Unraveling the influence of Interleukin-5 on eosinophil development: Contribution to a better understanding of precision therapies for severe eosinophilic asthma

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Abstract

Eosinophils are key suspects in the pathogenesis of various type 2 immune disorders. Particularly in type 2 asthma, eosinophils contribute to airway inflammation, bronchial hyperresponsiveness, and tissue remodeling. Interleukin (IL)-5 is a cytokine central for promoting the accumulation of eosinophils in blood and tissues, a condition known as eosinophilia. Consequently, precision therapies targeting IL-5 or its receptor have been developed for treating eosinophil-associated diseases. Despite the widespread use of these anti-eosinophil therapies, the exact influence of IL-5 on eosinophils remains incompletely understood. Notably, while anti-IL-5 neutralizing antibodies effectively alleviate eosinophilia, they leave a residual population of eosinophils. It is not yet clear whether these residual, IL-5-deprived eosinophils exhibit different biological activities compared to eosinophils at homeostasis.

In this study, we aimed to investigate the influence of IL-5, or its depletion, on the development of eosinophils in the steady-state and in contexts of eosinophilia in murine models and in patients receiving anti-IL-5 therapy.

In a first study, we combined single-cell proteomics and transcriptomics in humans and mice, generated transgenic IL-5 receptor alpha (Il5ra) reporter mice, and used IL-5 deficient mice as well as mice treated with anti-IL-5 neutralizing antibodies to assess the impact of IL-5 on eosinophilopoiesis. In doing so, we developed an easily transferable toolbox to study human and murine eosinophilopoiesis, which includes thoroughly defined cell surface immunophenotypes and transcriptomes at different stages along the continuum of eosinophil maturation in human and murine bone marrow.

In this same study, we observed that eosinophil lineage expansion during eosinophilia relied on IL-5-dependent transit amplification driven by increased cell cycling activity, prolonged proliferation, and delayed maturation of eosinophil progenitors. On the other hand, deletion or neutralization of IL-5 downregulated eosinophil transit amplification but did not impair their maturation. Additionally, by using our Il5ra reporter mouse strain, we found that Il5ra was only expressed after eosinophil lineage commitment in mice. In human in contrast, IL5RA was already expressed in eosinophil/basophil progenitors, which likely explains the impact of anti-IL-5 and anti-IL5RA biologicals on both lineages in patients.

In a second study, we assessed the consequences of IL-5 depletion on residual eosinophils. To do so, we compared the transcriptomes of eosinophils arising in IL-5-depleted or IL-5-replete human or murine hosts. In humans, 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. In mice, we compared bone marrow eosinophils from mice genetically deficient or sufficient for IL-5.

We observed that the neutralization or complete absence of IL-5 did not cause any detectable transcriptional response in steady state residual eosinophils in mepolizumab-treated patients or IL-5 deficient mice. Likewise, the neutralization or absence of IL-5 influenced only a handful of genes in the response of eosinophil to the alarmin cytokine IL-33 *ex vivo*. These results indicate that the restriction of IL-5 has no detectable impact on the gene expression programme of steady-state eosinophils, and only minimally influences their response to activation. From a clinical perspective, these findings suggest that treatment with IL-5-neutralising antibodies spares a pool of circulating residual eosinophils resembling those of healthy individuals.

Collectively, our findings support the notion that the major effect of IL-5 on eosinophilopoiesis is promoting eosinophil expansion rather than in influencing their differentiation, maturation, or subsequent response to activation.

In conclusion, this work provides resources, methods and insights for understanding eosinophil ontogeny, the regulation of eosinophil development and numbers in health and disease, as well as the effects of currently used precision therapeutics for severe type 2 asthma.

Introduction

Asthma

Asthma is a heterogeneous chronic lung disease characterized by inflammation and constriction of the muscles surrounding the airways leading to difficulty breathing (*Global Strategy for Asthma Management and Prevention*, 2023). Asthma is known for its variability in clinical presentation, from patients having symptoms of mild intermittent episodes to patients with severe and persistent manifestations. The prevalence of asthma is increasing in many countries, especially among children. In 2021, asthma affected 42.7 million people in the WHO European region, including 13.7 million children and young people, with 16,000 deaths attributed to the disease. Asthma places a significant burden on both patients and healthcare systems, as it leads to considerable morbidity and mortality, with an estimated 1.9 million healthy life years lost (*International Respiratory Coalition*). The majority of asthma-related deaths occur in low- and middle-income countries (Meghji et al., 2021).

Asthma usually presents with multiple symptoms including wheezing, shortness of breath, chest tightness, and coughing (*Global Strategy for Asthma Management and Prevention*, 2023). The nature of these symptoms is highly variable, fluctuating in frequency and intensity, both within and between individuals. The symptoms are often triggered or exacerbated by factors such as exercise, allergen exposure, change in weather conditions, or viral respiratory infections. Asthma is also characterized by episodic flare-ups, characterized by sudden and severe worsening of symptoms. These exacerbations can be life-threatening and significantly impair the daily lives of those affected (Castillo et al., 2017; Green et al., 2003).

The diagnosis of asthma is complex involving a combination of patient-reported symptoms, evidence of reversible airflow limitation, response to bronchodilators, and exclusion of alternative diagnoses (Global Strategy for Asthma Management and Prevention, 2023; Louis et al., 2022). Airflow limitation in asthma is attributed to several factors, including airway inflammation, bronchoconstriction and structural changes within the airways, often referred to as airway remodeling (Global Strategy for Asthma Management and Prevention, 2023; Louis et al., 2022).

Airway inflammation is a hallmark of asthma that is usually evaluated through non-invasive methods. These methods include measurements of increased fractional exhaled nitric oxide (FeNO) or counts of inflammatory cells in induced sputum (Karrasch et al., 2017; Pin et al., 1992). Bronchial hyperreactivity (BHR), another hallmark feature of asthma, is characterized by an exaggerated response of the bronchial tubes airways to various stimuli. BHR can explain many clinical features of the disease, such as coughing, wheezing, chest tightness or dyspnea (Brannan, 2010). Airflow obstruction is most commonly evaluated by spirometry. Spirometry measures the amount (volume) and speed (flow) of air that can be inhaled and exhaled. The key parameters measured with spirometry are forced vital capacity (FVC), and forced expiratory volume in one second (FEV1) (Celli, 2000; Chung et al., 2014). FVC measures the total amount of air a person can exhale forcefully after deep inhalation. FEV1 measures the volume of air forcefully exhaled in the first second of the FVC assessment. Both FVC and FEV1 and the FEV1/FVC ratio are typically reduced in asthma patients (Celli, 2000). BHR is estimated by assessing FEV1 before and after bronchodilator administration. An increase of FEV1 following bronchodilator administration indicates a reversible airflow obstruction. The reversibility of airflow obstruction is a characteristic feature of asthma and helps differentiate it from other respiratory conditions.

Bronchoconstriction, occurs when the smooth muscles surrounding the airways contract excessively in response to stimuli, narrowing the airway lumen and limiting airflow (Bousquet et al., 2000). Persistent bronchoconstriction, inflammation and repeated exacerbations can lead to structural changes within the airways, a process known as airway remodeling. This remodeling involves several pathological changes, including epithelial hyperplasia and metaplasia, subepithelial fibrosis, smooth muscle cell hyperplasia, and angiogenesis. These changes are promoted by ongoing inflammatory processes, with cytokines and growth factors such as transforming growth factor-beta (TGF-β), IL-4, and IL-13 playing pivotal roles (Henriksson et al., 2015; Hough et al., 2020; Torrego et al., 2007; Wen et al., 2003). In airway remodeling, the extracellular matrix (ECM) undergoes significant alterations, with increased collagen deposition and other ECM components contributing to fibrosis. As a result, the airway epithelium becomes more fragile and prone to damage, perpetuating the cycle of inflammation and repair. These structural changes reduce airway elasticity

and luminal diameter, leading to fixed airflow obstruction and increased BHR (Hough et al., 2020). Airway remodeling can be assessed using advanced imaging techniques, such as high-resolution computed tomography in adults, or using bronchial biopsies, or the measurement of specific biomarkers in sputum and blood (Hough et al., 2020; Saglani et al., 2006).

In healthy lungs, mucus plays a crucial role in maintaining barrier function and ensuring efficient gas exchange by trapping and clearing inhaled irritants through the mucociliary escalator (Bakshani et al., 2018). Airway mucus is composed primarily of water, along with a mix of mucins, proteins, and other substances, which are tightly regulated to maintain an optimal balance between liquidity and viscosity (Aegerter & Lambrecht, 2023). However, in conditions like asthma, the composition of mucus changes significantly. There is increased production of *MUC5AC*, a mucin that makes the mucus more viscous and elastic, leading to the formation of tenacious mucus plugs (Dunican et al., 2018). These plugs are difficult to clear from the airways and can obstruct airflow, contributing to the severity of asthma symptoms. Recent research shows that the quality of mucus, particularly the ratio of *MUC5AC* to *MUC5B*, is a critical factor in the development of mucus plugs, rather than just the quantity of mucus produced (Lachowicz-Scroggins et al., 2016).

Standard treatments

Standard treatments for asthma aim to reduce symptoms and the rate of exacerbations, to preserve lung function. Medication strategies for asthma combine preventive controller medications aimed at reducing airway inflammation and minimizing daily symptoms with reliever medications providing immediate relief during episodes of exacerbations or worsening of symptoms.

Reliever medications target bronchoconstriction and work quickly by relaxing the muscles around the airways making it easier to breathe again. A common example of reliever medication are short-acting beta-agonist (SABA) inhalers such as albuterol, levalbuterol, and terbutaline (Stanford et al., 2012). SABA's are inhaled using a metered dose inhaler (MDI) or a nebulizer. Asthma patients should carry their MDI with them at all times and use it to treat acute symptoms in the short-term. Over-use off SABA's, however, may increase the risk of asthma exacerbations (Nwaru et al., 2020).

On the other hand, controller medications aim to reduce airway inflammation, control symptoms, and reduce risks of exacerbations whilst preserving lung function. Controller medications are prescribed based on the severity of asthma and may be adjusted over time to ensure sufficient management. Inhaled corticosteroids (ICS) such as fluticasone, budesonide, and beclomethasone are common controller medication prescribed to effectively reduce airway inflammation (*Global Strategy for Asthma Management and Prevention*, 2023). Other examples of controller medication include long-acting beta-agonist (LABA) such as salmeterol and formoterol. LABA's are bronchodilators that relax the airway muscles making it easier to breathe. LABA's are often prescribed in combination with ICS. Leukotriene receptor antagonists (LTRA) such as montelukast and zafirlukast block the action of leukotrienes, lipid-derived inflammatory mediators that may contribute to asthma symptoms and inflammation. LTRA's can be administered orally and are often used as an alternative in people that cannot tolerate LABAs well (*Global Strategy for Asthma Management and Prevention*, 2023). Long-acting muscarinic antagonist (LAMA) such as tiotropium and glycopyrrolate help relax smooth muscles of the airways, similarly to LABAs (*Global Strategy for Asthma Management and Prevention*, 2023). They are generally combined with ICS in people with asthma that is not well-controlled using other medications. Additionally, in people with asthma that is not well-controlled or during episodes of exacerbations, oral corticosteroids (OCS) can be prescribed for a short period to reduce airway inflammation and improve asthma symptoms (*Global Strategy for Asthma Management and Prevention*, 2023).

Despite the use of standard treatments, a significant number of patients continue to experience symptoms, frequent exacerbations, and impaired lung function. Studies have shown that around 5-10 per cent of asthma patients have severe asthma that is poorly controlled even with high doses of standard therapy (*Global Strategy for Asthma Management and Prevention*, 2023). The prevalence of patients with poorly controlled asthma may be partly attributed to the high reliance of standard therapies on therapy adherence (Stempel et al., 2005). Factors such as the complexity of treatment regimens, side effects, and patient perceptions of the disease may lead to poor treatment adherence thereby reducing efficacy.

Additionally, ICS and OCS medication can only be prescribed for the short-term. Long-term use of these medications may lead to serious side-effects including obesity,

diabetes, osteoporosis and bone fractures, cataracts, hypertension, and adrenal suppression (Chalitsios et al., 2021; Foster et al., 2017). Healthcare providers should personalize the asthma treatment plan appropriately by monitoring asthma symptoms and managing the disease.

