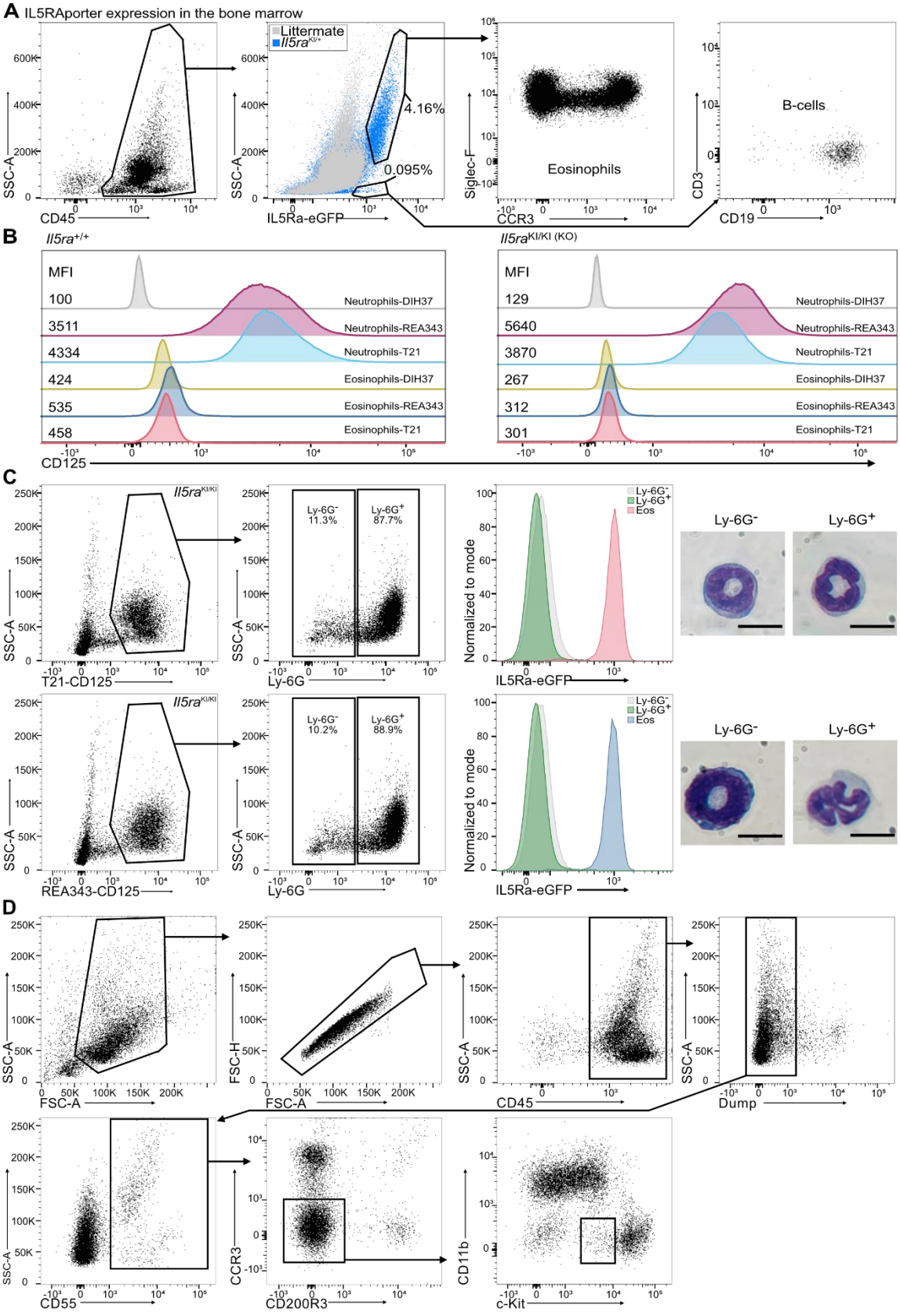

A IL5RAporter expression in the bone marrow
Littermate
Il5ra+/+
4.16%
0.095%
SSC-A
CD45
IL5Ra-eGFP
Siglec-F
CCR3
Eosinophils
CD3
CD19
B-cells
B
Il5ra+/+
Il5raKI/KI (KO)
MFI
100
Neutrophils-DIH37
3511
Neutrophils-REA343
4334
Neutrophils-T21
424
Eosinophils-DIH37
535
Eosinophils-REA343
458
Eosinophils-T21
129
5640
3870
267
312
301
CD125
C
Il5raKI/KI
T21-CD125
Ly-6G
Ly-6G- 11.3%
Ly-6G+ 87.7%
Normalized to mode
Ly-6G-
Ly-6G+
Eos
Ly-6G- 10.2%
Ly-6G+ 88.9%
REA343-CD125
D
FSC-A
FSC-H
Dump
CD55
CCR3
CD200R3
CD11b
c-Kit

Figure S6 (related to Figure 7).Identification of bona fide Il5ra-expressing cells using IL5RAporter mice.

A. Representative gating strategy in flow cytometry of $eGFP^{+}$ eosinophils and of a subset of B cells in the BM of $IL5RAporter^{KI/+}$ mice. Overlay with a littermate wild-type sample is provided for comparison. **B.** Representative flow cytometric histograms of staining with anti-CD125 antibody clones DIH37, REA343 and T21 of neutrophils and eosinophils of wild-type (left) and Il5ra deficient $IL5RAporter^{KI/KI}$ mice (right). **C.** Representative gating strategy in flow cytometry of cells staining brightly with anti-CD125 antibody clones T21 and REA343, comparison with eGFP reporter fluorescence intensity of eosinophils in $IL5RAporter^{KI/KI}$ mice, and light microscopic pictures of $Ly6G^{-}$ and $Ly6G^{+}$ cells brightly staining with T21 or RE343 showing their neutrophilic identity (scale bar: 10µm). **D.** Gating strategy of eosinophil/basophil progenitors upstream of Figure 7D, as in Figure 1K. (Eos: eosinophil, KI: knock-in, KO: knockout).

4.2.5 Discussion

Eosinophils remain one of the least understood immune cells when it comes to their biological functions and development. Notably, the rapidly expanding clinical implementation of precision therapies targeting eosinophils directly or indirectly in EADs calls for a refined understanding of the ontogeny, expansion mechanisms and responses to treatment of eosinophils in preclinical models and human beings. The primary aim of this work was to provide easily transposable flow cytometric methods and immunophenotypic and transcriptomic resources for the translational study of eosinophilopoiesis.

Eosinophils are evolutionarily versatile cells [50] and attention has been devoted to highlighting differences between murine and human eosinophils [51]. We show that the ontogeny of eosinophils may be more conserved than previously estimated. One notable difference between murine and human eosinophilopoiesis regards the expression of IL5RA. Human basophils express IL5RA [52], whereas their murine counterparts do not. We show that this difference is wired in the development of these lineages in each species. Noticeably yet, IL5RA expression was still lower in human basophil/mast cell progenitors than in their eosinophil-committed counterparts. This could contribute to the fact that basophils are comparatively less reduced than eosinophils in patients treated with the anti-IL5RA depleting antibody benralizumab [53].

Resolving eosinophilopoiesis is important for a correct understanding of the functioning of the eosinophil lineage, and we identified several pervasive and often cumulative prior limitations in this regard. First, the use of different phenotyping strategies for the identification of the earliest identifiable eosinophil progenitors on the one hand and their progeny on the other hand precluded an integrated view of eosinophil lineage development and dynamics. Second, popular anti-murine IL5RA/CD125 antibodies used for identifying eosinophil progenitors are shown here to generate unspecific staining. This calls for a reinterpretation of murine studies based on these reagents due to the risk of contamination of eosinophil lineage cells by neutrophil lineage cells. Finally, assumptions were often made based on models in which neutrophils and eosinophils share developmental proximity within the highly

heterogeneous "GMP" or "CMP" compartments. These hypotheses should also be reevaluated. For instance, it was previously proposed that *Irf8* is important in the GMP or CMP to upregulate *Gata1* and promote eosinophil maturation and fate divergence away from the neutrophil and monocyte lineages [18,54]. Rather, we show that *Irf8* deficiency is damaging to eosinophil development mainly because it negatively impacts on the HSPC progenitors of all Gata1-expressing lineages, which are on a trajectory distinct from that of monocytes and neutrophils. Our phenotyping strategies and transcriptomic data will hence be useful to reevaluate prior models of eosinophilopoiesis.

The resources provided herein can also be used to better understand the cellular dynamics of eosinophil lineage expansion in eosinophilia, or the response of the lineage to therapeutic interventions. We show that eosinophil progenitor expansion during eosinophilia involves enhanced transit amplification. Our transcriptomic and functional analyses of the regulation of eosinophil lineage expansion concur with a model in which IL-5 bioavailability determines the amplitude of eosinophil progenitor transit amplification as a major mechanism of regulation of eosinophil output from the BM in the steady-state or in eosinophilia. We did not observe cellular or transcriptional signs of maturational arrest in eosinophil progenitors following IL-5 depletion, which argues against the frequently purported requirement of IL-5 for eosinophil maturation. This is also in line with the observation that residual eosinophils in $Il5^{-/-}$ mice and in asthmatic patients treated with anti-IL-5 mepolizumab do not show overt signs of perturbed development [55]. Hence, antibodies that neutralize IL-5 essentially reduce BM and blood eosinophilia by inhibiting eosinophil lineage expansion, without compromising eosinophil maturation.

It is proposed based on the study of erythropoiesis that transit amplification of committed progenitors in hematopoiesis is a balancing act between pro-proliferative gene expression programs that antagonize terminal maturation, and anti-proliferative programs promoting terminal maturation [56]. Transit amplification in erythropoiesis notably increases in response to glucocorticoids by acting on this balance [56]. Eosinophils, which like erythrocytes belong to the Gata1 myeloid lineages, display a similar tunability of their transit amplification, of which IL-5 bioavailability is an essential rheostat. There is evidence that similar processes of post-commitment transit

amplification also control non-Gata1 lineage expansion, notably that of neutrophils in emergency granulopoiesis [57]. Another known mechanism for increasing neutrophil and monocyte output from the BM in inflammatory conditions is the promotion of the commitment of HSPCs toward non-Gata1 myeloid lineages [58–60]. It has been suggested that eosinophil, basophil and mast cell fates co-segregate in an early fate decision within the Gata1 arm of myelopoiesis [61], and that basophils and BM-derived mast cells share a common progenitor pool [33]. To our knowledge, to date, no physiological mechanism upstream of eosinophil lineage commitment has been uncovered *in vivo* that would regulate the commitment of HSPCs toward EBMs or the eosinophil lineage. We show herein that, at least in mice, the receptor to IL-5 is only expressed in committed eosinophil progenitors, which precludes a role of IL-5 in the balance between the commitment toward the eosinophil versus the basophil and mast cell lineages.