Therapy regimen strategies for asthma are a graduated approach designed to manage the disease based on its severity and symptom control. It begins with the use of a SABA's for mild intermittent asthma and progresses to the addition of low-dose ICS for mild persistent asthma. For moderate persistent asthma, the strategy commonly combines ICS with LABA's. As the severity increases, higher doses of ICS, additional LABA, and oral corticosteroids are introduced. This approach is dynamic, allowing for adjustments through regular monitoring to optimize asthma control and minimize medication-related side effects (*Global Strategy for Asthma Management and Prevention*, 2023). Add on biological therapies have also recently been introduced to complement ''blockbuster'' therapies and will be explained in a different section of this dissertation.

Asthma phenotypes

It is now recognized that asthma is a heterogeneous disease encompassing multiple phenotypes with different underlying disease processes. Identifying heterogeneity and categorizing asthma into distinct phenotypes forms a basis to understand the underlying causes of the disease and for improving treatments (Moore et al., 2010; Wenzel, 2012). Asthma phenotypes are defined based on various clinical, physiological, and inflammatory features, such as age of onset, atopic status, presence of comorbidities, and patterns of airway inflammation.

The most common phenotype of asthma, allergic asthma or atopic asthma, is characterized by high serum levels of immunoglobulin(Ig)E antibodies against one or more common environmental allergens such as house dust mites, animal dander, and mould (*Global Strategy for Asthma Management and Prevention*, 2023; Hamelmann, 2007). This phenotype often presents in childhood and is frequently associated with other allergic conditions such as eczema, allergic rhinitis, or food allergy. Induced sputum of allergic asthmatic patients most often shows signs of eosinophilic

inflammation and patients with this asthma phenotype generally respond well to ICS treatments (*Global Strategy for Asthma Management and Prevention*, 2023).

Non-allergic- or non-atopic asthma is a phenotype of asthma that, as the name suggests, is not associated with allergy but is rather triggered by other factors such as respiratory infections, exercise, cold air exposure, or irritants such as smoke. Induced sputum of patients with this asthma phenotype may be neutrophilic, eosinophilic, or paucigranulocytic. Patients with a non-allergic asthma phenotype usually exhibit a reduced short-term responsiveness to ICS (*Global Strategy for Asthma Management and Prevention*, 2023).

Adult-onset asthma presents for the first time in adult life. This phenotype is particularly seen in woman and the patients tend to be non-allergic. Patients can be refractory to corticosteroid therapy or require higher doses of ICS (*Global Strategy for Asthma Management and Prevention*, 2023).

In obesity-related asthma, excess body weight may lead to significant respiratory symptoms often without eosinophilic inflammation. Finally but not exhaustively, in patients with long-standing history of asthma, a persistent airflow limitation may develop that is incompletely reversible, possibly related to airway wall remodeling(*Global Strategy for Asthma Management and Prevention*, 2023).

Table 1. *Comparison of asthma phenotypes*

Phenotype	***Onset of disease***	***Corticosteroid Response***	***Clinical features***	***Cell counts***
Allergic asthma	Childhood or adolescence	Good	- Triggered by allergens - Associated with allergic rhinitis and eczema. - Elevated IgE levels and eosinophilic inflammation	- Sputum eosinophilia (≥2%) - Elevated blood eosinophil counts (≥150/μL)
Non-allergic	Common in adulthood	Poor	- No clear allergic trigger - May involve neutrophilic inflammation - Tends to be more severe and persistent	- Sputum neutrophilia (>60%) - Normal or low blood eosinophil counts
Adult-onset	Typically after age 40	Variable	- More severe persistent symptoms - Often eosinophilic or mixed inflammation	- Sputum eosinophilia (≥2%) or mixed granulocytic pattern - Elevated blood eosinophil counts
Obesity-related	Typically adulthood	Poor	- Often paucigranulocytic or neutrophilic nature - Associated with low lung volume and systemic inflammation	- Sputum paucigranulocytic or neutrophilic pattern - Normal or low blood eosinophil counts
Persistent airflow limitation	Following long-standing asthma or severe asthma	Poor	- Airway remodelling - Reduced lung function - May overlap with COPD-like features*	- Mixed granulocytic or neutrophilic sputum pattern - Variable blood eosinophil counts

*COPD: Chronic obstructive pulmonary disease

Asthma endotypes

In addition to the concept of asthma phenotypes, researchers introduced the concept of asthma endotypes (Pavord et al., 2018). Instead of categorizing asthma based on phenotypic features, this concept aims at categorizing asthma based on the central underlying immunological causes identified through distinct molecular drivers (Anderson, 2008).

The best-known asthma endotypes are the type 2 (T2) and non-T2 endotypes. The T2 asthma endotype is characterized by type 2 immune responses and will be discussed in more detail in the next section. The non-type 2 endotype underlies asthma phenotypes typically without eosinophilia that may be characterized by neutrophilic or paucigranulocytic sputum profiles. Examples of non-type 2 asthma phenotypes include neutrophilic and paucigranulocytic asthma (Figure 1). Other asthma endotypes are difficult to identify through point-of-care testing. As a result, asthma is currently mainly categorized based on the presence or absence of type 2 inflammation. In this dissertation, we will specifically focus on the T2 asthma endotype which accounts for roughly half of all asthma patients and up to 70 per cent of severe asthma patients (Woodruff et al., 2009).

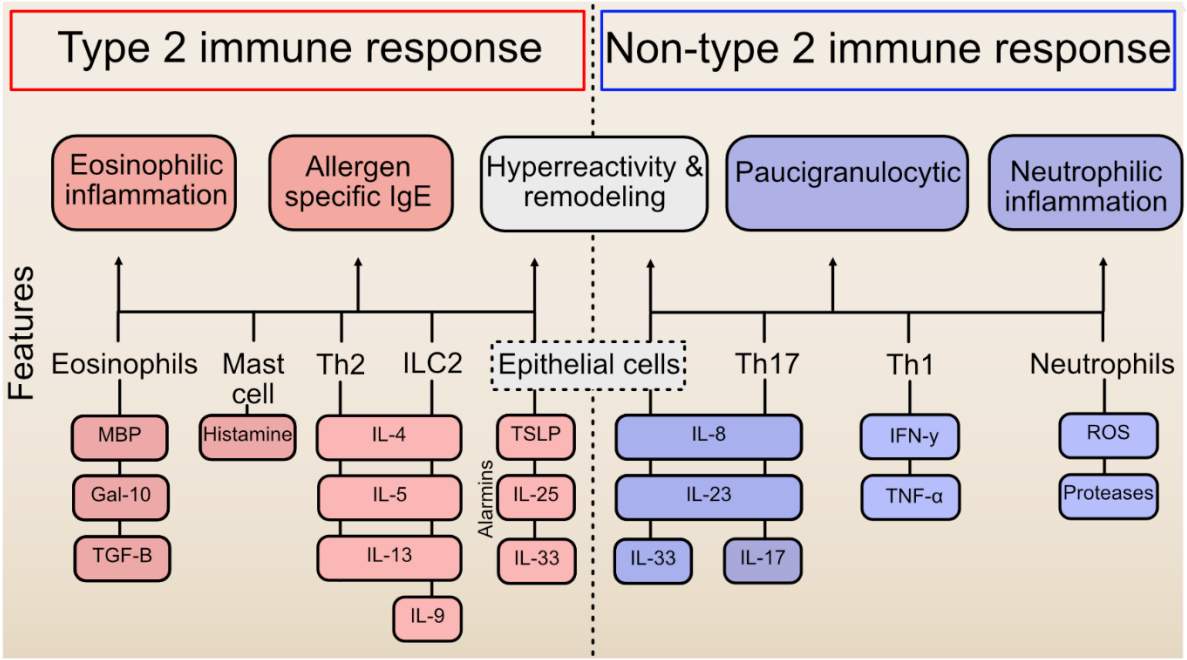

Figure 1. Asthma endotypes and their key immune features.

This figure illustrates the key immune cells, cytokines, and molecular mediators involved in asthma inflammatory responses in type 2 and non-type 2 asthma. Key cellular actors of type 2 immune responses include eosinophils, ILC2s, Th2 cells, and mast cells. Non-Type 2 Immune Responses in asthma are thought to primarily involve Th1, Th17 cells, and neutrophils.

T2 asthma

The T2 asthma endotype is driven by a complex interplay of cellular and molecular events within the immune system indicative of type 2 immunity. Type 2 immunity is a type of immunity characterized by the production of signature cytokines that include IL-4, IL-5, and IL-13 (Figure 2). Type 2 asthma is generally subcategorized into two distinct pathways leading to seemingly similar outcomes of type 2 inflammation in asthma, but yet differ in their initial triggers.

The first major type of T2 asthma is referred to as atopic or allergic asthma. Patients with atopic asthma often have a history of other atopic diseases such as eczema or food allergies and show positive results on skin prick tests or elevated serum specific IgE levels (*Global Strategy for Asthma Management and Prevention*, 2023). In atopic asthma, the immune system reacts to common environmental allergens leading to the production of IgE antibodies. Upon sensitizing exposure to the allergen, dendritic cells capture and process the allergens. Activated allergen-loaded dendritic cells interact with allergen-specific naïve $CD4^+$ cells and promote their activation into type 2 helper T cells (Th2) (Lambrecht & Hammad, 2009). Activated allergen-specific Th2 cells secrete IL-4, IL-5, and IL-13, promoting B cells to produce allergen-specific IgE(Lloyd & Snelgrove, 2018). These IgE antibodies bind to high-affinity IgE receptor (FcεRI) on mast cells, sensitizing them to the allergens. Re-exposure to the allergen triggers cross-linking of IgE on mast cells, causing degranulation and the release of histamine, leukotrienes, and other mediators, which lead to acute inflammatory responses, and bronchoconstriction (Froidure et al., 2016). Additionally, in response to allergens or epithelial damage, epithelial cells can produce thymic stromal lymphopoietin (TSLP), IL-25, and IL-33. These alarmin cytokines can activate innate lymphoid cells (ILCs), a specific group of immune cells (Hammad & Lambrecht, 2021). Among the different types of ILCs, type 2 innate lymphoid cells (ILC2s) are involved in both allergic and non-allergic type 2 asthma pathways. Following their activation, ILC2s produce large amounts of IL-5 and IL-13, which promote eosinophilic inflammation and tissue remodelling (Klein Wolterink et al., 2012). ILC2's in peripheral tissues maintain serum IL-5 levels, thereby maintaining eosinophil numbers (Nussbaum et al., 2013).

In a second endotype of T2 asthma, known as non-allergic or intrinsic type 2 asthma, the inflammatory response closely resembles that of atopic asthma. However, unlike

atopic asthma, the initial trigger is not an allergen, and therefore it does not involve an allergic response or IgE sensitization. Instead, intrinsic type 2 asthma can be triggered by factors such as viral infections, pollutants, or smoke (Fahy, 2015). Rhinoviruses (RV's) are most frequently associated with asthma exacerbations in both children and adults (Jackson & Johnston, 2010; Jartti et al., 2020). Other examples of viruses linked to asthma exacerbations include respiratory syncytial virus (RSV), influenza virus, and parainfluenza viruses. All respiratory viruses infect and replicate within airway epithelial cells, damaging both ciliated and non-ciliated cells. This can lead to cell death, loss of cilia, and reduced mucociliary clearance. The symptoms are likely caused not only by direct viral damage but also by the release of pro-inflammatory cytokines from the damaged epithelial cells such as TSLP, IL-25 and IL-33 (Jackson & Johnston, 2010). These cytokines may directly activate ILC2's, thereby bypassing the requirement for allergen sensitization (Jarick et al., 2022; Roan et al., 2019). Clinically, patients with non-atopic asthma typically have no history of allergic diseases, show negative skin prick tests, and have normal IgE levels (*Global Strategy for Asthma Management and Prevention*, 2023).

More generally, asthma comorbidities, such as chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) and allergic bronchopulmonary aspergillosis (ABPA), also exhibit features of type 2 inflammation. CRS in particular, shares a similar chronic inflammation affecting the upper airways and has an estimated prevalence of up to 50 per cent of patients with severe asthma. Furthermore, approximately 65 per cent of individuals with CRSwNP also have comorbid asthma (Bachert et al., 2021). The persistence of type 2 inflammation is a key driver of asthma exacerbations and chronic symptoms. The cytokines released in type 2 asthma lead to airway hyperresponsiveness, mucus overproduction, and structural changes such as subepithelial fibrosis. Other innate immune cells such as mast cells and dendritic cells may additionally release pro-inflammatory mediators including histamine, leukotrienes, and prostaglandins, known to further exacerbate airway inflammation and bronchoconstriction (Lambrecht & Hammad, 2015). Recently, alarmin-activated basophils have also been proposed to favour type 2 immune cell infiltration in the lung in murine models of asthma (Schuijs et al., 2024).

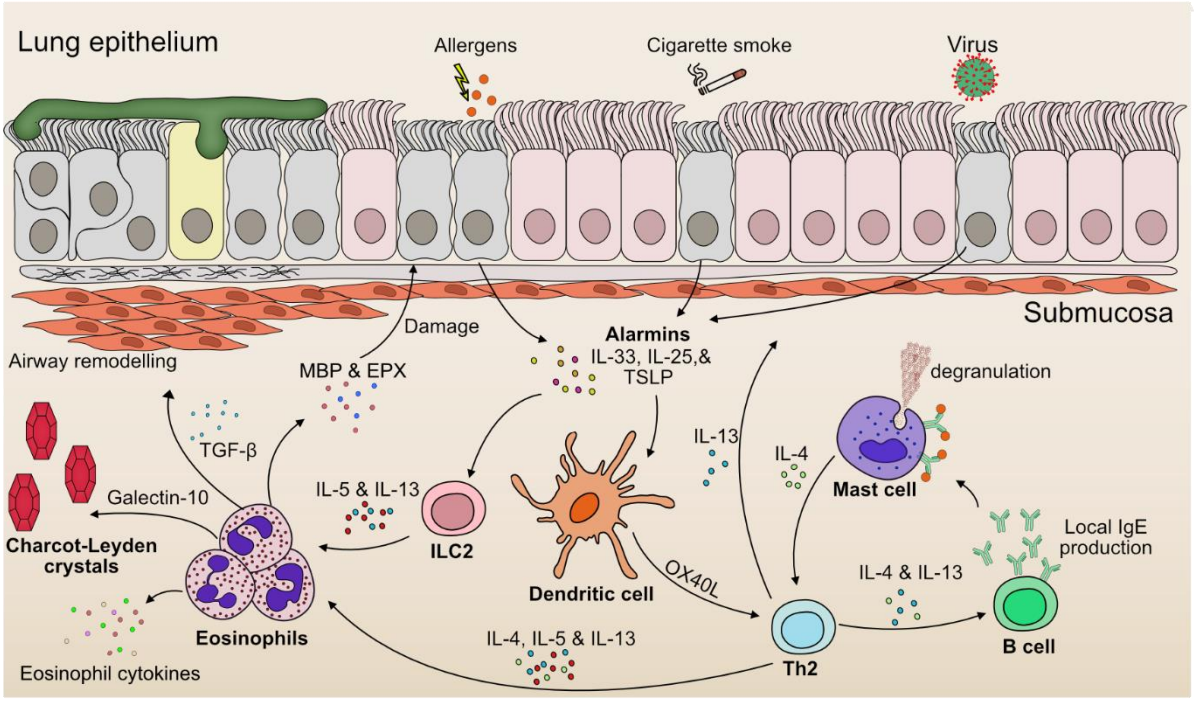

Figure 2. Key components and immune pathways in type 2 inflammation.

This diagram illustrates the immune signalling pathways involved in type 2 inflammation, with a focus on key cytokines, immune cells, and their interactions in response to allergens and viruses. The epithelial cells, upon exposure to allergens, smoke, or viruses, produce the cytokines TSLP, IL-25, and IL-33. These cytokines activate various immune cells. ILC2s respond to epithelial-derived cytokines such as TSLP, IL-25, and IL-33 by producing type 2 cytokines IL-4, IL-5, IL-9, and IL-13, promoting allergic inflammation. Dendritic cells present antigens to T cells promoting their activation into Th2 cells. Th2 cells produce IL-4, IL-5, and IL-13, which in turn promotes allergic inflammation, mucus production, and recruitment of eosinophils. B cells start producing IgE antibodies in response to IL-4 and IL-13 signalling. Mast cells degranulate upon interaction with IgE-bound allergens, releasing histamine and other inflammatory mediators that exacerbate allergic inflammation. Eosinophils are recruited to the site of inflammation via IL-5 and IL-13, releasing their granule contents and thereby contribute to tissue damage, charcot-leyden crystals, and chronic inflammation. MBP: Major Basic protein, EPX: eosinophil peroxidase, TSLP: Thymic stromal lymphopoetin, TGF- β: transforming growth factor β,

Add on precision medicine

Although asthma is well-controlled in most patients, the disease remains uncontrolled or sometimes even worsens in some patients, in spite of good compliance. The prevalence of severe asthma is estimated between 3-10 per cent of patients (*Global Strategy for Asthma Management and Prevention*, 2023). In 2015, a Dutch study found that 3.6 per cent of asthmatic adults qualified for a diagnosis of severe refractory asthma (Hekking et al., 2015). These patients experience a heavy burden of symptoms as well as adverse effects from long-term and frequent use of OCS (Lefebvre et al., 2015). For uncontrolled severe asthma patients with a non-type 2 endotype, additional strategies are currently limited to improving their current therapy regimens by, for instance, improving inhaler technique, adherence, comorbidity management, and the use of non-pharmacological measures.

For type 2 asthma in contrast, thanks to the additional understanding of the underlying molecular and cellular actors of the disease, an increasing number of biological therapies were developed to better manage disease severity and reduce OCS use. These precision therapies selectively inhibit pathways implicated in the pathogenesis of the disease more specific to the patient's asthma phenotype/endotype. Some major such therapies are briefly presented below, while precision therapies that more specifically target eosinophils will be presented in further sections

Anti-IgE therapy (omalizumab) is a monoclonal antibody that binds to IgE and prevents its interaction with surface IgE receptors. By blocking the activation of mast cells by allergen-specific IgE, omalizumab prevents the hypersensitivity reactions that initiate allergic asthma (Humbert et al., 2005). Omalizumab has been shown to significantly reduce the frequency and severity of asthma exacerbations (Busse et al., 2001; Pelaia, Calabrese, Terracciano, et al., 2018). Patients are eligible for omalizumab therapy following confirmation of allergic sensitization through a skin prick and high specific serum IgE to relevant allergens. Indicators for a good response to omalizumab therapy include a high blood eosinophil count (>260/μL), FeNO≥20 ppb, allergen driven symptoms, and onset of the disease in childhood (*Global Strategy for Asthma Management and Prevention*, 2023; Humbert et al., 2018).

Anti-IL-4/IL-13 (dupilumab) is a monoclonal antibody that specifically inhibits signalling through the IL-4 and IL-13 receptor, a cornerstone of type 2 immunity. Dupilumab treatment significantly reduces the need of use of oral glucocorticoid dose and rate of severe exacerbations in otherwise glucocorticoid-dependent severe asthma patients (Rabe et al., 2018). Indicators for a good response to dupilumab include high blood eosinophils and high FeNO (*Global Strategy for Asthma Management and Prevention*, 2023; Rabe et al., 2018).

Anti-Thymic Stromal Lymphopoietin (Tezepelumab) blocks the action of TSLP. TSLP promotes the production of signature T2 cytokines by activating ILC2s and Th2 cells (Cianferoni & Spergel, 2014). Tezepelumab significantly reduced exacerbations and improved lung function, asthma control, and quality of life in patients with severe, uncontrolled asthma (Menzies-Gow et al., 2021). Indicators for a good response to Tezepelumab include high blood eosinophils and high FeNO (*Global Strategy for Asthma Management and Prevention*, 2023).

Anti-IL-5 biological add-on therapies have emerged as a significant advancement in the treatment of severe eosinophilic asthma. These therapies, include mepolizumab, reslizumab, and benralizumab and specifically target IL-5 or its receptor to reduce eosinophils (Castro et al., 2015; FitzGerald et al., 2016; Pavord et al., 2012). These therapies are commonly reserved for patients with high eosinophil counts who do not respond adequately to standard corticosteroids and bronchodilators. In the upcoming subsection on IL-5, a more detailed discussion will explain the mechanisms, clinical efficacy, and application of these therapies in severe eosinophilic asthma.

Understanding asthma endotypes and phenotypes is crucial for tailoring treatment strategies and optimizing therapeutic outcomes. For instance, patients with the allergic asthma phenotype, which is driven by a type 2 allergic response, typically respond well to ICS and allergen-specific therapies, such as omalizumab (Humbert et al., 2005). In contrast, patients with neutrophilic asthma, a phenotype characterized by non-type 2 inflammation, do not respond effectively to ICS, necessitating alternative treatment approaches (Gibson & Simpson, 2009). Patients with severe allergic asthma, but also patients with severe non-allergic eosinophilic asthma, are expected to respond well to targeted biologics like mepolizumab, reslizumab, and benralizumab, which reduce eosinophil levels (Bel et al., 2014; Ortega et al., 2014). In addition, these

patients are also expected to respond well to dupilumab, which targets the IL-4 receptor alpha, and blocks IL-4 and IL-13 signalling (Wenzel et al., 2016). In contrast, non T2 asthma, often associated with neutrophilic inflammation and Th1/Th17 pathways, might better respond to therapies targeting non-type 2 inflammation, although most of the potential targets remain to be formally investigated (Lambrecht et al., 2019). Unfortunately, inhibition of IL-17 receptor did not produce a significant treatment effect in moderate to severe asthma patients (Busse et al., 2013).

Eosinophils

Eosinophils are relatively rare, comprising approximately 1-3 per cent of circulating leukocytes, which equates to about 100-400 cells per microliter of blood (Rosenberg et al., 2013). Eosinophils are a distinct type of granulocytic leukocyte, easily recognized by their unique morphology and staining characteristics. When viewed under bright-field microscopy, eosinophils appear as spherical cells with a bilobed nucleus and large cytoplasmic granules that stain prominently pink/red with eosin dye (Gleich et al., 1993). Eosinophils can be specifically identified by surface markers such as C-C chemokine receptor (CCR) 3 and sialic acid-binding Ig-like lectin (SIGLEC)-8 (in humans) or Siglec-F (in mice), which are useful in flow cytometry and immunohistochemistry for distinguishing these cells from other leukocyte populations (Bochner, 2009; Zhang et al., 2007).

Production of eosinophils in the bone marrow

Hematopoiesis, the process of blood cell formation, is a highly conserved and hierarchical process between mice and humans, with a few notable species-specific differences. This process begins with hematopoietic stem cells (HSCs), which reside in the bone marrow and are characterized by their ability to self-renew and differentiate into all blood cell lineages (Orkin & Zon, 2008a). HSCs give rise to multipotent progenitors (MPPs), which lose self-renewal capacity but retain multilineage potential. In the traditional view of hematopoesis, MPPs diverge into two major branches. Firstly, Common Myeloid Progenitors (CMPs) able to differentiate into megakaryocyte-erythroid progenitors (MEPs) for platelet and erythrocyte production. Additionally,

CMPs give rise to granulocyte-monocyte progenitors (GMPs), which further differentiate into granulocytes and monocytes (Orkin & Zon, 2008b). Secondly, common lymphoid progenitors (CLPs) give rise to B cells, T cells, and natural killer (NK) cells (Kondo, 2010; Orkin & Zon, 2008a, 2008b).

Eosinophils are produced in the bone marrow from hematopoietic stem and progenitor cells through complex differentiation processes that are only partly resolved (Jacobsen & Nerlov, 2019). While both human and mouse eosinophils originate from hematopoietic stem cells, there are notable differences in their proposed developmental pathways in the two species. In mice, eosinophil-committed progenitors, known as eosinophil progenitors (EoPs), have been identified within a heterogeneous pool of GMPs (Iwasaki et al., 2005). In contrast, in humans, eosinophils are proposed to emerge directly from the CMP pool, bypassing a distinct EoP stage (Drissen et al., 2016).