Many open questions remain about the biology of eosinophils, which could be rooted in their development. For instance, could eosinophil progenitors be imprinted and could this later affect the activity of their mature progeny [62]? Eosinophils have also been shown to be phenotypically and functionally diverse in different organs, but the potential contribution of developmental processes to subsequent said diversity remains unexplored. We anticipate that the resources provided herein will help in answering these questions.

Limitations of the study

Limitations of the current study include the absence of analyses in human BM in eosinophilia-promoting conditions. Is eosinophil lineage expansion a sustained process or does it involve dynamic, time-resolved pulses of progenitor expansion, as observed in our murine models, in different EADs? Our study also focused mostly on events affecting the eosinophil lineage in the BM. Dynamic assessment of the entire eosinophil compartment from the BM to the blood to peripheral tissues in the steady-state and eosinophilia-promoting conditions would complement this work and allow mathematical modelling of the flux of eosinophils. Finally, because human EBM express IL5RA unlike their murine counterparts, the effect of IL-5 on eosinophil versus basophil or mast cell

lineage commitment, as well as its role in the potential transit amplification of basophil and mast cell progenitors, remain to be assessed.

4.2.6 References

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4.3 Study 2: Anti-IL-5 mepolizumab minimally influences residual blood eosinophils in severe asthma

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4.3.1 Abstract

Neutralising antibodies against the cytokine interleukin (IL)-5 have become widely used for the control of severe eosinophilic asthma. Remarkably, patients receiving neutralising anti-IL-5 biological therapies retain a stable population of residual blood eosinophils. Whether these residual eosinophils are endowed with particular biological activity has not yet been studied, but is of importance in predicting potential long-term effects of IL-5 neutralisation in patients. To tackle the effect of IL-5 depletion on residual eosinophils, we used a comparative RNA-sequencing approach and compared the gene expression programme of eosinophils arising in IL-5-depleted or IL-5-replete human or murine hosts, at steady-state *in vivo* and following *in vitro* stimulation with the eosinophil-activating alarmin IL-33. We compared blood eosinophils from patients with severe allergic eosinophilic asthma treated with anti-IL-5 mepolizumab therapy to those of healthy controls and matched asthma patients receiving anti-IgE omalizumab therapy. We made similar comparisons on bone marrow eosinophils from mice genetically deficient or not for IL-5. We report that restriction of IL-5 availability did not elicit any detectable transcriptional response in steadystate residual eosinophils in mepolizumab-treated patients or IL-5-deficient mice, and influenced only a handful of genes in their response to IL-33. Together, these results support the notion that treatment with IL-5 neutralising antibodies spares a pool of circulating residual eosinophils largely resembling those of healthy individuals.

4.3.2 Introduction

Eosinophils, evolutionarily conserved granulocytes characterised by their elevated content in acidophilic granule proteins [1], have become a cellular target of biological therapies in the precision treatment of so-called human eosinophilic diseases [2], in particular, severe eosinophilic asthma [3–5]. Indeed, in eosinophilic asthma, eosinophilic airway inflammation is associated with disease severity, and there is a positive correlation between blood and tissue eosinophilia and the rate of exacerbations and risk of irreversible airway obstruction [6, 7].

Eosinophilia heavily depends on the bioavailability of a particular cytokine called interleukin (IL)-5 [8], as demonstrated initially in mouse models of asthma [9, 10]. Eosinophilia results from increased production of eosinophils from bone marrow progenitors, increased eosinophil transit through the bloodstream and eosinophil extravasation in target tissues [11]. The unique dependency of eosinophilia on IL-5 instigated the introduction of neutralising anti-IL-5 monoclonal antibody-based biological treatments, namely mepolizumab and reslizumab. These biological therapies alleviate eosinophilia and consequently reduce disease exacerbations in severe eosinophilic asthma [3, 4, 12–14].

Remarkably, a stable and interindividually consistent population of residual eosinophils persists in the blood of patients receiving anti-IL-5 biological treatment, which amounts to approximately half the blood count of eosinophils in the general population [4]. Whether these residual eosinophils are endowed with particular biological activity has not yet been studied, but is of importance in predicting potential long-term effects of IL-5 neutralisation in patients. Indeed, because of its radical effect on eosinophil amplification, IL-5 is still widely believed to act as a maturation factor for eosinophils, favouring progenitor engagement and progression along the eosinophil lineage [8, 15]. In this line of thought, an early report examining the effect of mepolizumab on eosinophil development concluded that IL-5 neutralisation induces a maturational arrest of eosinophils in human bone marrow [16].

Therefore, even though the role of IL-5 in eosinophil maturation has been less thoroughly studied, it is of high clinical relevance in the context of anti-IL-5 biological therapies. In addition to reducing their numbers, withdrawing IL-5 during eosinophil development might alter their biological activities as well. This could have unforeseen long-term consequences given the various potential immune and homeostatic roles experimentally assigned to eosinophils [17, 18] and their putative heterogeneity [19, 20].

Here, through a comparative transcriptomic approach in mice and humans, we studied whether residual eosinophils developing in conditions of IL-5 restriction *in vivo* display alterations in their gene expression programme.

4.3.3 Materials and methods

Human subject characteristics and study design

We recruited 26 patients from the university asthma clinic of Liege (Centre Hospitalier Universtaire de Liege, Liege, Belgium) between February 2019 and May 2020. 10 healthy volunteers were enrolled by advertisement among the hospital and staff and were nonsmokers, nonasthmatic and nonatopic. Asthma patient characteristics are presented in tables 1 and 2. Asthma was diagnosed following the Global Initiative for Asthma (GINA) guidelines (http://ginasthma.org/). Severe asthma was defined according to American Thoracic Society (ATS) criteria [21]. All patients had a history of at least one serious exacerbation requiring hospitalisation and two or more exacerbations requiring systemic corticosteroid treatments. In addition, patients presented with airflow limitation 300 cells·mm−3 , poor symptom control defined as Asthma Control Questionnaire (ACQ) consistently $\geqslant$1.5, Asthma Contol Test (ACT)<20 or not controlled by National Asthma Education and Prevention Program (NAEPP) or GINA guidelines. Patients receiving methylprednisolone up to 4 weeks prior to blood sampling were excluded from the study. Mepolizumab was administered as 100mg subcutaneously every 4 weeks. Dosage and frequency of omalizumab administration was determined by the patient's age, pre-treatment serum total immunoglobulin (Ig)E level (IU·mL−1) and body weight.

The study was approved by the local ethics committee (institutional animal care and use committee, University of Liège) and written informed consent was obtained from all study participants. This research was undertaken in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and followed the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals of the International Committee of Medical Journal Editors.

Mice

C57BL/6J and $Il5^{-/-}$ (C57BL/6-$Il5^{tm1Kopf/J}$) mice were purchased from The Jackson Laboratory. The two strains were interbred and heterozygous $Il5^{+/-}$ progeny was further bred for generating littermates of the genotypes of interest. All mice were housed and bred in institutional specific pathogen-free facilities. Age and sex-matched (female or male) mice were used at 8–16 weeks of age. All animal experiments were approved by the animal ethics committee of the University of Liege and complied with the Animal Research: Reporting of In Vivo Experiments guidelines, the European Union directive 2010/63/EU and the Declaration of Helsinki for the use and care of animals.

Isolation of eosinophils from human blood for cell sorting

Human blood polymorphonuclear cells were isolated by double-layer density centrifugation and blood eosinophils were purified using EasySep Human Eosinophil Isolation kit (Stemcell Technologies) following the manufacturer's instructions. Pre-sort cell viability was 95 per cent or superior as assessed by trypan blue exclusion. Isolated cells were stained with CCR3, CD3, CD19, SIGLEC-8 (Miltenyi Biotec), and CD16 (BD Biosciences). Human blood eosinophils ($CCR3^{+}$ $SIGLEC\text{-}8^{+}$) were sorted (purity $\geqslant$95 per cent) into TRIzol® (ThermoFisher) and stored at −80°C for downstream RNA applications.

Ex vivo activation of eosinophils

Mouse bone marrow eosinophils were stimulated for 4h at 37°C and 5 per cent carbon dioxide (CO2) in culture medium and 100 ng·mL−1 purified IL-33 (BioLegend), and 10 ng·mL−1 purified IL-5 (Peprotech). Human blood eosinophils were stimulated for 6h at 37°C and 5 per cent CO2 in culture medium and 100 ng·mL−1 purified IL-33 (PeproTech). Stimulated mouse bone marrow eosinophils and human blood eosinophils were resuspended in TRIzol (ThermoFisher) and stored at −80°C for downstream RNA extraction.

RNA isolation

Eosinophil RNA from human blood and mouse bone marrow was isolated using phenol-chloroform phase separation RNA extraction procedure. Isolated RNA was treated with DNase (Zymo Research) for 15 min at room temperature. Treated RNA was purified with the RNA Clean & Concentrator-5 kit (Zymo Research). Purified RNA integrity and quantity was assessed using the RNA 6000 Pico kit (Agilent) for the presence of 18s and 28s rRNA peaks. All human samples had RNA integrity number (RIN) >7.9.

RNA sequencing and data processing

Full-length cDNA was prepared using SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio) following the manufacturer's instructions. Purified cDNA integrity and quantity was assessed using the High Sensitivity DNA kit (Agilent). cDNA libraries were prepared for sequencing using Nextera XT DNA library preparation kit (Illumina) using the manufacturer's instructions and samples were sequenced on a NovaSeq 6000 sequencing system (Illumina). If samples were sequenced in different batches, groups were kept equal within every batch.

Differential gene expression analyses

Sequenced reads were aligned to the mouse genome (UCSC mm10) or the human genome (HG19) with RNA-seq Alignment (v2.0.2) using STAR aligner (version 2.6.1a) on BaseSpace (https://basespace. illumina.com). Differential gene expression was calculated using DESeq2 (1.26.0) in R (3.6.3 and 4.0.3) [22]. If samples were sequenced in different batches, sample batch was taken into account in the DESeq2 design. GSEAR analyses on differentially expressed genes were performed on pre-ranked list of significantly differentially expressed genes with baseMean >50 ordered according to their log2 fold change. Online GSEAR v7.2.1 (https://genepattern.broadinstitute.org/gp/pages/index.jsf) was used with the "h.all. v7.2.symbols" (Hallmarks) gene sets and default parameters, except for a "classic" scoring scheme and minimal gene set size of 20.

Statistical analyses

All statistical analyses were performed in R (3.5.0). All mouse experiments followed a randomised design. Sample sizes were determined by power analysis. Respect of tests assumptions and model fit were evaluated using diagnostic plots. Raw data were transformed when needed and back-transformed for graphical presentation. A p-value <0.05 was considered significant.

Data deposition

RNA-sequencing data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/ arrayexpress) under accession numbers E-MTAB-10188, E-MTAB-10189 and E-MTAB-10190 Additional details and methods are available in the supplementary material.