Eosinophils have been considered to develop closely alongside other granulocytes, such as neutrophils and basophils, due to their shared origins from GMPs or CMPs. However, more recent studies indicate that myelopoiesis may occur along two distinct pathways: one pathway, characterized by the expression of the *GATA1* transcription factor in progenitors, gives rise to eosinophils, basophils, mast cells, erythrocytes, and megakaryocytes, while the other pathway leads to the development of neutrophils and monocytes (Drissen et al., 2016; Jacobsen & Nerlov, 2019; Tusi et al., 2018; Weinreb et al., 2020). In line with this notion, deletion of the GATA-binding enhancer site within the mouse *Gata1* gene results in the absence of eosinophils (McNagny & Graf, 2002; Rosenberg et al., 2013).

Eosinophil development and differentiation in the bone marrow is a tightly regulated process orchestrated by a complex network of transcription factors and signalling pathways. Eosinophilopoiesis begins with the commitment of hematopoietic stem cells (HSCs) to the myeloid lineage, which is influenced by several lineage-specific transcription factors. Initially, IL-5 was proposed to promote the commitment of cells toward the eosinophil lineage (Foster et al., 1996; Stolarski et al., 2010). However, *in vitro* enforced retroviral expression of *Il5ra* in GMPs does not increase EoPs in the presence of IL-5. In addition, IL-5 deficient mice still retain residual levels of eosinophils, suggesting that other factors promote commitment to the eosinophil

lineage (Iwasaki et al., 2005; M. Kopf et al., 1996). One of the earliest and most critical transcription factors in this lineage specification is *GATA-1*. *GATA-1*, in conjunction with *PU.1*, promotes the differentiation of CMPs into EoPs (McNagny & Graf, 2002). The interplay between *GATA-1* and *PU.1* is crucial for the precise control of eosinophilopoiesis (McNagny & Graf, 2002)

The CCAAT/enhancer-binding protein (C/EBP) family, particularly C/EBPα and C/EBPε, are also involved in the differentiation of eosinophils (Yamanaka et al., 1998). These transcription factors work in conjunction with *GATA-1* to regulate the expression of eosinophil-specific genes. C/EBPε, in particular, promotes the differentiation of eosinophils and regulates the expression of proteins necessary for granule formation such as major basic protein (MBP) and eosinophil peroxidase (EPX) (Gombart et al., 2003; Nerlov et al., 1998).

Eosinophils at homeostasis

Eosinophils are an evolutionarily conserved cell type that dates back at least 350 million years, as they are present across all vertebrate lineages (Stacy & Ackerman, 2021). Their preservation throughout evolution strongly suggests a role in promoting evolutionary fitness. However, their exact physiological and pathophysiological roles are still not fully understood (Jacobsen et al., 2021; Klion et al., 2020). Eosinophils possess the ability to secrete a diverse array of cytokines, chemokines, lipid mediators, and granule components, including eosinophil cationic protein (ECP), EPX, eosinophil-derived neurotoxin (EDN), Galectin-10, and MBP, in response to various stimuli. This diverse secretory capacity allows eosinophils to influence a broad spectrum of biological processes (Rothenberg & Hogan, 2006). *In vitro* studies have shown that eosinophil-derived MBP can activate basophils, mast cells, and neutrophils, while EDN can activate dendritic cells (Kita, 2011; Rothenberg & Hogan, 2006). Additionally, eosinophils are capable of releasing cell-free intact granules and Galectin-10, as well as generating eosinophil extracellular traps (EETs). These EETs, along with MBP and ECP, are implicated in the clearance of extracellular bacteria (Simon et al., 2011). Despite these known functions, the complete range of eosinophils' roles in health and disease remains an ongoing area of research (Ackerman & Bochner, 2007; Klion et al., 2020).

Under homeostatic conditions, significant populations of eosinophils are present in various human organs, including the lungs, gastrointestinal tract, adipose tissue, and thymus (Marichal et al., 2017). The secretory products of eosinophils, including cytokines, chemokines, and growth factors, are linked to various physiological processes such as metabolism, fat deposition, glucose homeostasis, tissue remodeling and repair, neuronal regulation, epithelial integrity, microbiome composition, and immunoregulation (Jacobsen et al., 2012; Rosenberg et al., 2013). In adipose tissue, eosinophils have been shown to regulate glucose metabolism and fat deposition, which are key factors in maintaining energy balance and metabolic health (Wu et al., 2011). Eosinophils also play a role in tissue repair and remodeling through the secretion of growth factors like transforming growth factor-beta (TGF-β) (Kay et al., 2004). In addition to their roles in metabolism and tissue repair, eosinophils have been involved in neuronal regulation (Lebold et al., 2020). They interact with sensory neurons, potentially influencing pain perception and other neurological functions (Drake, Lebold, et al., 2018; Drake, Scott, et al., 2018). Furthermore, eosinophils are involved in immunoregulation, interacting in both innate and adaptive immune responses. Their ability to secrete a wide range of immunomodulatory molecules allows them to influence the activity of other immune cells, contributing to immune homeostasis and possibly prevention of chronic inflammation (Klion et al., 2020; Wechsler et al., 2021)

Most evidence suggesting that eosinophils participate in multiple homeostatic processes, originates from murine studies. While murine models provide valuable insights, the translation of these findings to humans often requires careful validation of analogous human eosinophil functions. Intriguing and not in direct line with the aforementioned homeostatic eosinophil functions, is the fact that, to date, there is no conclusive evidence of disrupted homeostatic processes in patients undergoing eosinophil-targeted therapies (Jackson & Pavord, 2023).

Roles of eosinophils in anti-pathogen immunity

Eosinophils and their granule proteins, such as MBP and ECP, have traditionally been associated with strong antiparasitic functions. These views were primarily established through histological observations of eosinophils and parasites in

tissue specimens, as well as through *in vitro* studies demonstrating the antiparasitic effects of these granule proteins (Rothenberg & Hogan, 2006). However, the role of eosinophils in antiparasitic defence is more complex and remains controversial, particularly because patients receiving anti-eosinophil therapies do not appear to be at increased risk for helminth infections, even in endemic regions (Khatri et al., 2019; Manfred Kopf et al., 1996).

Besides anti-parasitic defence, preclinical data suggest that eosinophils are activated in response to certain bacterial infections and exhibit bactericidal capabilities. Eosinopenia —an abnormally low number of eosinophils— has been identified as a feature of acute bacterial infections in experimental models and clinical observations. A reduction in eosinophil blood count is thought to be due to the rapid migration of eosinophils to sites of infection, reflecting their active involvement in the immune response against bacterial pathogens (Bass, 1975; Ravin & Loy, 2016; Svensson & Wennerås, 2005). More recently, a study reported that eosinophil derived IL-4 inhibits apoptosis of $CD8^+$ T cells, thereby enhancing their survival and memory formation. In addition, eosinophil-deficient mice have impaired memory $CD8^+$ T cell responses and therefore reduced resistance to Listeria monocytogenes (*L.m.*) infection (Zhou et al., 2024). Clinical observations indicate an inverse relationship between bacterial load and peripheral blood eosinophil counts, suggesting that eosinopenia could serve as a predictive marker for bacterial infection in sepsis patients (Davido et al., 2017; Lipkin, 1979; Shaaban et al., 2010).

Eosinophils have also been proposed to play a role in antiviral defence. A dose-dependent inhibition of respiratory syncytial virus (RSV) was observed when adding eosinophils in increasing concentrations to a fixed number of viruses *in vitro.* This inhibition was reversed when a ribonuclease inhibitor was added before the eosinophils, indicating secreted ribonucleases from the eosinophils were responsible for the antiviral effect (Domachowske et al., 1998). *In vivo* work in guinea pigs infected with Sendai virus showed that reducing eosinophils in bronchoalveolar lavage fluid via systemic anti-IL-5 antibody led to a significant increase in lung virus titre (Adamko et al., 1999). Another study demonstrated enhanced RSV clearance in *IL-5* transgenic mice compared to wild types, further revealing that the antiviral effects were dependent on intact MyD88 signalling through toll like receptor (TLR) 7 (Phipps et al., 2007). These findings suggest eosinophils can promote antiviral activity, particularly through

TLR7 signalling pathways. Eosinophil degranulation products have also been detected in respiratory secretions during RSV infections, and such degranulation is associated with better outcomes in mice infected with pneumonia virus of mice, a model for human RSV (Rosenberg et al., 2009). Intriguingly, a recent study observed that severe asthma patients who receive dupilumab, mepolizumab or benralizumab, have lower SARS-CoV-2-specific antibody titers, neutralizing activity, and virus-specific- B- and $CD8^+$ T cell counts following vaccination (Runnstrom et al., 2024).

Eosinophils as biomarkers and actors in asthma

In patients with T2 asthma, circulating eosinophil levels often increase well beyond the normal reference range of 50-300 cells/mm^3, leading to a condition known as eosinophilia (Rothenberg, 1998) . Eosinophilia in T2 asthma is generally believed to result from increased eosinophil production in the bone marrow (Jacobsen et al., 2012). Eosinophilia is an important biomarker of T2 asthma, with well-established evidence showing that elevated eosinophil levels positively correlate with disease severity (Graff et al., 2019). This correlation supports the hypothesis that eosinophils play a central role in the pathophysiology of T2 asthma and other eosinophil-associated disorders (EADs) (Deykin et al., 2005; Fulkerson & Rothenberg, 2018; Green et al., 2002).

How eosinophils contribute to asthma pathogenesis is probably multifactorial. Following tissue infiltration, eosinophils can become activated, which is expected to trigger pathogenic responses in T2 asthma. The release of cytotoxic proteins such as MBP, EPX, ECP, Galectin-10, and EDN can contribute to tissue damage, attract other immune cells and exacerbate disease (Rothenberg & Hogan, 2006; Van Hulst et al., 2020). Released Galectin-10 can also autocrystallize into Charcot-Leyden crystals (Persson et al., 2019; Ueki et al., 2018). These Charcot-Leyden crystals have been shown to actively promote type 2 inflammation (Persson et al., 2019).

Furthermore, recent research has uncovered a diverse range of activities performed by activated eosinophils, extending beyond the classical view of eosinophils as merely bulk releasing toxic granule contents to kill invaders or cause tissue damage in EADs. Firstly, eosinophils can degranulate using different mechanisms. These include compound and classical exocytosis, both mechanisms leading to the release

of the full granule contents. Additionally, when using piecemeal degranulation, eosinophils can release specific mediators in a more controlled manner through so-called sombrero vesicles or exosomes (Melo et al., 2008). In a process called cytolysis, a non-apoptotic form of cell lysis, eosinophils can release cell-free granules, which remain functional and capable of responding to stimuli extracellular (Muniz et al., 2013). Cytolysis may also release large amounts of galectin-10 crystallizing into Charcot–Leyden crystals (Persson et al., 2019). Eosinophils can also form EETs in a process known as EETosis (Ueki et al., 2016).

Beyond their signature granule proteins, activated eosinophils are important sources of cytokines and growth factors, including key mediators of type-2 immunity and tissue remodeling such as IL-4, IL-13, and TGF-β1 (Davoine & Lacy, 2014). These molecules play significant roles in the pathogenesis of asthma and other allergic conditions. Therefore, a better understanding of the functional activities of eosinophils could provide critical insights into the mechanisms driving EADs.

Interleukin-5

IL-5 is a homodimeric cytokine characterized by an atypically intertwined four-helix bundle structure (Milburn et al., 1993). IL-5 binds with high affinity to the IL-5-specific receptor (IL5RA) expressed on the surface of eosinophils. IL-5 was initially identified as a cytokine produced by T cells and able to promote the terminal differentiation of activated B cells into antibody-secreting plasma cells, at least in murine models (Takatsu et al., 1980). As research advanced, it became clear that IL-5 had a broader role beyond just acting on B cells since studies showed that IL-5 can be produced by Th2 cells which linked IL-5 to type 2 immunity. This shifted the understanding of IL-5 as primarily a Th2 cytokine rather than a pro-B cell factor.