4.3.4 Results

Because, much like in patients receiving mepolizumab, mice deficient for IL-5 ($Il5^{-/-}$mice) retain residual eosinophils [10], we first tested whether residual eosinophils in $Il5^{-/-}$ mice displayed alterations in their development or potential biological activities. We reasoned that such alterations should be reflected to some extent in the mature eosinophil gene expression programme. In line with previous reports [10], $Il5^{-/-}$ mice raised in specific pathogen-free conditions displayed reduced numbers of eosinophils in their blood, lung, spleen and bone marrow (BM) compared with wild-type $Il5^{+/+}$ and heterozygous $Il5^{+/-}$ littermates (figure 1a–d). We sorted BM eosinophils from $Il5^{+/+}$ and $Il5^{-/-}$ mice to very high purity (figure 2a), retrieved high-quality RNA and performed high-throughput RNA-sequencing of their poly-adenylated RNAs. In this analysis, samples from $Il5^{+/+}$ and $Il5^{-/-}$ mice did not segregate according to their genotype (figure 2b). Furthermore, analysis for differential gene expression returned no gene significantly regulated (adjusted $p<0.05$) according to mouse genotype (figure 2c, d and supplementary figure S1). Hence, the gene expression programme of steady-state mature BM eosinophils in mice is largely unperturbed by the total absence of IL-5 during their development.

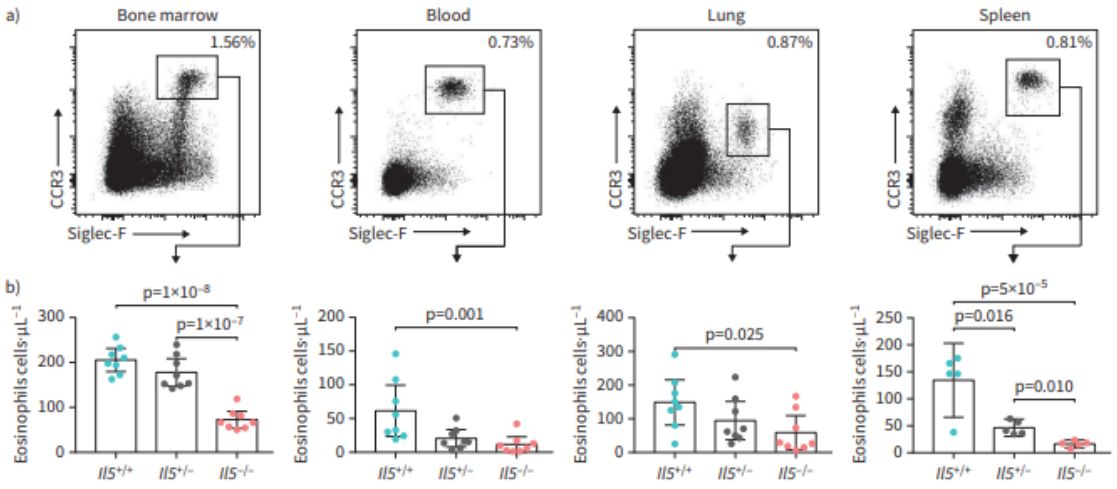

Figure 1. IL-5 deficient mice retain residual eosinophils. a) Representative plots of flow cytometric gating strategy with percentage of eosinophils in indicated organs. **b)** Quantification of eosinophils in specified organs of $Il5^{+/+}$, $Il5^{+/-}$ and $Il5^{-/-}$ mice as in a). Data were pooled from two to three independent experiments, presented as mean (95 per cent CI) and analysed by one-way ANOVA followed by Tukey honestly significant difference tests. Only significant differences of interest are indicated for clarity of presentation.

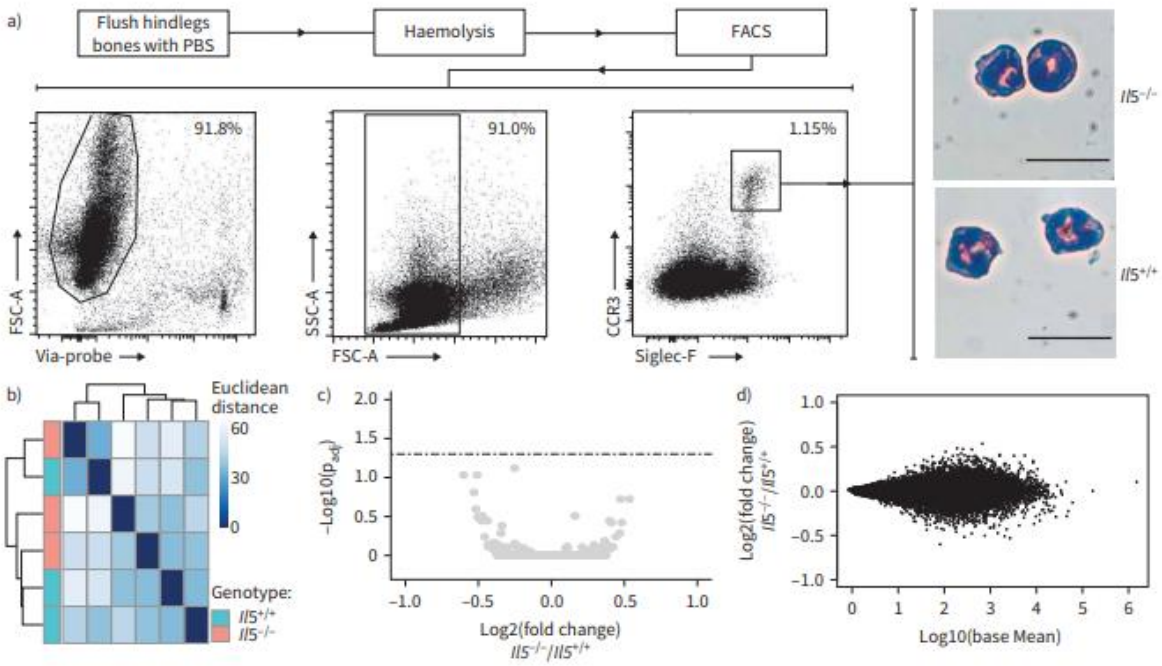

Figure 2. Genetic deficiency in *IL-5* has no detectable impact on mouse residual eosinophils. a) Isolation strategy of IL-5$^{+/+}$ and IL-5$^{-/-}$ mouse bone marrow eosinophils and representative post-sort light microscopy picture. Scale bars=15µm. **b)** Sample clustering, **c)** volcano plot and **d)** MA plot based on RNA-sequencing of biological triplicates in a).

It may be argued that BM eosinophils in the steady state are quiescent cells with minimal levels of gene transcription, as reflected by their low RNA content. Alterations in gene expression of residual IL-5$^{-/-}$ eosinophils could consequently only become apparent following their activation. To address this possibility, we stimulated BM eosinophils from IL-5$^{-/-}$ and IL-5$^{+/+}$ mice ex vivo with both IL-5 and the alarmin IL-33, two very potent activating signals of eosinophils [23–25] and compared their transcriptome (figure 3a). We observed that stimulation elicited a potent transcriptional response in eosinophils from both IL-5$^{-/-}$ and IL-5$^{+/+}$ mice as evidenced by the separation in a principal component analysis (PCA) of unstimulated and stimulated samples along the first principal component that captured 98 per cent of variance in gene expression (figure 3b). Compared with their unstimulated counterparts, 2660 genes were differentially expressed (adjusted p1) in stimulated eosinophils from both IL-5$^{-/-}$ and IL-5$^{+/+}$ mice (figure 3c). Ranked gene-set enrichment analyses (GSEAR) identified “hallmark_TNFA_signaling_ via_NFKB” as the most significantly upregulated hallmark process (figure 3d, e), probably reflecting the fact that IL33 activated the *Nfkb* pathway through its St2 receptor [25]. Notably, PCA suggested that the response of eosinophils from IL-5$^{-/-}$ and IL-5$^{+/+}$ mice to stimulation was highly similar. This was confirmed by the fact that only one gene, suppressor of cytokine signalling 3 (Socs3), was differentially expressed ($p_{adjusted}<0.05$) in the response to stimulation of IL-5$^{-/-}$ versus IL-5$^{+/+}$ eosinophils (figure 3f).

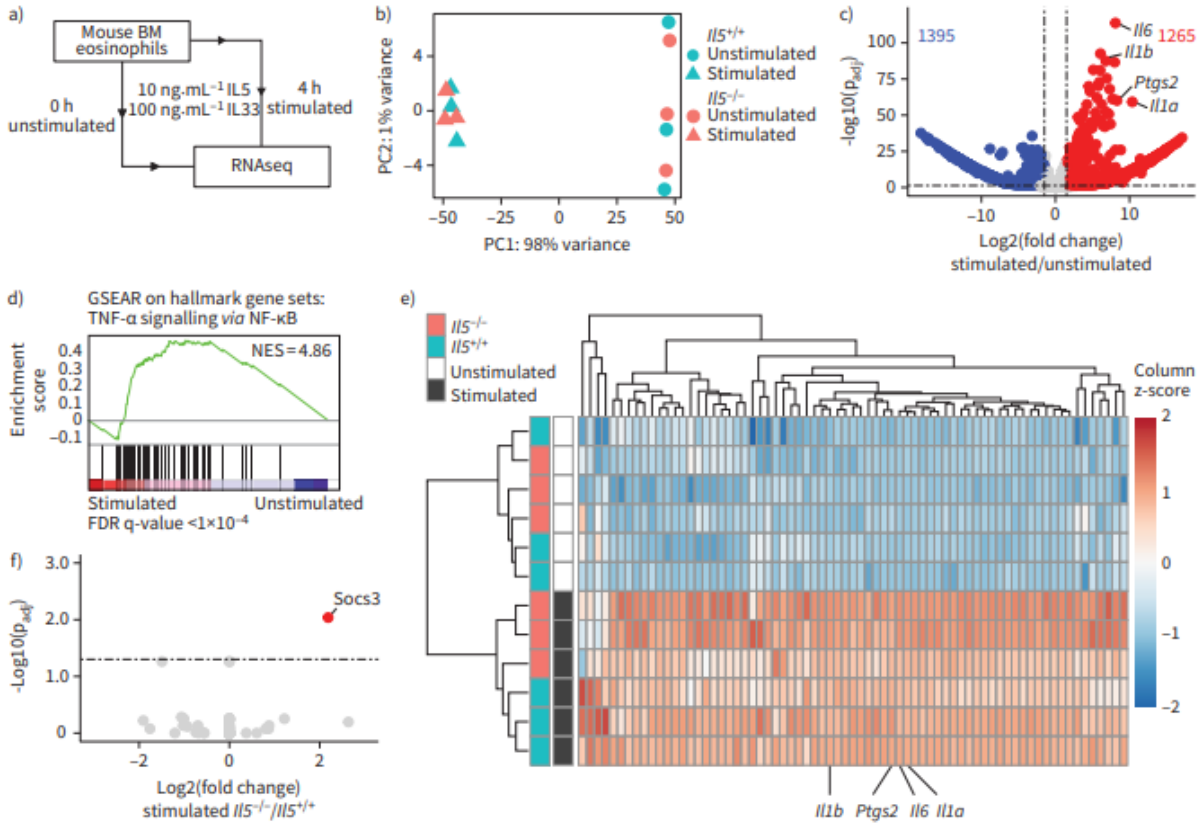

Figure 3. Response to stimulation of IL-5$^{+/+}$ and IL-5$^{-/-}$ bone marrow (BM) eosinophils. a) Experimental outline; **b)** principal component (PC) analysis; **c)** changes in gene expression of IL-5$^{+/+}$ and IL-5$^{-/-}$ eosinophils in response to stimulation; **d)** ranked gene set enrichment analysis (GSEAR) plot for the indicated Molecular Signatures Database (MisgDB) hallmark gene set; **e)** heatmap of changes in expression of genes in d); **f)** changes in gene expression of IL-5$^{-/-}$ versus IL-5$^{+/+}$ eosinophils in response to stimulation. TNF: tumour necrosis factor; NES: normalised enrichment score; FDR: false discovery rate.

These observations suggested that deprivation of IL-5 has limited consequences on the development of eosinophils in mice. Next, we tested whether these observations would translate to human eosinophils. Toward this aim, we recruited 10 severely asthmatic patients with eosinophilic allergic asthma who received mepolizumab for ⩾6 months, as well as 10 severely asthmatic patients with allergic asthma who received anti-IgE omalizumab for ⩾6 months and 10 healthy patients. Mepoluzimab- and omalizumab-treated patients were matched for maintenance nonbiological treatments in order to allow identifying potential treatment-related effects compared with healthy patients (table 1 and supplementary table S1). As expected, blood of mepolizumab-treated patients contained only residual eosinophils (figure 4a), which were approximately the typical 50 eosinophils·μL−1 average [4]. We sorted blood eosinophils of the 30 subjects to high purity and retrieved high-quality RNA (figure 4b). Subsequently, we compared polyadenylated RNA expression by RNA-sequencing. Sample clustering and PCA analyses indicated that eosinophil gene expression profiles failed to aggregate in function of the patient groups (figure 4c, d). Pairwise differential gene expression analyses retrieved no differentially expressed genes (adjusted p1) between subject groups, including in mepolizumab-treated versus healthy control patients (figure 4e). Together, these results indicate that gene expression profiles of residual blood eosinophils from severely asthmatic patients receiving mepolizumab did not differ detectably from that of eosinophils from healthy patients or omalizumab-treated patients.

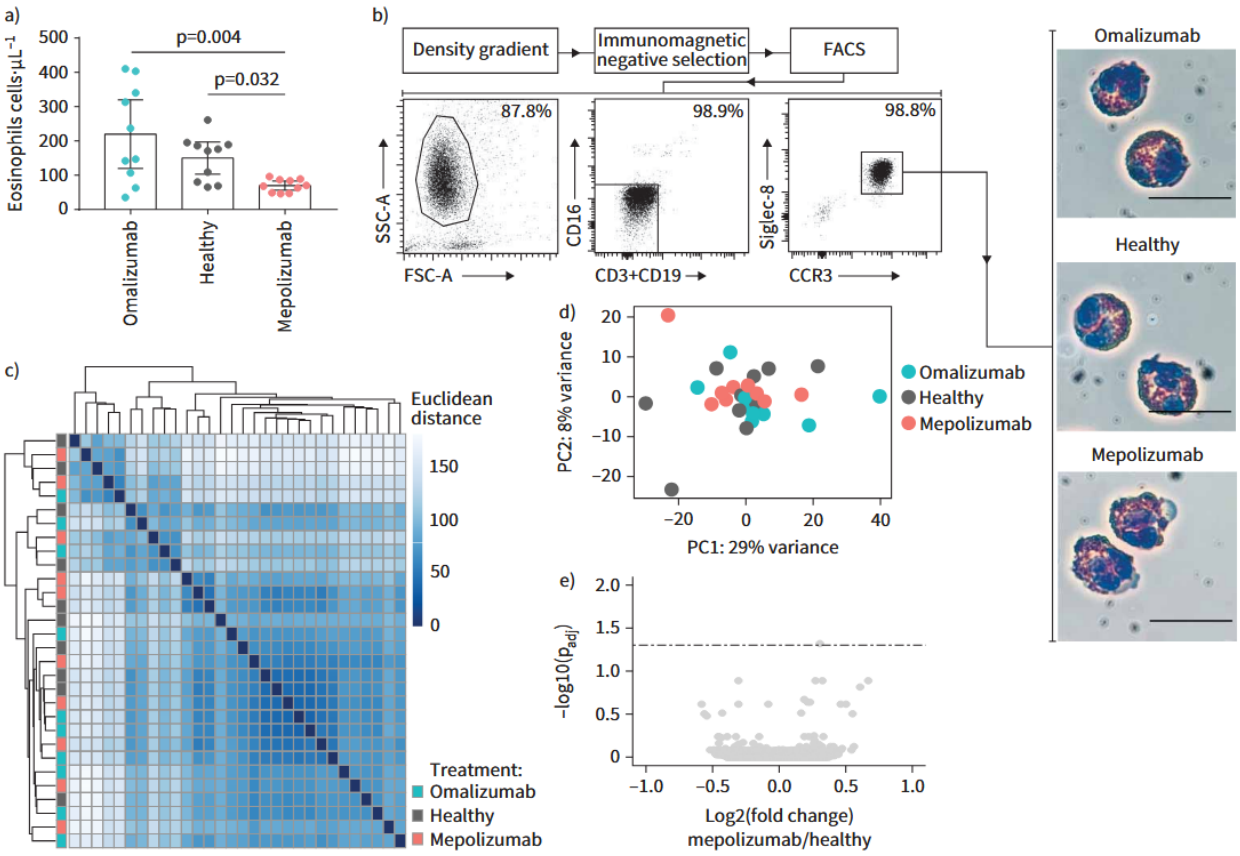

Figure 4.Transcriptomic impact of mepolizumab on human blood eosinophils.

a) Eosinophil blood counts in healthy controls, and in severely allergic asthmatic patients receiving mepolizumab or omalizumab treatment (one-way ANOVA followed by Tukey honestly significant difference tests); **b)** blood eosinophil sorting strategy and representative post-sort light microscopy pictures of eosinophils from the three groups of donors in a); **c)** sample clustering by RNA-sequencing; **d)** principal component (PC) analysis; **e)** differentially expressed genes in eosinophils from mepolizumab-treated versus healthy control donors. Scale bars=15 µm. FACS: fluorescence-activated cell sorting.erimental outline;

Like in our experiments with murine eosinophils, we compared the response to activation of human eosinophils that developed in IL-5-depleted versus IL-5-replete conditions. To this end, we collected blood eosinophils from an additional three mepolizumab-treated and three omalizumab-treated severely asthmatic patients (table 2). Half of each patient's sample was immediately processed for RNA-sequencing, while the other half of the eosinophils were stimulated for 6 h with IL-33 before processing. We stimulated human eosinophils with IL-33 alone to stay closer to the *in vivo* environment encountered by eosinophils in mepolizumab-treated patients, in whom IL-5 is neutralised. Individual patients' RNA samples were subsequently sequenced and submitted to differential gene expression analysis using a paired design (figure 5a). Like in murine eosinophils, culture in the presence of IL-33 had a very marked impact on human eosinophil gene expression. The first principal component in a PCA captured 56 per cent of variance in gene expression and separated IL-33-cultured samples from their unstimulated counterparts in each patient, whereas PC2 did not separate patient samples based on treatment and captured only 23 per cent of the variance. This suggested that IL-33 stimulation, but not the patients' biological treatment, had a predominant effect on the eosinophil transcriptome (figure 5b). Further substantiating this notion, the gene expression changes induced by culture in the presence of IL-33 correlated highly between eosinophils from mepolizumab- and omalizumab-treated patients, as 1015 genes were significantly co-regulated (adjusted $p<0.05$; figure 5c and supplementary table S2). GSEAR analysis for hallmark gene sets returned "hallmark_TNFA_signaling_via_NFKB" as the most significantly upregulated hallmark process (figure 5d), consistent with our results in murine eosinophils. In contrast, only 14 genes were differentially regulated in the response to IL-33 between eosinophils from mepoluzimab- and omalizumab-treated patients (figure 5e, f). Finally, based on differences in expression of these 14 genes, samples clustered first according to IL-33 treatment, and only second according to the fact that samples came from mepolizumab- or omalizumab-treated patients (figure 5f). In other words, only the magnitude of changes in gene expression induced by IL-33 differed depending on the patients' treatment. Of potential interest still, one gene, SOCS3, was more robustly induced by IL-33 in IL-5-depleted eosinophils in both mice and human.

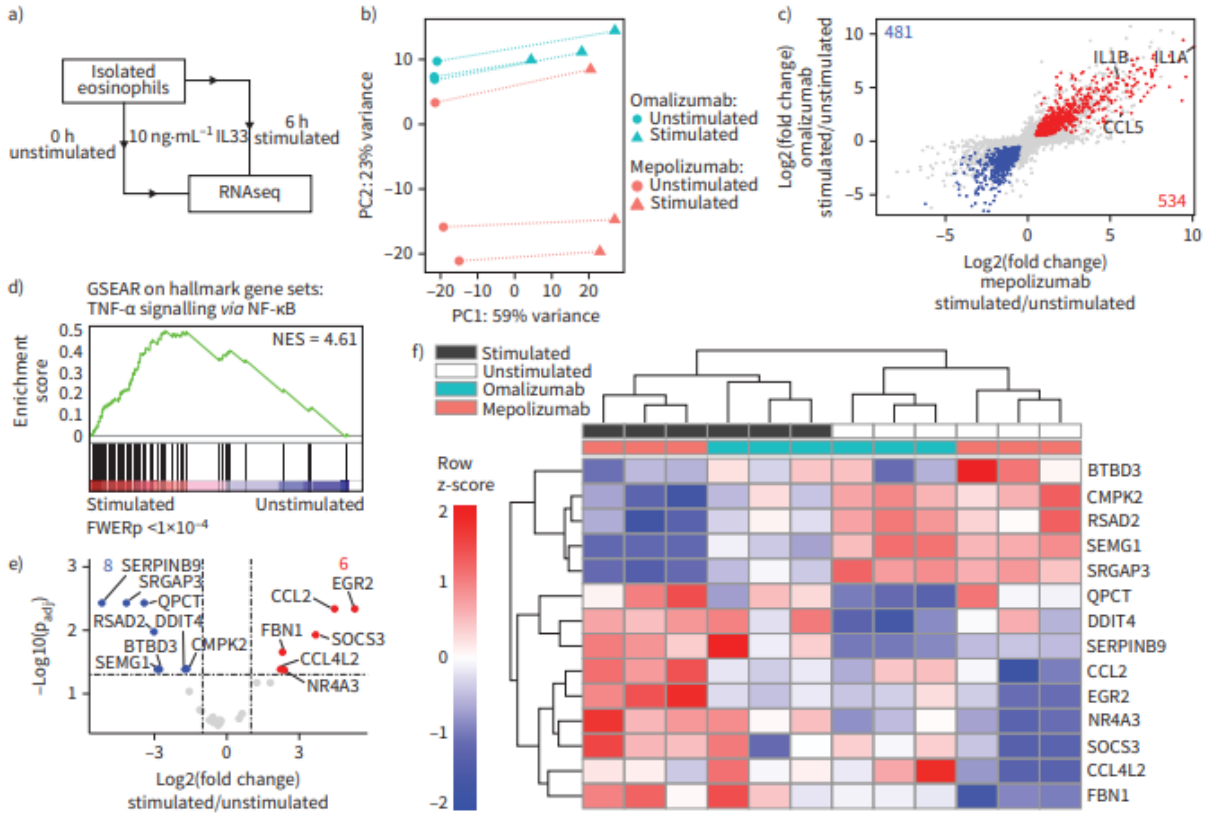

Figure 5. Transcriptomic response to stimulation of blood eosinophils from mepolizumab- or omalizumab-treated patients. **a)** Experimental outline; **b)** principal component (PC) analysis; **c)** correlation of changes in gene expression in response to IL-33 in eosinophils from both patient groups; **d)** ranked gene set enrichment analysis (GSEAR) plot for the indicated Molecular Signatures Database hallmark gene set; **e, f)** differentially expressed genes in response to stimulation of eosinophils from mepolizumab- versus omalizumab-treated patients presented as e) a volcano plot or f) a heatmap. TNF: tumour necrosis factor; NES: normalised enrichment score; FWER: family-wise error rate.