Production of IL-5

In the context of severe asthma, IL-5 is known to be released by Th2 cells in response to allergens and by ILC2s in response to alarmins such as IL-25, IL-33, and TSLP. Th2 cells produce IL-5 mainly following encounter with their cognate allergen. Several studies characterizing memory Th2 cells demonstrated that pathogenic IL-5-

producing memory Th2 subpopulations promote allergic inflammation and chronic skin inflammation (Endo et al., 2011; Islam et al., 2011). Memory Th2 cells are generally subdivided into two subsets; $CD62L^{low}CCR7^{low}$ effector memory type T cells (T_{EM}) located in peripheral tissues, and $CD62L^{hi}CCR7^{hi}$ central memory type T cell (T_{CM}) in lymphoid tissues (Sallusto & Lanzavecchia, 2009). In addition, a substantial portion of memory Th2 cells express chemokine C-X-C motif receptor (CXCR) 3. Upon antigen restimulation, only memory Th2 cells with $CXCR3^{low}CD62L^{low}$ surface expression produced IL-5 and were responsible for the asthmatic phenotype including eosinophilic infiltration into the airways, airway hyperresponsiveness, and mucus hyperproduction (Endo et al., 2014).

ILCs are a relatively recent discovery in the immune system, but our understanding of these lineage-negative cells has advanced rapidly over the past decade. ILCs provide early immune protection against infectious agents, are involved in lymphoid organogenesis and tissue repair, mediate the transition from innate to adaptive immunity, contribute to inflammation and autoimmunity, repair tissue damage and regulate metabolic homeostasis (McKenzie et al., 2014). ILCs are often subdivided into two main categories; namely, killer ILCs and helper-like ILCs. Helper-like ILCs are distinguished from killer ILCs by surface expression of IL-7Rα and encompass various effector cytokine-producing subsets, including IL-13 and IL-5 producing ILC2 cells (Diefenbach et al., 2014). ILC2 cells reside in many different tissues including spleen, liver, lung, intestinal lamina propria, skin, bone marrow and adipose tissue (Brestoff et al., 2015; Mchedlidze et al., 2013; Roediger et al., 2013; Salimi et al., 2013). ILC2 cells produce effector cytokines in response to stimulation by alarmin cytokines. ILC2s produce large amounts of IL-5 when stimulated with PMA/ionomycin *in vitro* (McKenzie et al., 2014; Spooner et al., 2013). In the lungs, ILC2s significantly contribute to the production of IL-5 and IL-13 in experimental asthma. Notably, in ovalbumin (OVA) -induced asthma, the proportion of ILC2s within the total intracellular IL-5 expressing cells in the lung was similar to Th2 cells (Klein Wolterink et al., 2012).

In mice, IL-5 reporter mouse strains have made it possible to identify innate IL-5-producing cells residing in the small and large intestines, peritoneal cavities, and the lung (Ikutani et al., 2012). Of note, $CD4^+$ T cells were not the major producers of IL-5 at steady state as well as under Il-25 and IL-33 promoting conditions. Innate IL-5

producing cells shared key characteristics with innate lymphoid cell types such as surface markers, namely, lineage^{-} c-Kit^{+} Sca-1^{+}, and responsiveness to Il-25 and IL-33. *In vitro* experiments revealed that these innate lymphoid cells were able to produce IL-5, IL-13, or both, suggesting that the production of these cytokines is regulated independently (Ikutani et al., 2012). Innate IL-5 producing cells are more abundant than IL-13 producing cells in lungs and peritoneum of unprimed mice (Price et al., 2010). IL-33, one of the most critical cytokines to activate ILC2s, was more effective at inducing IL-5-producing cells in the lungs than Il-25 which was more potent at stimulating the production of IL-13 (Drake & Kita, 2017; Ikutani et al., 2012). These findings indicate that ILC2s may be the primary source of IL-5.

IL-5 receptor signalling in Eosinophils

IL-5 exerts its biological effects through its interaction with the IL-5 receptor (IL5R), a complex consisting of a specific α subunit (IL5RA) and a common βc subunit, the latter also shared with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Murphy & Young, 2006; Rossjohn et al., 2000). The α chains provide specificity for the respective cytokines but bind with low affinity (0.2–100 nanomolar)(Broughton et al., 2012). IL-5 binds as a homodimer to IL5RA. This intermediate IL-5/IL5RA complex recruits the common β chain (βc) receptor (CD131) into a ternary complex (Patino et al., 2011). In the absence of IL-5, IL5RA is associated with the intracellular tyrosine kinase JAK2, while the βc subunit is linked with JAK1 (Caveney et al., 2024; Kouro & Takatsu, 2009; Ogata et al., 1998). When IL-5 binds to IL5RA, it facilitates the assembly of a functional IL5RA/βc receptor complex and the activation of JAK2, which then phosphorylates specific tyrosine residues on the IL5RA subunit (Konrad Pazdrak et al., 1995). Phosphorylated tyrosine residues serve as docking sites for STAT proteins, particularly STAT5 (Figure 3). These STAT proteins are then phosphorylated, dimerize, and translocate to the nucleus to regulate the transcription of target genes (Caveney et al., 2024; Stout et al., 2004).

The tyrosine kinases Lyn, Syk, Jak2, and Pi3k, are essential for transmitting signals to downstream molecules and were linked to eosinophil survival (Bates et al., 1996; Coffer et al., 1998; K Pazdrak et al., 1995; Yousefi et al., 1996). However, this connection is largely based on studies using Lyn knockout mice, which exhibit

eosinopenia and reduced eosinopoiesis in the bone marrow. (Hibbs et al., 1995). The question whether Lyn kinase's effect on eosinophils is directly related to their survival or perhaps more linked to their production remains unresolved.

Another study synthesized a mutant form of IL-5, IL-5-E12K, which does not activate eosinophils or stimulate cell growth but effectively blocks these functions (McKinnon et al., 1997). Despite having similar binding properties to the IL-5 receptor as the wild type IL-5, IL-5-E12K does not trigger tyrosine phosphorylation in eosinophils and inhibits this process when induced by wild-type IL-5. Interestingly, IL-5-E12K still promotes eosinophil survival, although less effectively than the normal protein. The E12 site appears essential for activating the IL-5 receptor βc chain to signal eosinophil activation. However, since IL-5 (E12K) still supports eosinophil survival, it indicates that other regions of IL-5, beyond E12, are responsible for triggering the survival signalling pathway.

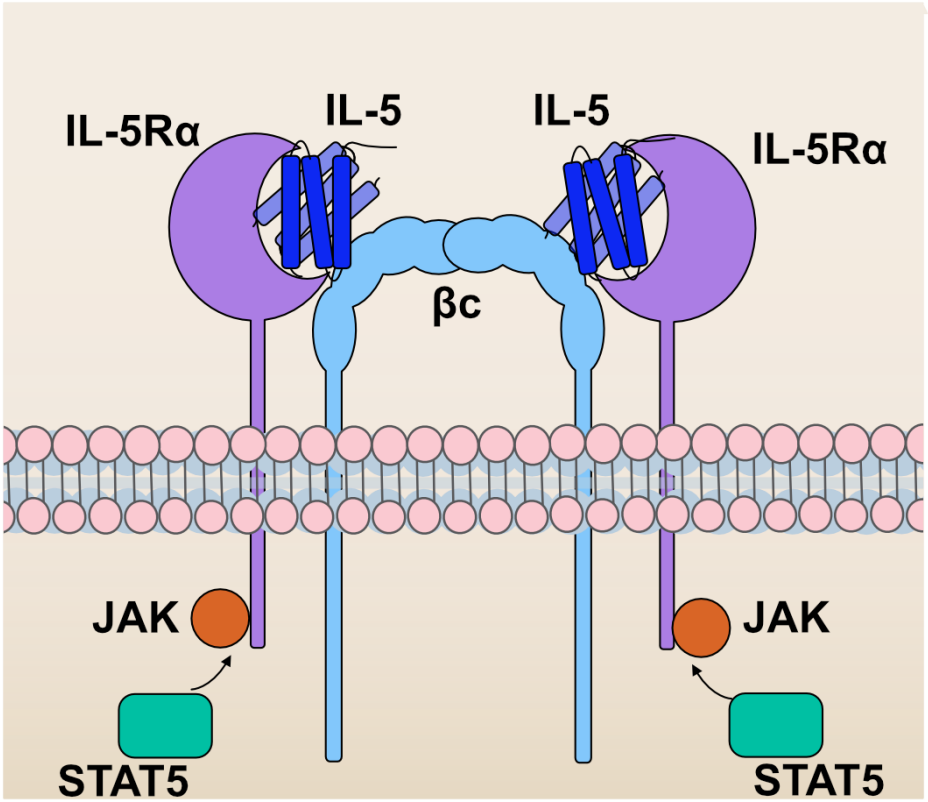

Figure 3. ***IL-5 Signalling Pathway.*** Interaction of IL-5 with the IL-5Rα, and STAT5 phosphorylation through JAK.

Difficulties in interpreting the function of interleukin-5

Despite the efforts in understanding the functions of IL-5 and its relationship with eosinophils, several key questions still remain unanswered. The exact mechanisms by which IL-5 regulates eosinophil numbers in various tissues and diseases are still poorly resolved. How does IL-5 influence eosinophil production in the bone marrow and can IL-5 directly prolong eosinophil survival in the tissues? How does neutralizing IL-5 alleviate eosinophilia in context of eosinophilic diseases? These questions are not easily investigated mainly because eosinophils are particularly difficult cells to manipulate in the laboratory. They are fragile, easily activated, have low content in messenger ribonucleic acid (mRNA) and express large amounts of ribonuclease (RNase) which are not inhibited by commonly available RNase inhibitors. This considerably complicates functional studies of eosinophils.

IL-5 is often linked to eosinophil maturation. A study by Menzies-Gow et al, concluded that mepolizumab induces eosinophil maturational arrest in the bone marrow of treated patients (Menzies-Gow et al., 2003). We argue that this interpretation may be incorrect. The term "maturational arrest" suggests a disruption in a well-defined sequential developmental process with a known origin, which has not yet been fully characterized or thoroughly investigated in eosinophils. To reach their conclusion, the investigators relied on an increase in the relative proportion of immature versus mature eosinophils in the bone marrow of patients receiving mepolizumab. However, one may argue that accelerated maturation in an open system, in which mature cells can exit the bone marrow, could lead to a "left shift" misleadingly appearing as a block of maturation. Relative cell counts (e.g., percentages of total cells) do not provide information about the absolute number of cells. A change in the relative proportion of a cell type might reflect a change in the total cell count, the count of other cell types, or both. In situations where the total cell population changes (as observed in patients treated with mepolizumab), the relative proportions of different cell types can shift in misleading ways.

IL-5 is often described to be crucial for eosinophil proliferation. To our knowledge, there have not been studies that have directly measured proliferation in eosinophils and related their findings to IL-5. Most studies have indirectly linked their findings to proliferation by measuring changes in cell counts. Early experiments involved culturing eosinophils *in vitro* with and without IL-5 and other cytokines

(Clutterbuck et al., 1989). Researchers observed that IL-3 and GM-CSF induced a greater number of eosinophil colonies compared to IL-5, possibly because IL-5 does not impact on eosinophil lineage commitment. When IL-5 was combined with either IL-3 or GM-CSF, it exhibited an additive, rather than a synergistic effect, in promoting eosinophil colony formation. This observation still only suggests that IL-5 may stimulate the proliferation of eosinophil progenitors.

The presence of IL-5 has also been linked to increased eosinophil survival and negatively correlated to apoptosis. Early research suggests that IL-5 maintains eosinophil survival by inhibiting apoptosis, supported by the observation that eosinophils cultured with IL-5 exhibit prolonged viability compared to those without IL-5 (Yamaguchi et al., 1991; Yamaguchi et al., 1988). These studies indicate an association between IL-5 and the inhibition of cell death, but it does not provide direct evidence linking IL-5 to specific anti-apoptotic pathways or proteins. Another study investigated the role of IL-5 in eosinophil apoptosis more specifically in the context of asthma (Xu et al., 2007). This study found a negative correlation between IL-5 levels and eosinophil apoptosis in sputum from asthmatic patients, indicating that IL-5 might prolong eosinophil survival. However, the observation that sputum of some asthmatic patients had generally high levels of apoptotic eosinophils complicates interpretation. It suggests that while IL-5 may inhibit eosinophil death in some cases, other factors may override this effect.

IL-5 has been widely proposed as a key cytokine involved in the activation of eosinophils. The evidence supporting IL-5 as an activation molecule for eosinophils comes from both *in vitro* and *ex vivo* studies. *In vitro* studies co-cultured purified eosinophils with IL-5 and subsequently assessed changes in cell surface markers such as CD18, CD69, and CD11b (Sedgwick et al., 1995). CD18 and CD11b are integral components of the integrin complex known as MAC-1 or complement receptor 3 (CR3). The MAC-1 complex enhances eosinophil adhesion to endothelial cells by binding to intercellular adhesion molecule-1 (ICAM-1) on endothelial surfaces. This is a critical step in eosinophil migration from the bloodstream into tissues, particularly at sites of inflammation. Upregulation of these markers is therefore better related to a function in extravasation rather than activation. In addition, eosinophils already express these markers at steady state, again raising questions about the specific role of IL-5 in "activating" these surface proteins beyond baseline. In humans, anti-IL-5

therapy (mepolizumab) was found to reduce *ex vivo* eosinophil activation (Stein et al., 2008). This claim is made using an eotaxins-induced eosinophil shape change assay. It remains yet unclear whether this assay captures actual activation.

Another interesting observation, giving some insight into IL-5's function, is that blood eosinophil counts of anti-IL-5 mepolizumab-treated patients typically amount to approximately half the normal reference values (Haldar et al., 2009). Similarly, in IL-5 deficient mice, eosinophil levels are significantly reduced but not completely absent. When IL-5 deficient mice are infected with helminths, they fail to develop the characteristic blood and tissue eosinophilia that is typically observed in such infections (M. Kopf et al., 1996). These findings together suggest that while IL-5 is not essential for the initial production of eosinophils, it plays a key role in maintaining normal eosinophil levels and facilitates their expansion during inflammatory responses. Whether eosinophils in IL-5 depleted conditions are functionally or phenotypically similar to eosinophils in IL-5 replete condition is not known.

IL-5 targeting anti-eosinophil biological therapies

Since eosinophils were suspected key drivers in the pathogenesis of severe eosinophilic asthma, several biologic therapies have been developed to specifically target these cells by interfering with the IL-5 "master regulator" pathway. In the early stages of clinical development, researchers did not fully understand the importance of using biomarkers, such as blood or sputum eosinophil levels, to identify which patients would benefit most from anti-IL-5 therapy. As a result, early trials included patients who did not have significant eosinophilic inflammation and thereby diluted the benefits (Flood-Page et al., 2007). Subsequent studies targeting severe eosinophilic asthma demonstrated marked improvements in managing asthma exacerbations with anti-IL-5 biologics (Ortega et al., 2014). These findings, along with observations from eosinophil-depleting biological therapies, have solidified the understanding of eosinophils as a key contributor to the pathogenesis of severe eosinophilic asthma, affirming their role in exacerbating the disease.

Currently, three precision medicines —mepolizumab, reslizumab, and benralizumab— are available as add-on therapies specifically in managing severe eosinophilic asthma. These monoclonal antibodies (mAbs) are designed to disrupt the IL-5 pathway. While all three biologics are approved for treating severe eosinophilic

asthma, they differ in their mechanisms of action, routes of administration, dosing schedules, and have several other clinical considerations (Table 2).

Table 2. *Key Characteristics and Clinical Applications of Mepolizumab, Benralizumab, and Reslizumab*

Characteristic	Mepolizumab	Reslizumab	Benralizumab
Mechanism of action	Humanized IgG1 inhibiting interaction with the IL-5Ra	Humanized afucosylated IgG1 targeting the L-5Rα, inducing ADCC	Humanized IgG4 inhibiting interaction with the IL-5Ra
Route of administration	Subcutaneous injection.	Intravenous infusion	Subcutaneous injection.
Dosing	- Adults and Adolescents (≥12 years): 100 mg every 4 weeks.	- Adults (≥18 years): 3 mg/kg every 4 weeks.	- Initial Doses: 30 mg every 4 weeks for the first 3 doses. - Maintenance: 30 mg every 8 weeks thereafter.
Age group	6 years and older.	18 years and older.	12 years and older.
Efficacy	- Reduces asthma exacerbations. - Improves lung function (FEV1). - Decreases reliance on oral corticosteroids. - Effective in treating EGPA	- Reduces asthma exacerbations. - Improves lung function. - More beneficial in patients with higher baseline eosinophil counts	- Reduces asthma exacerbations. - Rapid and near-complete depletion of blood eosinophils. - Improvement in lung function and asthma control. - Longer dosing interval after initial treatment period.
Onset of action	Improvements observed within weeks	Clinical improvements typically observed after several doses	Rapid eosinophil depletion observed within 24h post-administration.
Adverse effects	- Headache. - Injection site reactions. - Back pain. - Fatigue.	- Oropharyngeal pain. - Increased creatine phosphokinase. - Myalgia. - anaphylactic reactions	- Headache. - Pharyngitis. - Injection site reactions. - hypersensitivity reactions
Other considerations	- Also indicated for EGPA.	- Requires intravenous infusion over 20–50 minutes, necessitating healthcare facility visits. - Weight-based dosing.	- The afucosylated antibody boosts ADCC, enabling rapid eosinophil depletion. - Less frequent maintenance dosing improves compliance.

Mepolizumab targets IL-5 directly, inhibiting its interaction with the IL-5 receptor, leading to a reduction of eosinophil numbers (Figure 4) (Ortega et al., 2014). It is administered via subcutaneous injection every 4 weeks, offering a convenient option for patients. Mepolizumab is approved for patients as young as 6 years old, making it particularly suitable for pediatric cases (Gupta et al., 2019). Additionally, it is uniquely approved for the treatment of eosinophilic granulomatosis with polyangiitis (EGPA), expanding its therapeutic utility beyond severe eosinophilic asthma. While it effectively reduces eosinophil counts, the reduction may occur more gradually compared to benralizumab (FitzGerald et al., 2016; Pavord et al., 2012). Its safety profile is well-established, although patients should still be monitored for injection-related reactions. Mepolizumab is most effective in patients with an eosinophil-driven inflammation endotype, indicated by increased blood eosinophils, adult-onset asthma, and/or nasal polyps .

Reslizumab also targets IL-5 directly, preventing it from binding to the IL-5 receptor, thereby reducing eosinophil numbers (Figure 4) (Castro et al., 2015). Unlike mepolizumab, reslizumab is administered via intravenous (IV) infusion, which requires a clinical setting and takes longer, making it less convenient for some patients. It is approved only for adult patients aged 18 and older. Reslizumab is highly effective in reducing eosinophil counts, but its administration is associated with a higher risk of anaphylactic reactions, necessitating careful monitoring during and after infusions (Manka et al., 2021). The dosing schedule is every 4 weeks, similar to mepolizumab, but its requirement for IV administration may influence the choice based on patient preference and clinical considerations. Reslizumab is particularly beneficial in patients with a pronounced eosinophilic endotype and corticosteroid-dependent asthma (Nair et al., 2020).

Benralizumab differentiates itself by targeting the IL-5 receptor, expressed on the surface of eosinophils, and by leading to the depletion of eosinophils through antibody-dependent cellular cytotoxicity (ADCC) (Figure 4) (Pelaia, Calabrese, Vatrella, et al., 2018). This mechanism allows for rapid and near-complete eosinophil depletion in bone marrow and blood, which can be particularly beneficial in cases requiring swift intervention (Jackson et al., 2020). Benralizumab is administered via subcutaneous injection, with a maintenance dosing schedule of every 8 weeks after initial loading doses, offering greater convenience and potentially better patient

adherence compared to the more frequent dosing required for mepolizumab and reslizumab (Korn et al., 2021). It is approved for patients aged 12 and older. Like other biologics, benralizumab carries a risk of injection-related reactions, but its overall profile is favourable, mostly for patients seeking less frequent treatments. Benralizumab is particularly effective in patients with severe eosinophilic asthma, often with a history of frequent exacerbations, nasal polyps, and adult onset disease (Kavanagh et al., 2021).

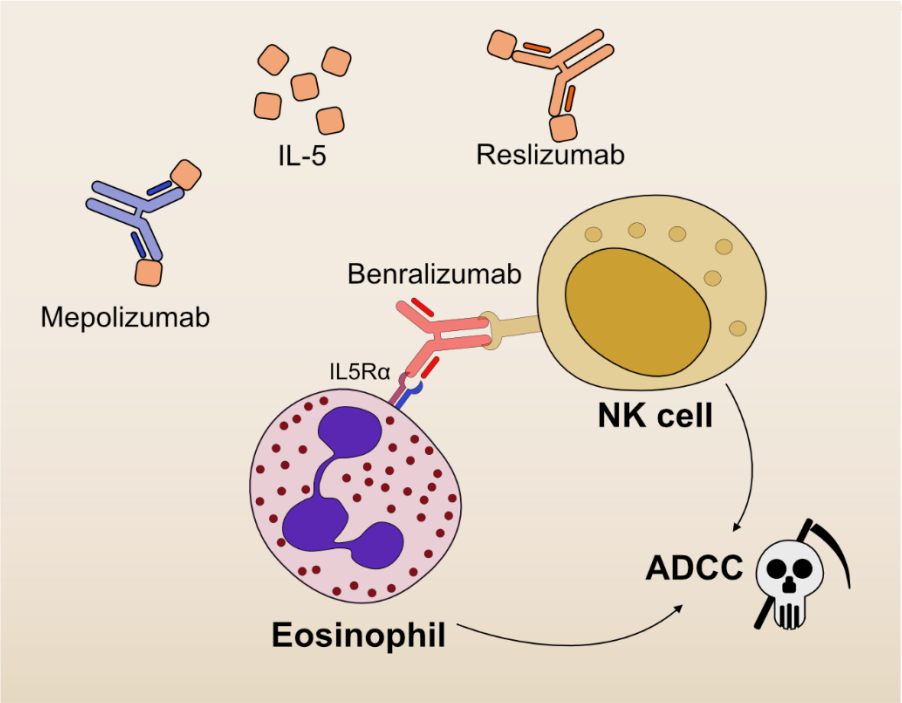

Figure 4. **Mechanism of action of anti-IL-5 biologicals**.

Biological drugs targeting IL-5 or its receptor operate through distinct mechanisms. Mepolizumab and reslizumab bind to IL-5, inhibiting its interaction with eosinophils and preventing its biological effects. Benralizumab, on the other hand, targets the IL-5 receptor (IL-5Rα) through its Fab region, leading to antibody-dependent cellular cytotoxicity (ADCC).

In current clinical practice, its not uncommon that patients are switched between mepolizumab, reslizumab, and benralizumab. These therapy switches are considered in cases of inadequate response, adverse effects, patient preferences, or specific clinical needs. For example, if a patient continues to experience frequent asthma exacerbations or persistent symptoms despite optimal use of one biologic (e.g., mepolizumab), switching to another (e.g., benralizumab or reslizumab) may be considered. The different mechanisms of action and dose regimens might provide better control. If a patient has difficulty with IV infusions (as required for reslizumab) or prefers home administration, switching to a subcutaneous therapy like mepolizumab or benralizumab may be advantageous. A successful switch in biologic therapy often unfortunately still relies on trial-and-error. Interestingly, despite differences in their mechanisms of action, all of these biologic therapies have demonstrated significant efficacy in reducing asthma exacerbations. All 3 currently used anti-eosinophil therapies lead to a significant decrease in circulating eosinophil levels. The significant reduction in blood eosinophils observed with IL-5 neutralization therapies is strongly suggestive of a decreased eosinophil output from the bone marrow.

Objectives

IL-5 was identified as a cytokine central to the development and regulation of eosinophils, and indispensable for eosinophilia. However, its exact mechanisms of action are not fully resolved, despite the widespread use of biological therapies targeting IL-5 or its receptor. This thesis aimed to investigate how IL-5 neutralizing antibodies may lead to decreased eosinophil output from the bone marrow, focusing on whether IL-5 depletion reduces proliferation, causes maturational arrest, or reduces eosinophil commitment.