4.3.5 Discussion

In this work, we show that depletion of IL-5, through genetic deficiency in mice or through the administration of anti-IL-5 neutralising antibodies in human, results in only minimal perturbations in the gene expression programme of residual eosinophils in the steady state or following acute activation. As such, our study supports the notion that anti-IL-5 biological therapies leave residual circulating eosinophils largely unaltered, albeit in reduced numbers.

This conclusion is based on congruent observations of the role of IL-5 in two distant organisms. First, we studied syngeneic mouse strains differing only for their genetic proficiency or deficiency at producing IL-5. Second, we compared severe allergic asthmatic patients receiving anti-IL-5 or anti-IgE biological treatments. In both cases, depletion of IL-5 had no detectable effect on the gene expression programme of steady-state residual eosinophils. In addition, eosinophils in both organisms responded almost uniformly to acute cytokine stimulation. Indeed, only the response of a handful of genes differed between eosinophils that developed in IL-5-depleted versus IL-5-replete conditions, namely one gene in murine eosinophils and 14 genes in human eosinophils. Remarkably, the sole differentially expressed gene in stimulated murine eosinophils experiencing IL-5 restriction, namely Socs3, was also more robustly induced in stimulated eosinophils from mepolizumab-treated patients. Altogether, these results suggest that IL-5 only plays a minimal role in priming the eosinophil gene expression programme per se, but that this role, minimal as it is, is conserved between humans and mice.

SOCS3 encodes a negative regulator of signalling by different cytokines and growth factors, including IL-12, a key regulator of auxiliary T-cell polarisation [26]. SOCS3 is a suspected driver of asthma risk in genetic association studies [27]; its expression correlates with asthma severity [28]. This is probably explained by the fact that SOCS3 is a marker of auxiliary type 2 T-cells and facilitates their polarisation in airway allergy [28]. Yet, the role of SOCS3 in eosinophils themselves remains to be established. Hence, determining whether increased stimulation-induced expression of

SOCS3 in eosinophils in IL-5-depleted conditions has biological consequences would be worth pursuing.

The absence of a major impact of the absence of IL-5 on residual eosinophils may seem at odds with its previous proposal as an eosinophil maturation factor. However, the uniform reduction in maturing eosinophil progenitors that was observed in the bone marrow of mepolizumab-treated patients [16] may be more consistent with a reduction in eosinophil amplification, rather than with an impairment of eosinophil maturation. This notion is consistent with the observation that IL-5 is dispensable for mouse eosinophil maturation after differentiation is initiated [29]. From a fundamental standpoint, our results are mainly in line with the notion that the major effect of IL-5 on eosinophilopoiesis is in promoting eosinophil expansion rather than in influencing their differentiation per se.

In this study, we relied solely on gene expression profiling for determining the effect of IL-5 on residual eosinophil function. We do not exclude that IL-5 has activities not directly related to gene expression control. Yet, we argue that major changes in differentiation or activity in any cell are reflected, at least indirectly and to some extent, in its gene expression programme. However, we could not detect any gene expression signature of IL-5 depletion in steady-state eosinophils, and only very limited changes in gene expression following eosinophil activation. Our current findings are consistent with a report that mepolizumab does not alter the expression of activation markers on eosinophils in the bronchoalveolar lavage fluid or their release of eosinophil peroxidase in the lung mucosa of treated patients [30]. Our analysis in humans arguably comprised a limited number of patients (10 per group), and might thereby not have captured genes with elevated interindividual variability and low differences between groups. Nevertheless, as discussed earlier, our analyses in syngeneic mice, which differ only by the expression of IL-5, showed striking similarities with our analyses in human eosinophils.

Altogether, our results indicate that the restriction of IL-5 bioavailability has no detectable impact on the gene expression programme of residual quiescent steady-state eosinophils, and only minimally influences their response to activation. From a clinical

perspective, our work supports the notion that treatment with IL-5-neutralising antibodies spares a pool of circulating residual eosinophils largely resembling those of healthy individuals.

4.3.6 References

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5. Discussion and perspectives

While the main important questions have already been addressed within the discussion of the two above publications, I intend to use this section to emphasize certain critical points of this project and I will allow myself a bit of liberty discussing certain issues that we faced within the field.

Reconciliation of human and murine eosinophil ontogenies

Our data provides further evidence supporting to the latest model of hematopoiesis, in which GATA-1 promotes the development of the arm of myelopoiesis comprising erythrocytes, megakaryocyte, mast cells, basophils and their sister lineage eosinophils (S. E. W. Jacobsen & Nerlov, 2019). In this model, eosinophils are only very distantly related to neutrophils with which they share the morphological feature of having granules. We show that human and mouse eosinophils share similar ontogenies and development trajectories. This is important because it supports the relevance of mouse models of eosinophil associated diseases and can reassure us regarding the ability for this research to be translated to humans. We provide datasets and reliable methods to identify, sort and study eosinophil progenitors within the bone marrow of human and mice. We also created a reporter mouse strain for IL-5Rα in mice resolving previous issues with reagent aspecificity and poor resolution. IL5RAporter mice yet allowed the identification of a small but still important difference between human and mouse bone marrow progenitors. In mice, *Il5ra* is only expressed after eosinophil commitment, while in humans IL-5Rα is present at the surface of the common progenitors of eosinophils and basophils as well as on mature basophils, although a magnitude lower in basophil progenitors.

IL-5 as the rheostat of eosinophil lineage transit amplification

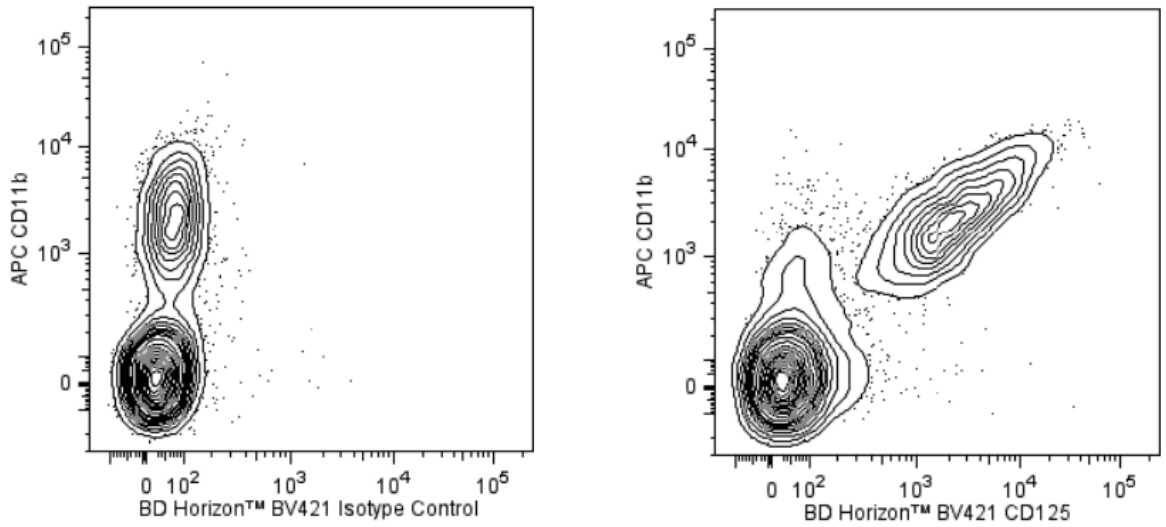
We proposed that IL-5 bioavailability regulates the intensity of the process of transit amplification in which maturation of eosinophil is delayed and their proliferative capacity extended. This could serve as a mechanism to explain the action of anti-IL-5 biologics on eosinophil numbers.

IL-5 the do-it-all eosinophil cytokine

As presented in the figure 14 of the introduction, IL-5 has been proposed to cover about every imaginable function regarding eosinophils. We argue that this pleiotropy finds its origin in the usage of eosinophil counts as a readout or proxy for implicating IL-5 in these processes. Since an increase in IL-5 induces an increase in eosinophil lineage expansion, it also ultimately means that more eosinophils will be available to be recruited, activated etc. IL-5 was notably thought to be required for eosinophil maturation and maturational arrest was the main proposed mechanism of action of anti-IL-5 biotherapies (Menzies-Gow et al., 2003). However, the combination of our two studies shows that, although IL-5 deficiency or depletion reduce bone marrow eosinophil output and peripheral blood eosinophil numbers, IL-5 is not required for eosinophil maturation. On the contrary, our data favors the hypothesis that IL-5 delays eosinophil maturation, granting eosinophil progenitors with heightened and prolonged proliferative capacity.

In mice, we also showed that IL-5Rα is not expressed before commitment to the eosinophil lineage. Our data hence rules out a direct impact of IL-5 on eosinophil lineage commitment, at least in mice.

Finally, only minor transcriptomic differences were observed between eosinophil having developed in the presence or absence of IL-5. Eosinophil developing without IL-5 were able of activation when exposed to IL-33 and again showed minimal transcriptomic differences with eosinophil developing with IL-5 being available.



Two-color flow cytometric analysis of CD125 expression on mouse bone marrow cells. *Mouse bone marrow cells were preincubated with Purified Rat Anti-Mouse CD16/CD32 antibody (Mouse BD Fc Block™) (Cat. No. 553141/553142). The cells were then stained with APC Rat Anti-Mouse CD11b antibody (Cat. No. 553312/561690) and either BD Horizon™ BV421 Rat IgG1, κ Isotype Control (Cat. No. 562868, Left Panel) or BD Horizon BV421 Rat Anti-Mouse CD125 antibody (Cat. No. 565015, Right Panel). Two-color contour plots showing the correlated expression of CD125 (or Ig Isotype control staining) versus CD11b were derived from gated events with the forward and side light-scatter characteristics of viable bone marrow cells. Flow cytometric analysis was performed using a BD™ LSR II Flow Cytometer System.*

Figure 17: Flow cytometry stain with the T21 antibody from its technical datasheet.