To address these questions, we first needed to address issues regarding eosinophilopoiesis, as the ontogeny and mechanisms of eosinophil lineage expansion were not sufficiently resolved. Hence, our first objective was to better characterize eosinophilopoiesis in the bone marrow (Figure 5[1]). To circumvent the limitations of single cell RNA-sequencing for eosinophils, we used a proteomic approach by flow cytometry. We screened human and mouse bone marrow progenitors for hundreds of surface markers and combined these markers into a single analysis using the Infinity Flow bioinformatic pipeline. This allowed us to precisely capture human and murine eosinophil development from the first committed eosinophil progenitor stage to fully mature eosinophils.

Leveraging these findings, we investigated the impact of IL-5 on eosinophil maturation stages, progenitor dynamics, and proliferation using IL-5 deficient mice or mice treated with anti-IL-5, at steady state and in the context of eosinophilia, employing murine models of exposure to the alarmin IL-33, the mould *Alternaria alternata*, and the parasitic helminth *Nippostrongylus brasiliensis* (Figure 5[2]).

We further assessed whether IL-5 influenced eosinophil commitment by determining at which point in the eosinophil maturation continuum eosinophils began to express Il5rα, thus becoming responsive to IL-5. This required the development of transgenic Il5ra reporter mice, as commonly available anti-murine CD125 monoclonal antibodies showed high off-target staining of neutrophil lineage cells. Using Il5ra reporter mice, we characterized true Il5ra expression along eosinophil maturation stages (figure 5[3]).

Finally, we assessed whether eosinophils haven intrinsically different biological activities when developing in IL-5-depleted conditions (Figure 5[4]). We compared the

transcriptomes of eosinophils arising in IL-5-depleted or IL-5-replete human or murine hosts, at steady state in vivo, and following ex vivo stimulation with the eosinophil-activating alarmin IL-33.

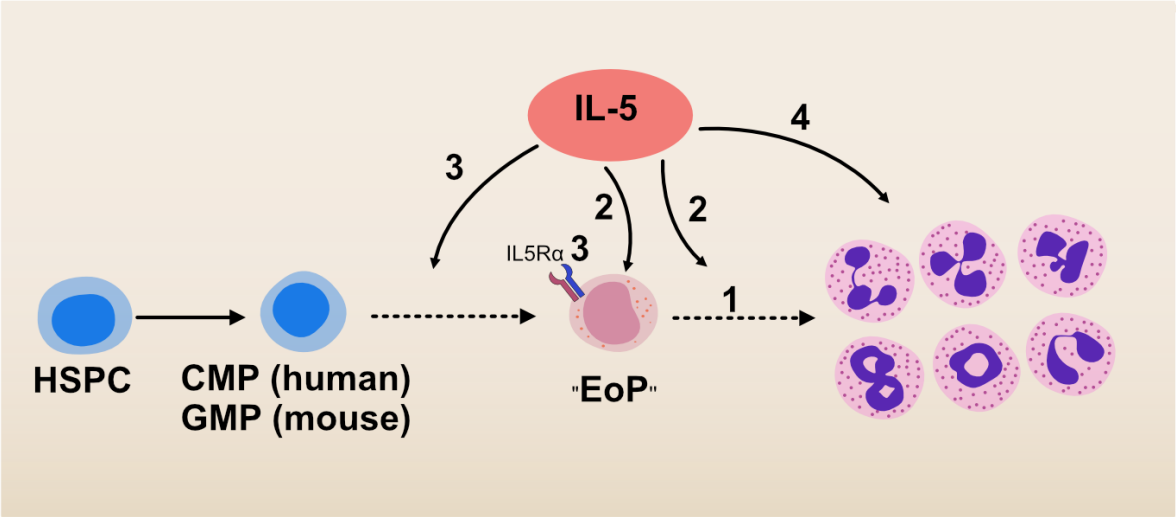

Figure 5. Graphical representation of the objectives investigated in this thesis.

Results

Personal contributions

Both studies presented in this thesis were conducted as part of a collaborative project between Glenn Van Hulst and Joseph Jorssen. 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. Joseph Jorssen 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. Both Joseph Jorssen and Glenn Van Hulst contributed equally to sample preparation for all experiments and to data interpretations.

The results presented below are derived from two key publications in which I served as (co-)first author. The first study examines eosinophil development in the bone marrow and the role of IL-5 in therein. The second study explores the effects of interleukin-5 depletion on residual circulating eosinophils. Both studies are formatted similarly to their original publications with minor adaptations. Additional supplemental tables and gene lists can be found online following the provided doi links of each study.

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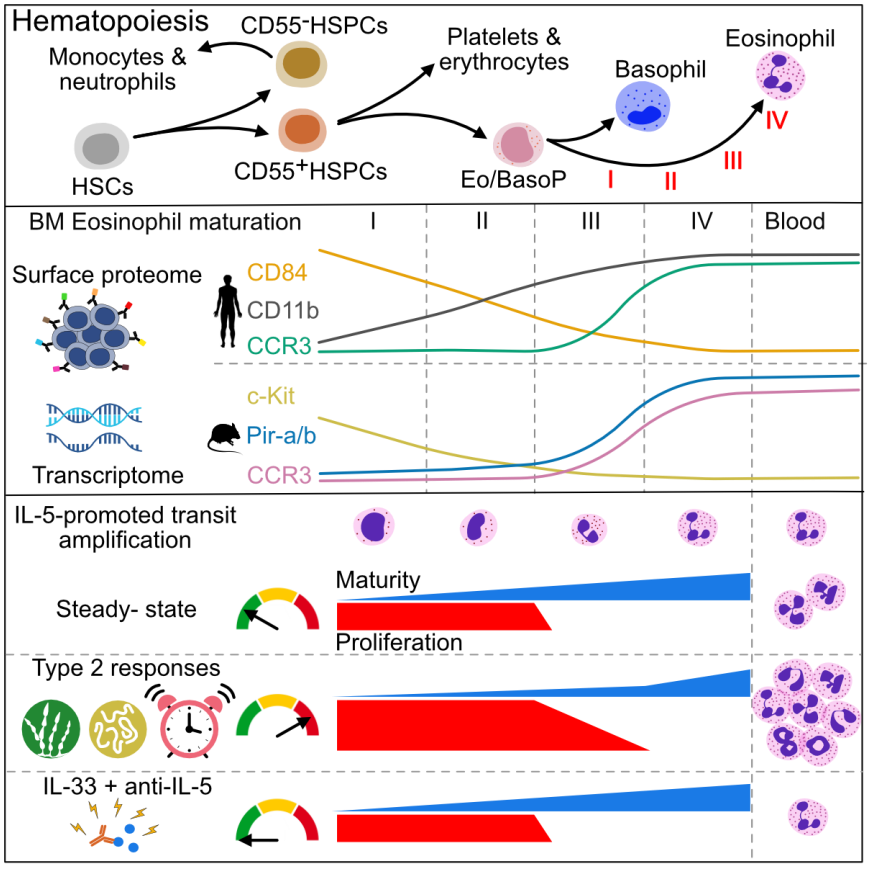
Note that figures in this section are numbered as in the original publication.

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.



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.

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.

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.

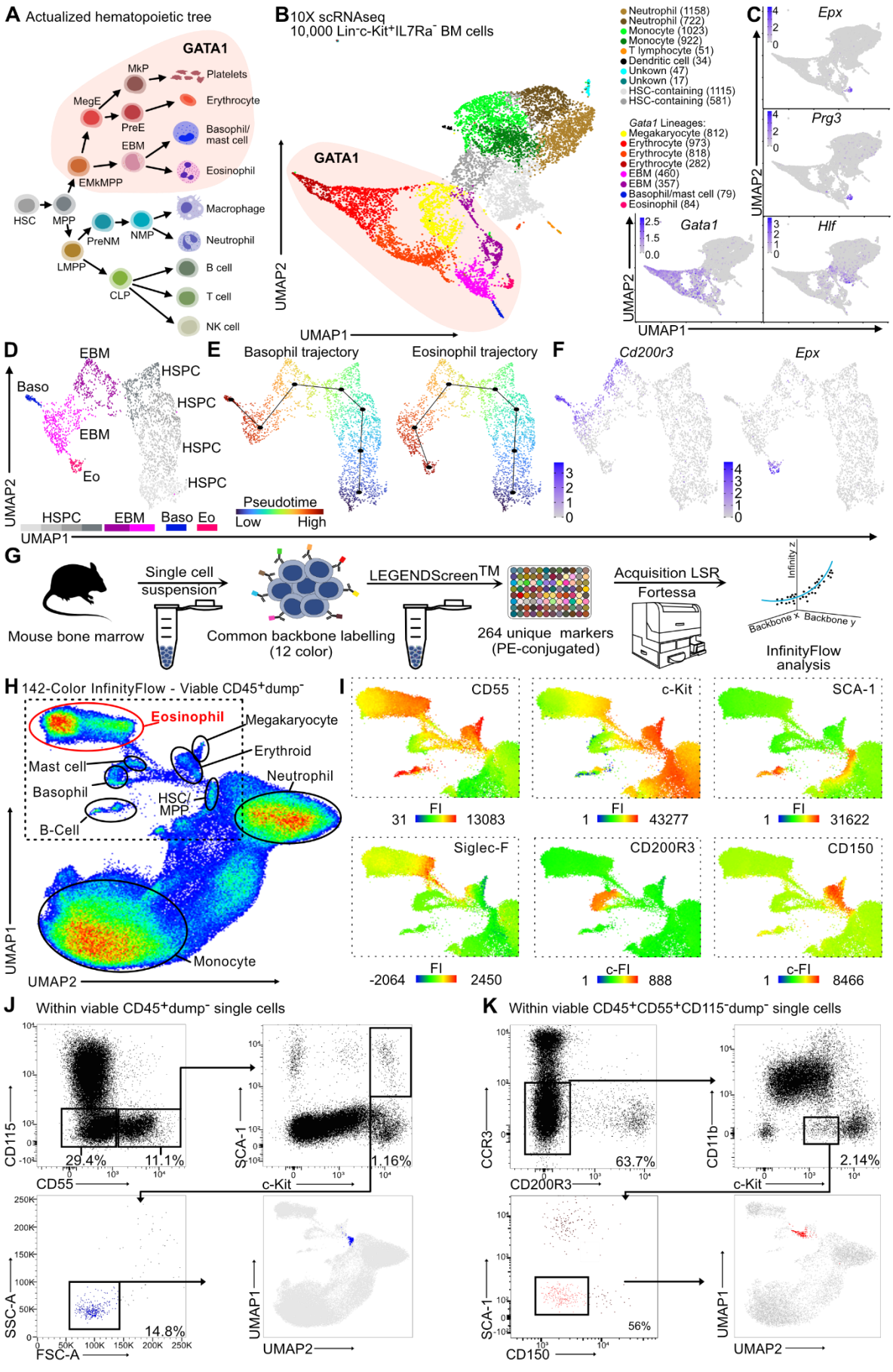

A Actualized hematopoietic tree
GATA1
MkP
Platelets
MegE
Erythrocyte
PreE
Basophil/ mast cell
EBM
Eosinophil
EMkMPP
HSC
MPP
Macrophage
PreNM
NMP
Neutrophil
LMPP
B cell
CLP
T cell
NK cell
B 10X scRNAseq
10,000 Lin-c-Kit+IL7Ra- BM cells
GATA1
UMAP2
UMAP1
Neutrophil (1158)
Neutrophil (722)
Monocyte (1023)
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)