In these plots, it can be observed that T21 anti CD125 (or IL-5Rα) labels an unexpectedly large population of $CD11b^+$ murine bone marrow cells.

Extracted from:
https://www.google.com/url?sa=t&source=web&rct=j&opi=89978449&url=https://www.bdbiosciences.com/content/dam/bdb/products/global/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/565xxx/5650xx/565015_base/pdf/565015.pdf

T21 and IL-5Rα; how an antibody fooled an entire field of research

In this section I will elaborate on this topic, which we had to fit only in a supplementary figure in one of our publications. In flow cytometry, one must keep in mind that granulocytes and especially eosinophils and their progenitors are auto fluorescent cells (Weil & Chused, 1981). By choosing channels sufficiently impacted by auto fluorescence such as the channels for FITC or BV510, one could gate human blood eosinophils in flow cytometry based on granularity and autofluorescence alone. IL-5Rα is not expected to be an abundant receptor at the cell surface, which directly challenges the possibility to obtain appropriate resolution. Moreover, in mice, the scientists who provided the most widely used monoclonal antibody for IL-5Rα, clone T21, already pointed out an unexpectedly bright labelling of a large myeloid population (Hitoshi et al., 1990). Unfortunately, T21, as well as REA343 that was engineered to match T21, became the most commonly used antibodies. This can be attributed to their bright staining of at least this myeloid population, which was probably preferred over other antibodies barely labelling anything. On the T21 datasheet from BD biosciences, we can already appreciate the vast majority of $Cd11b^+$ cells in murine bone marrow are labelled by T21 (fig17). It is to be mentioned that all mature granulocyte populations in mice are $Cd11b^+$. This includes basophils, macrophages, neutrophils and eosinophils. It hence appears that this T21 antibody labels way more than just eosinophils (Jeong et al., 2022). We therefore tested the all the commonly available antibodies labelling the murine IL-5Rα . Thanks to our IL5RAporter strain, which is deficient for IL-5Rα in the homozygous state, we showed that T21 and REA343 clones are actually not labelling IL-5Rα but an unknown antigen present at the surface of neutrophils (figure S6 of study 1). Indeed, T21 and REA343 labeled neutrophils and their progenitors even in the absence of IL-5Rα. This specific issue ended up causing a great deal of confusion in the literature. It notably led to the description of "Neos" (neutrophil-derived eosinophils) and multipotent myeloid cells (MMCs) with eosinophil and neutrophil potential claimed to make up to 50% of the bone marrow, of mice with a surprising disappearance of true neutrophils (Jeong et al., 2022). It also led to misidentification of eosinophil progenitors and unreliable counting of “EoPs” in different conditions. This originally surprising observation of the expression of IL-5Rα at the surface of murine neutrophils which we show is due to antibody aspecificity is

unfortunately making its way as accepted knowledge in the community. We argue that if true, expression of IL-5Rα by neutrophils would have led to serious side effects for patients treated with benralizumab due to the ADCC induced depletion of IL-5Rα- bearing cells. IL5rα expression is now also discussed in other places such as at the surface of some epithelial cells (Barretto et al., 2020). We expect that the use of our IL5RAporter mice will allow to clarify the expression of this receptor but, up to this day, we did not find any other IL-5Rα expressing cells beyond the already known eosinophils and some specific B cell subtypes.

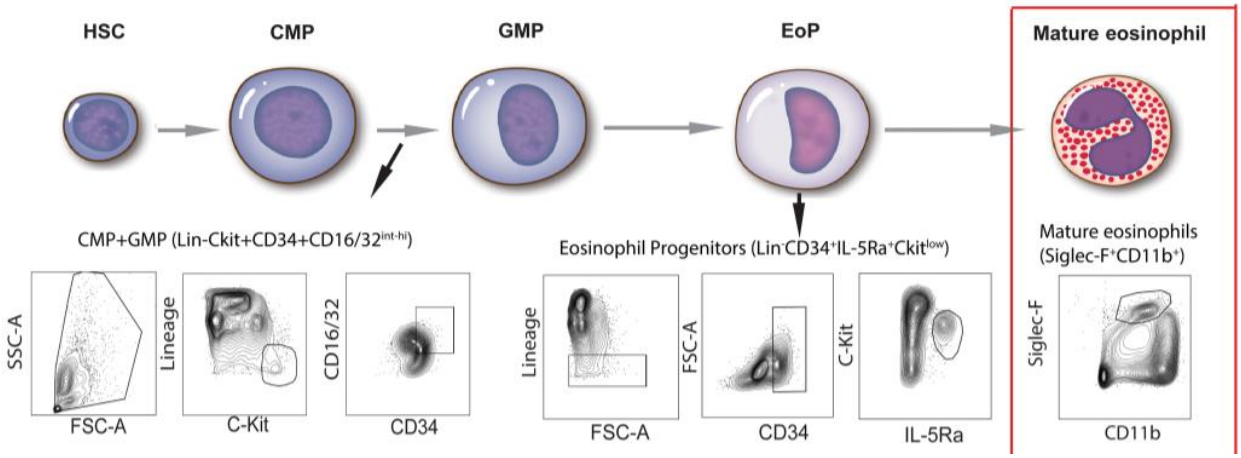
CMPs and GMPs: time for an update.

I will start this section with a citation of a well-known researcher of the eosinophil field.

James J Lee et al : "Unfortunately, the strengths of dogmas are also their greatest weaknesses."(Kevin Range, 2012)

Then I will continue with a small analogy that came to my mind:
One should not mix phenotype and ontogeny. We could hypothetically group all the animals that have wings and that are able to fly. This group would obviously contain some birds but also some insects and bats and even species of flying fish. However, this group would not be used in phylogenetic because it is a paraphyletic group combining species that don't have their most recent ancestor in common. In the same way, granulocytes, comprising eosinophils neutrophils and basophils, have a common morphological feature, their granules, and with it the ability to degranulate. As in the analogy above, this does not allow us to make the interpretation that they come from a direct common progenitor. Although it seemed very logical and convenient that all the granulocytes would originate from a common granulocyte progenitor, we provide further evidence supporting the model in which eosinophils and basophils are closely related but in fact very distant of neutrophils.

A



B

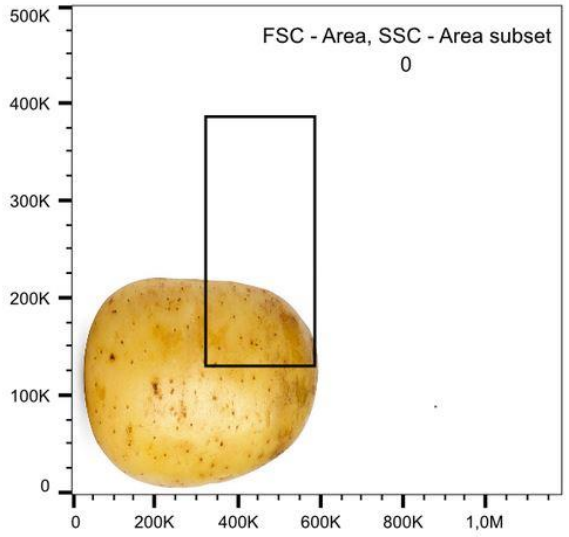


Figure 18: Last nail in the GMP's coffin.

A: The GMP and the model in which EoPs would originate from this progenitor are unfortunately still in use and published even in highly regarded journals despite ancient gating strategies with poor resolution. As in (Mei et al., 2024). However, one can appreciate a second time that the EoP is still not gated within the GMP population but that the strategy is restarted from the beginning. Despite this, authors still continue to expect the EoP to originate from the GMP.

B: Potato gating, a plot imagined by Joseph Jorssen and Glenn van Hulst and illustrated by Glenn van Hulst as a lighthearted joke is parodying the CD16/32 CD34 plot. Similarly, we also refer to potato clustering in instances where the continuous search of new populations in the name of heterogeneity leads to similar issues in data projections from ScRNAseq data.

It is not difficult to conceptualize that the GMP, identified via such a small amount of markers displaying poor resolution could be heterogeneous We and others have discussed the heterogeneity and obsolescence of the GMP progenitors(Kwok et al., 2020). We have now at our disposal large datasets of mRNA and surface protein expressions. We hope that these resources will be used in the future as an opportunity to provide better characterization and sorting strategies of bone marrow progenitors, definitely putting an end to the GMP and CMP era. The concept of GMP impaired the progression of eosinophil research since the first attempt at identifying EoPs. The authors of the study that first attempted to identify murine eosinophil progenitors provided the community with an important tool, the GATA1-reporter mice, that would a decade later reshape the hematopoietic tree (Iwasaki et al., 2005). Unfortunately, the GMP concept misled their interpretation. Figure 18A from a publication in Science Translational Medicine no sooner than 2024 should be the last evidence we needed to move forward. With the in-depth methods and ever improving equipment available to us we should strive to improve the quality of available flow cytometry datasets and not rely on outdated data acquired with limited parameters and resolution.

To refer to Dr. Lee again: My intention to be “inflammatory” and challenge a common perspective resides in the hope that this will shed a light on these topics and open more questions leading to a faster revision of the current accepted dogma.(Kevin Range, 2012)

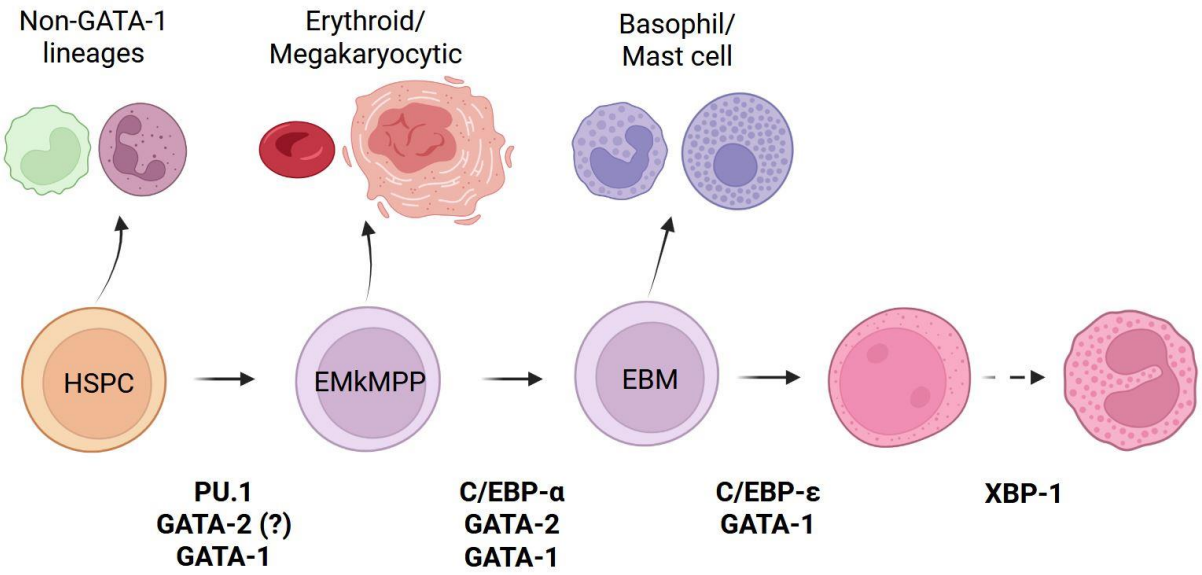


Figure 19: Transcription factors involved in eosinophil development.

Drawn with BioRender

We hereby propose an updated overview of the different transcription factors involved in eosinophil lineage specification and eosinophil development. Further research will be required to precisely pinpoint the exact roles and timely orchestrated interactions of each of these factors involved in eosinophilopoiesis.

Transcription factors in eosinophil development: Beyond the CMP/GMP dogma

In figure 13, we reviewed how eosinophil lineage commitment relies on a complex and synchronized network of transcription factors, some with synergistic or antagonistic effects, each expressed in the right amount at the right moment. We could classify these transcription factors in a hierarchical or temporal fashion. First, "pioneering" transcription factors bind closed chromatin in promoter and enhancer regions of lineage-specific genes in HSPCs, rendering these regulatory regions more accessible to other transcription factors and the transcriptional machinery. PU.1, GATA-2 are considered such essential pioneering factors in myelopoiesis and hematopoiesis (Heinz et al., 2010; Katsumura et al., 2017). Early hematopoietic failure in GATA-2-deficient HSCs complicates studies, but our SCENIC analyses of murine progenitors concur with the notion that GATA-2 might be a pioneering factor of "GATA-1" lineages in general. GATA-1 is not considered a pioneering factor since it has not been shown yet to bind and open closed chromatin.

Downstream of the activity of pioneering factors, "lineage-specifying" transcription factors enforce expression of lineage-specific genes such as granule genes. Importantly, the combined activity of different pioneering factors in HSPCs determines what genes become accessible to a given "lineage-specific" transcription factor (Heinz et al., 2010). In consequence, that same transcription factor might affect different sets of genes in different progenitors. Several such "lineage-specifying" transcription factors are required for activating the eosinophil gene expression program. One critical such factor is GATA-1, whose partial downregulation in Gata1dbl mice suffices to completely block eosinophil development (Yu et al., 2002). We argue that IRF-8 was wrongly attributed a specific role in eosinophilopoiesis, since we showed its complete depletion does not block eosinophil differentiation. Other "eosinophil lineage-specifying" factors include C/EBP family members, especially C/EBP-α which is essential to eosinophilopoiesis but also to the basophil/mast cell lineage. Another interesting C/EBP family member is CEBP-ε, which our SCENIC analyses suggest is acting downstream of the lineage bifurcation between eosinophils and basophils. CEBP-ε is also an essential promoter of

neutrophil maturation (Kwok et al. 2020). We hypothesize that “convergent” activation of a CEBP-ε-dependent gene expression program along eosinophil development explains the evolutionary divergence of basophils and eosinophils, as well as the previously assumed developmental proximity of eosinophils and neutrophils. In this view, “convergent” cooption of CEBP-ε-dependent neutrophil/macrophage leukocyte program in the otherwise ontogenically distant "GATA1” arm of myelopoiesis might explain the expression in eosinophils of genes also expressed in mature neutrophils (e.g. Lyz2, S100a9, Mpo) along eosinophil-specific expression of genes originating from ancient duplication events of neutrophil/monocyte genes such as Epx, which originated from a duplication of Mpo (Noia et al., 2021). In simple terms, eosinophils might have been evolutionarily shaped from basophil/mast cell progenitors that “hijacked” a monocyte/neutrophil gene expression program, which progressively diverged. It is tempting to speculate that the selection advantage early eosinophils conferred to the ancestors of all jawed verterbrates originate from their mixed features of “GATA-1” and “non-GATA-1” myeloid leukocytes.

A final group of transcription factors involved in eosinophilopoiesis could be referred to as “housekeeping” transcription factors, required for maintaining basic vital functions during eosinophil maturation without directly being involved in lineage specific gene expression programs. We would argue that XBP-1 is such a transcription factor. XBP-1 indeed seems required mostly to allow eosinophil-committed progenitors to cope with the intense endoplasmic reticulum stress caused by their massive production of lineage-specific proteins (Chen et al., 2023) .

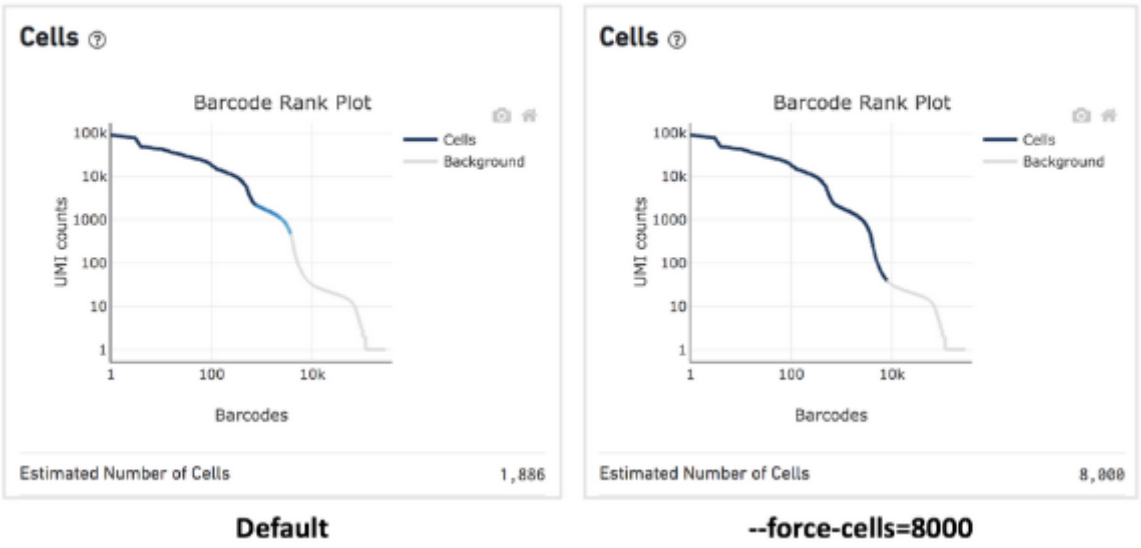
Based on the above considerations, we propose a revised version of the classical view of the transcription factors regulating eosinophilopoiesis (fig19). Obviously, more experimental evidence is required to test this model. We hope novel developments in the field, such as provided in this work, will help shed new light on the development and possibly the function of the still elusive cell type that are eosinophils.

Run Cell Ranger

For this dataset, when using the default Cell Ranger cell calling algorithm, there are only 1,886 cells identified (left plot below). This is because the RNA profile of neutrophils is more similar to the background (low UMI, low gene count) and the cell calling algorithm cannot readily distinguish between the neutrophils and background.

Therefore, to capture the neutrophils, we need to override the cell calling algorithm to ensure that low-UMI cells such as neutrophils are included in the filtered feature-barcode matrix. At this stage, there is no need to be concerned about including some background GEMs (Gelbeads-in-Emulsion) to the filtered matrix because we can filter them out later using Loupe Browser or other third-party tools. To override the cell calling algorithm, run `cellranger count` with the `--force-cells` option. 10x Genomics recommends starting with the number of cells targeted. If you are unsure about the number of cells expected, it is better to overestimate at this stage.

Given that 8,000 cells are expected to be recovered for this sample, use `--force-cells=8000` to analyze this dataset. In the barcode rank plot (right plot below), note a group of lower UMI count barcodes (second "knee") being included as cells, which are likely the neutrophils of interest.



Default **--force-cells=8000**

Figure 20: How to recover poor quality data within the noise that did not pass QCs.

Extracted from:

https://www.10xgenomics.com/support/software/cell-ranger/latest/tutorials/cr-tutorial-neutrophils

When to ScRNAseq and when not to ScRNAseq ?

ScRNAseq can be a very useful tool to build new hypotheses and to decipher cellular ontogenies. However, my journey was sprinkled with more or less failed attempts at using ScRNAseq on mature murine eosinophils. There was, and still is, in the field a kind race to who would produce ScRNAseq data on eosinophils. I would argue that everyone involved in this should stop, at least for a second, and evaluate the usefulness of this data even if they were to be successful at obtaining it. We actually obtained scRNAseq data on eosinophils on several occasions but did not think that the quality as well as the depth of information of said data would support any useful interpretation. This might actually be a conclusion that we should have been reporting. On top of the presence of numerous RNAses which we discussed previously, eosinophil have low RNA content which gets lower the more they mature. Facing those issues, adepts of ScRNAseq of eosinophils have tried to develop countermeasures. These include reducing the quality thresholds for what is considered analyzable data or overloading the system, literally calling it “super-loading“ delivering a percentage of cell recovery that barely reaches the percentage of expected multiplets (Gurtner et al., 2023).

I argue that these methods do not constitute viable options. Since the depth of sequencing is very limited, assembling the data contained in several cells in a kind of pseudobulk could still be the best of the non-ideal options. To illustrate my point, we will now have a look at recommendations which were recently provided by 10X Genomics to identify and recover neutrophils in ScRNAseq datasets (fig20). Dealing with neutrophils is also complex but not to the level of eosinophils. They propose to force the detection of cells up to the number of expected cells. This will leave you with cells only expressing a handful of genes with a handful of UMIs. In this specific example, they went from less than 2000 cells passing quality checks (QCs) to 8000. This comes at a cost, if you pay attention to the log scales you will observe that the depth of sequencing is very shallow (around 100 UMIs in most of the cells) and, after removing house keepings genes, this technique might just barely allow to identify the cell type. If ScRNAseq is only allowing the identification of a handful of genes of interest with a handful of UMIs, one might prefer flow cytometry or another proteomic technique that will provide more information within more cells at a lower cost. Finally, I will share an important graph from an article I co-

authored, which displays a correlation between bulk mRNA and protein expression (fig21). What stood up to me in this graph is that at least around 2 log2 fold changes in mRNA expression are required in that case to be able to accurately infer any change in protein expression.

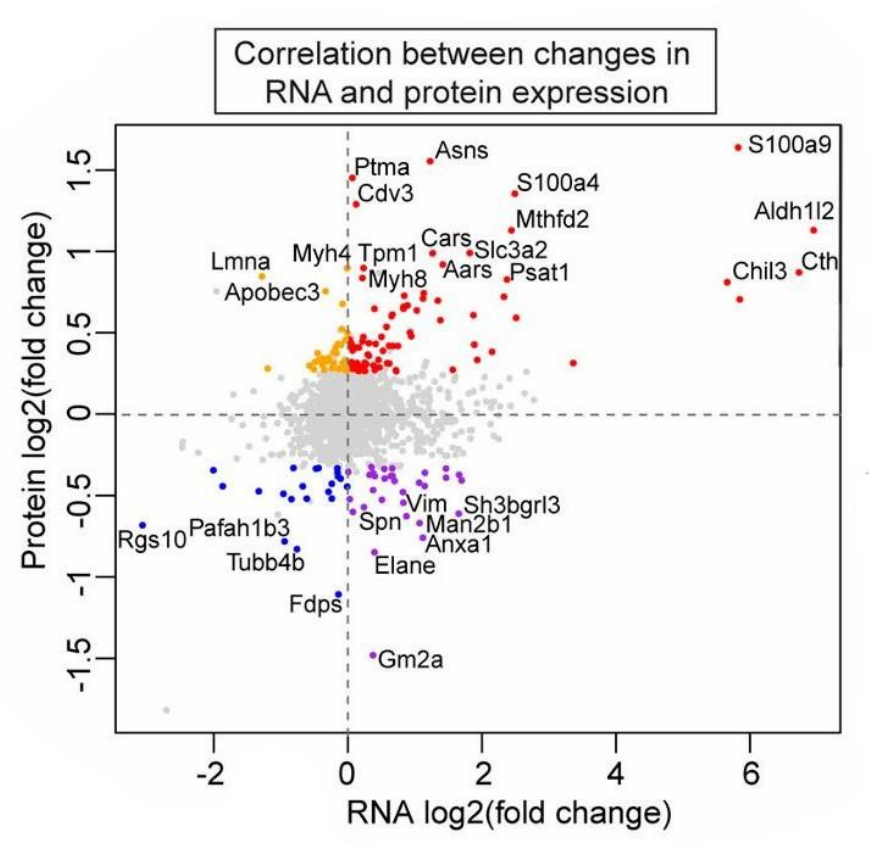


Figure 21: Correlation between mRNA expression variation and protein expression variation in bone marrow progenitors deficient or not for the Elp3 tRNA-modifying enzyme.

From this graph we may extrapolate a reasonable threshold for a change in mRNA to reflect a change in its corresponding protein expression. I would consider this threshold to be around 2 log2 fold changes in mRNA abundance to be conservative.

Plot as in (Rosu et al., 2021)

How might we improve the collection of scRNAseq data from eosinophils? In study one, we leveraged our gating strategies for eosinophil developmental stages and used them in Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq). CITE-Seq is a technique in which nucleic acid probes are coupled to antibodies instead of fluorophores for them to be used in ScRNAseq to allow protein and mRNA detection. This technique allowed us to retrieve or 4 eosinophil stages in ScRNAseq despite their low amount of mRNA by fishing for droplets expressing the surface marker proteins. We actually show the amount of RNA that can be recovered in each stage in figure S2 from study 1. Only stage I passed traditional QCs after which the quantity of UMIs rapidly declined and droplets were assigned mainly from surface protein data. So we show that early progenitors are somewhat easier to sequence and this has been useful in terms of ontogeny inference. It is also possible that activated eosinophils, as well as human opposed to mouse eosinophils, could provide more data. In any case the depth of sequencing that can be achieved with bulk RNAseq, although also challenging on eosinophils, will be orders of magnitude superior to ScRNAseq. We argue that in case in-depth characterization is needed of a population that can be identified and purified in flow cytometry, bulk RNAseq should be preferred. If single cells or a population can be investigated using proteomics, it should be preferred as well.

We have been using a very promising technique called Infinity Flow to investigate the surface proteome of eosinophils and other bone marrow cells(Becht et al., 2020). This technique could prove instrumental in uncovering heterogeneity and variations in surface marker expression in many other contexts. Regarding ScRNAseq, we argue that it should always be coupled at least with anti-CD45 antibodies but preferentially also with other antibodies for all cells of interest in CITE-Seq. Reduction in the stringency of QCs can then be avoided while still being able to observe the cells than suffered from mRNA degradation or have low RNA quantity. Coupling mRNA to protein in big datasets is, in any case, a very interesting step towards validation.

Confusing in vitro results

During my thesis, I also wasted a lot of time trying to culture eosinophils in vitro and my thesis committee members might remember that sentence I finally came up with: “in vivo veritas , in vitro oppositas”. Regarding early progenitor culture and as good as it can be set up, sorting is not 100% accurate. With highly expanding progenitors in the right medium, one can obtain some eosinophils after so many days only due to initial contamination. The 1.5% of the cultured cells leading to eosinophils in the original murine EoP article is more probably due to contamination than actual eosinophil potential within the Gata1-negative GMP (Iwasaki et al., 2005). Furthermore, testing lineage potential with high amounts of cytokines in vitro is very far from physiologic conditions and can generate a whole bestiary of artefactual cell types as well as results conflicting with in vivo data (Dyer et al., 2010). Let’s take a minute to compare in vitro to in vivo (fig22). On the right you have a mature murine eosinophil, on the left however; I have no idea what that is, is the size even correct? What about the vacuolization? It has some pinkish stain to it, is it even in some granules? One could rightfully ask the relevance of that cell which grew in a plate compared to eosinophils that grew in a complex bone marrow environment modulated by cell-cell interactions and numerous cytokines, nutrients and growth factors of which the amounts are tightly regulated. I would argue that we are not yet able to reproduce the bone marrow conditions in a way that would allow to obtain interpretable data on eosinophils differentiated in vitro. Terminally mature eosinophils can however be put in culture for short periods of time to test their activation, degranulation or other functional phenotype but directly relating whatever is differentiated in eosinophil promoting conditions in vitro to mature eosinophils in vivo requires careful consideration.

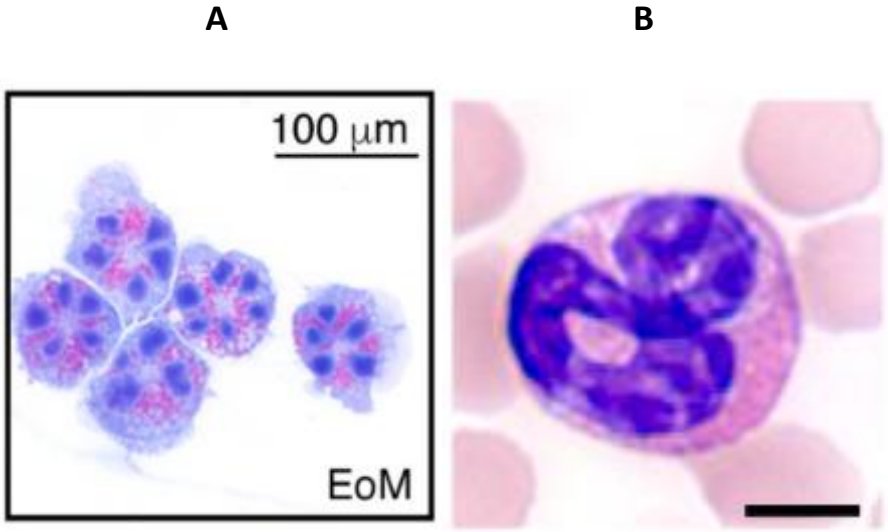


Figure 22: Morphology of in vitro differentialted eosinophil VS a mature mouse eosinophil.

A: on the left, in vitro differentiated mouse eosinophils as in (Johnston et al., 2016)

B: on the right, mature mouse eosinophil as in (J. J. Lee et al., 2012), scale bar = 5µm.

Perspectives

In this work we contributed to better resolve eosinophil ontogeny and provided new methods and datasets for the study of eosinophils. We also modelled a mechanism for the expansion of the eosinophil lineage painting IL-5 as the rheostat of eosinophil transit amplification. This is an important step in the understanding of the mechanism of action of anti-eosinophil biotherapies. Several biotherapies are now available and there is a crucial need for reliable markers orienting the choice towards the optimal therapy. Indeed, a significant portion of patients still are being empirically switched between different therapies. We will expand our work which is currently lacking data on human eosinophils during active eosinophil expansion. We plan to study HES patients as well as dupilumab-induced eosinophilia. We also plan on studying potential feedback mechanism regulating total eosinophil numbers. The data obtained in our models could also indicate that the moment at which a patient is sampled or treated could be of high relevance. Indeed, the amount of time since the last exacerbation of the EAD or the last encounter with a trigger could mean that one would or would not observe eosinophil expansion in the bone marrow at a given time point. This could also have an impact on the choice of biotherapies: Would it really be useful to treat with anti-IL-5 in the absence of active expansion or would it be wiser to test another treatment? Could the eosinophil be bystander or rather be involved in repairing tissues in some cases? More research will be required on the different mechanism involved in EADs and notably HES to properly orient the choice of biologics. We can hope that new biomarkers could be identified to inform this decision.

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7. Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
CCR3	C-C chemokine receptor 3
CITE-seq	Cellular indexing of transcriptomes and epitopes
CMP	Common myeloid progenitor
CLP	Common lymphoid progenitor
CXCR	Chemokine C-X-C motif receptor
C/EBP	CCAAT/enhancer-binding protein
EADs	Eosinophil-associated disorders
EBM	Eosinophil, basophil, or mast cell
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EdU	5-ethynyl-2'-deoxyuridine
EETs	Eosinophil extracellular traps
EoPs	Eosinophil progenitors
EoMP	Eosinophil mast-cell progenitors
EMkMPP	Erythroid–megakaryocyte-primed multipotent progenitors
EMR1	EGF-like module containing mucin-like hormone receptor 1
EPX	Eosinophil peroxidase
FcεRI	High-affinity IgE Receptor
GMPs	Granulocyte/monocyte progenitors
G-CSF	Granulocyte- colony stimulating factor
HSCs	Hematopoietic stem cells
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate lymphoid cells
ILC2s	Type 2 innate lymphoid cells
IL-5Rα	IL-5 receptor subunit α
IRF8	Interferon-response factor-8
IV	Intravenous
Lin	Lineage
LMPP	Lymphoid myeloid primed progenitor
mAbs	Monoclonal antibodies
MBP	Major basic protein
MegE	pre-Megakaryocyte-erythroid progenitor
MEP	Myeloid erythoid progenitor
MkP	Megakaryocyte progenitor
MPP	Multipotent progenitor
NMP	Neutrophil-monocyte progenitor
mRNA	Messenger ribonucleic acid
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
RSV	Respiratory syncytial virus
ScRNAseq	Single cell RNA sequencing
SIGLEC	Sialic acid-binding Ig-like lectin

SSC	Side scatter
TGF-β	Transforming growth factor-beta
Th2	Type 2 helper T cells
TLR	Toll like receptor
TSLP	Thymic stromal lymphopoietin
T2	Type 2
UMAP	Uniform manifold approximation and projection

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The cousins

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Odon, It was a though call and we both got temperament and huge personalities. If people think we are brothers, it is because we are mentally. I grew along you in these last years, and I had a great time. I can’t wait to drive more with you and repair whatever comes in our way.

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