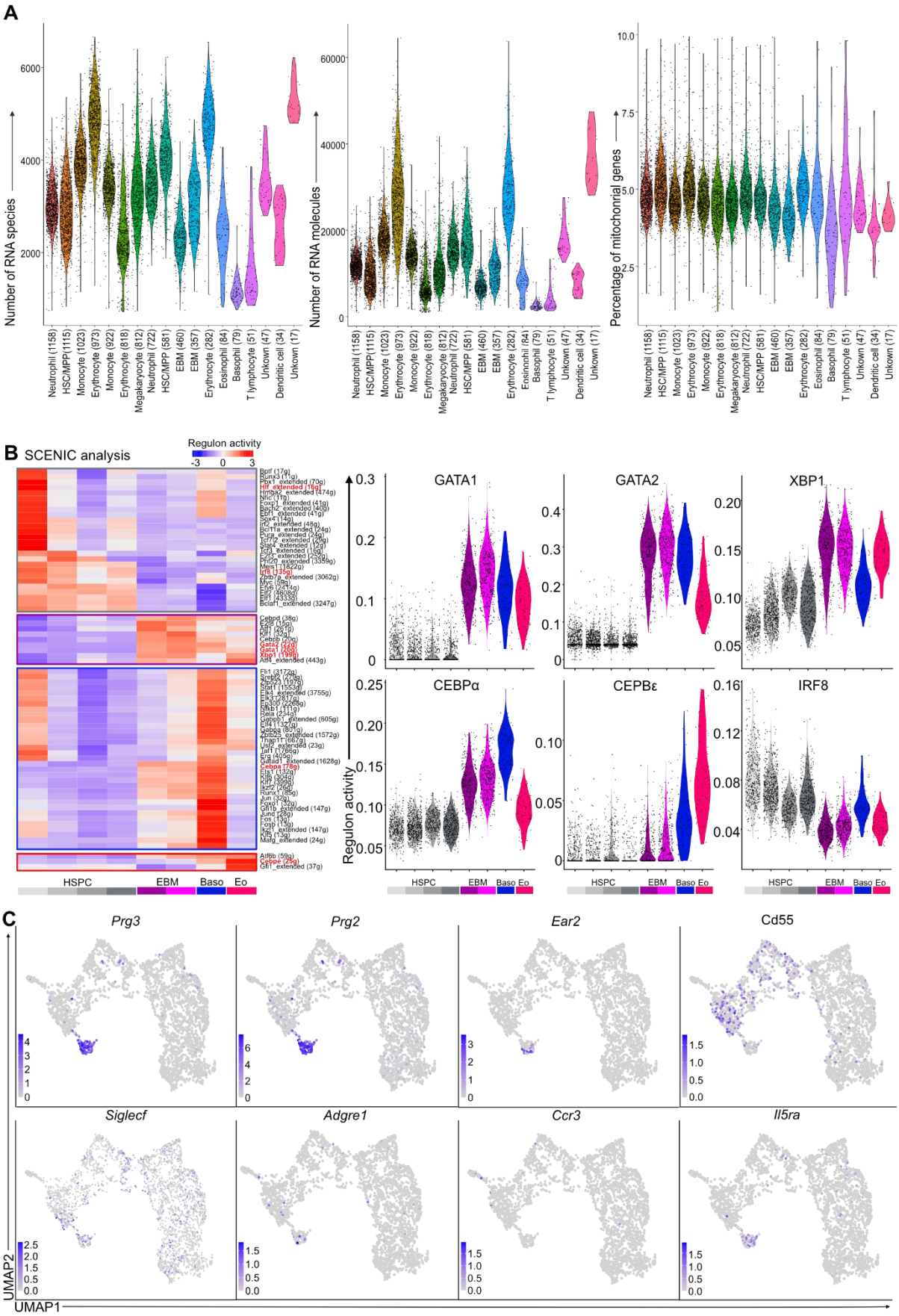

A
Number of RNA species
Number of RNA molecules
Percentage of mitochonrial genes
Neutrophil (1158)
HSC/MPP(1115)
Monocyte (1023)
Erythrocyte (973)
Monocyte (922)
Erythrocyte (818)
Megakaryocyte (812)
Neutrophil (722)
HSC/MPP (581)
EBM (460)
EBM (357)
Erythrocyte (282)
Eosinophil (84)
Basophil (79)
T 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].

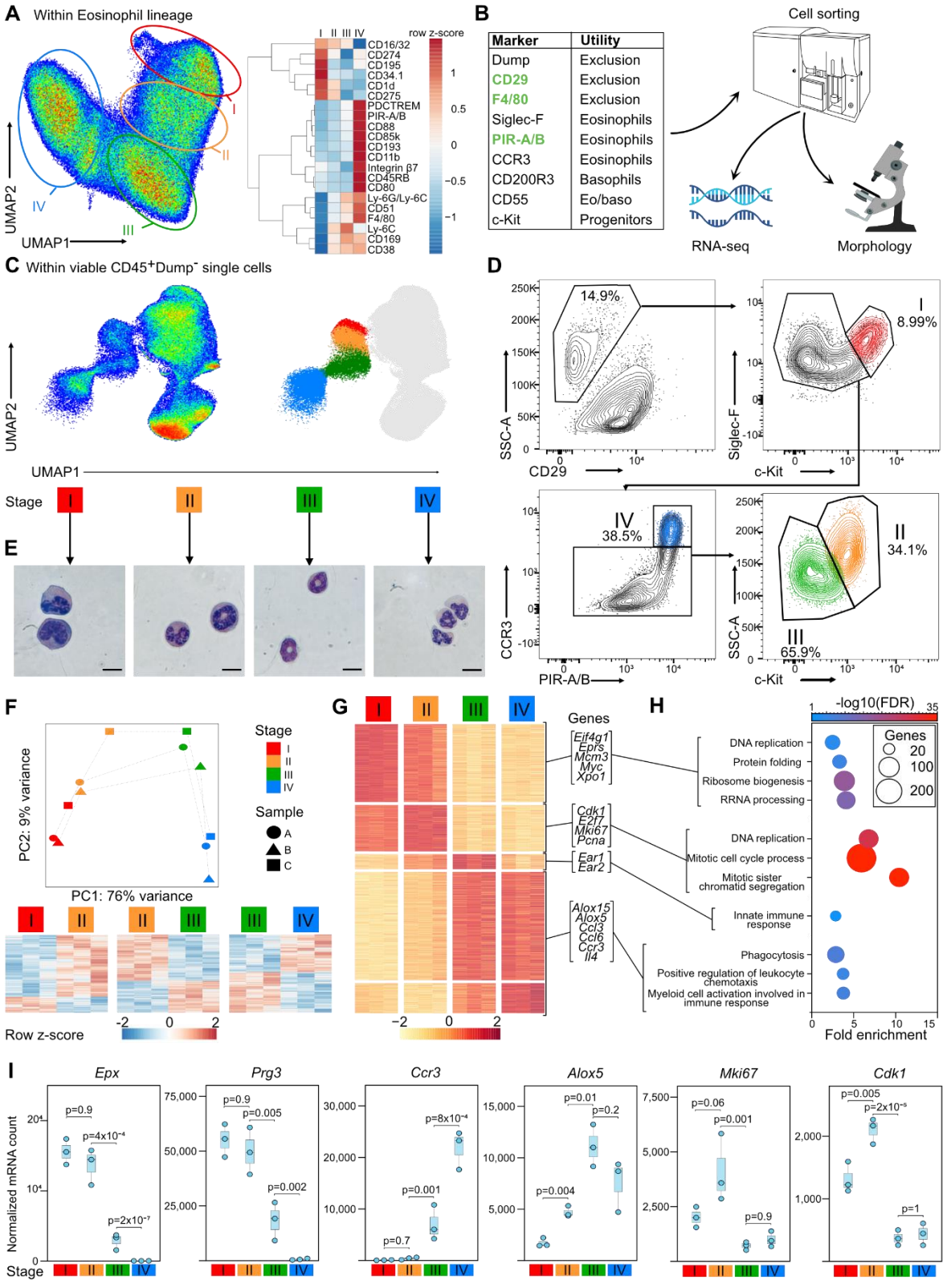

A
Within Eosinophil lineage
UMAP2
UMAP1
I
II
III
IV
I II III IV
row z-score
CD16/32
CD274
CD195
CD34.1
CD1d
CD275
PDCTREM
PIR-A/B
CD88
CD85k
CD193
CD11b
Integrin β7
CD45RB
CD80
Ly-6G/Ly-6C
CD51
F4/80
Ly-6C
CD169
CD38
B
Marker
Utility
Dump
Exclusion
CD29
Exclusion
F4/80
Exclusion
Siglec-F
Eosinophils
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
E2f1
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
p=0.9
p=4x10-4
p=2x10-7
p=0.9
p=0.005
p=0.002
p=0.7
p=0.001
p=8x10-4
p=0.004
p=0.2
p=0.01
p=0.9
p=0.001
p=0.06
p=1
p=2x10-5
p=0.005

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).

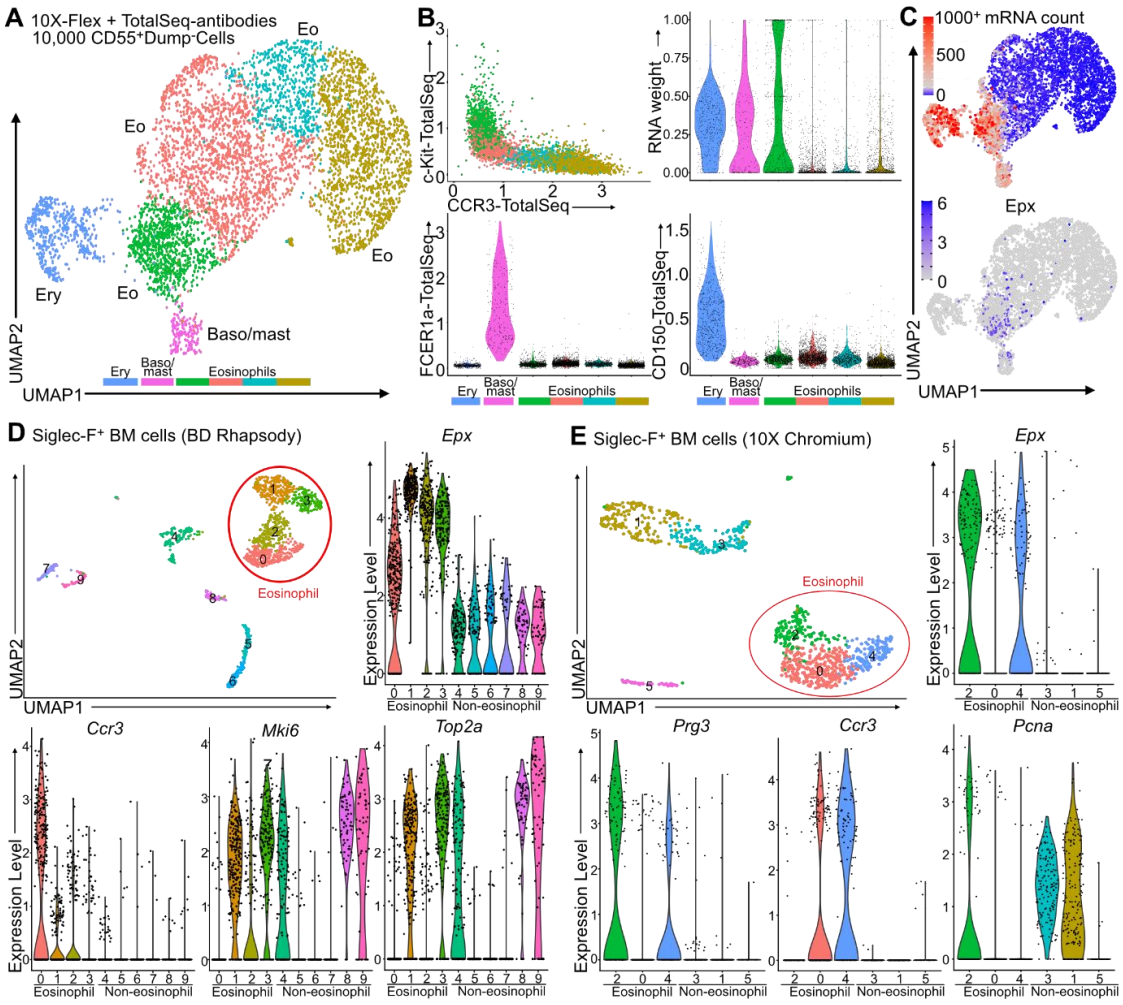


Figure S2. Single-cell RNA sequencing analysis of murine bone marrow progenitors using different platforms.

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.

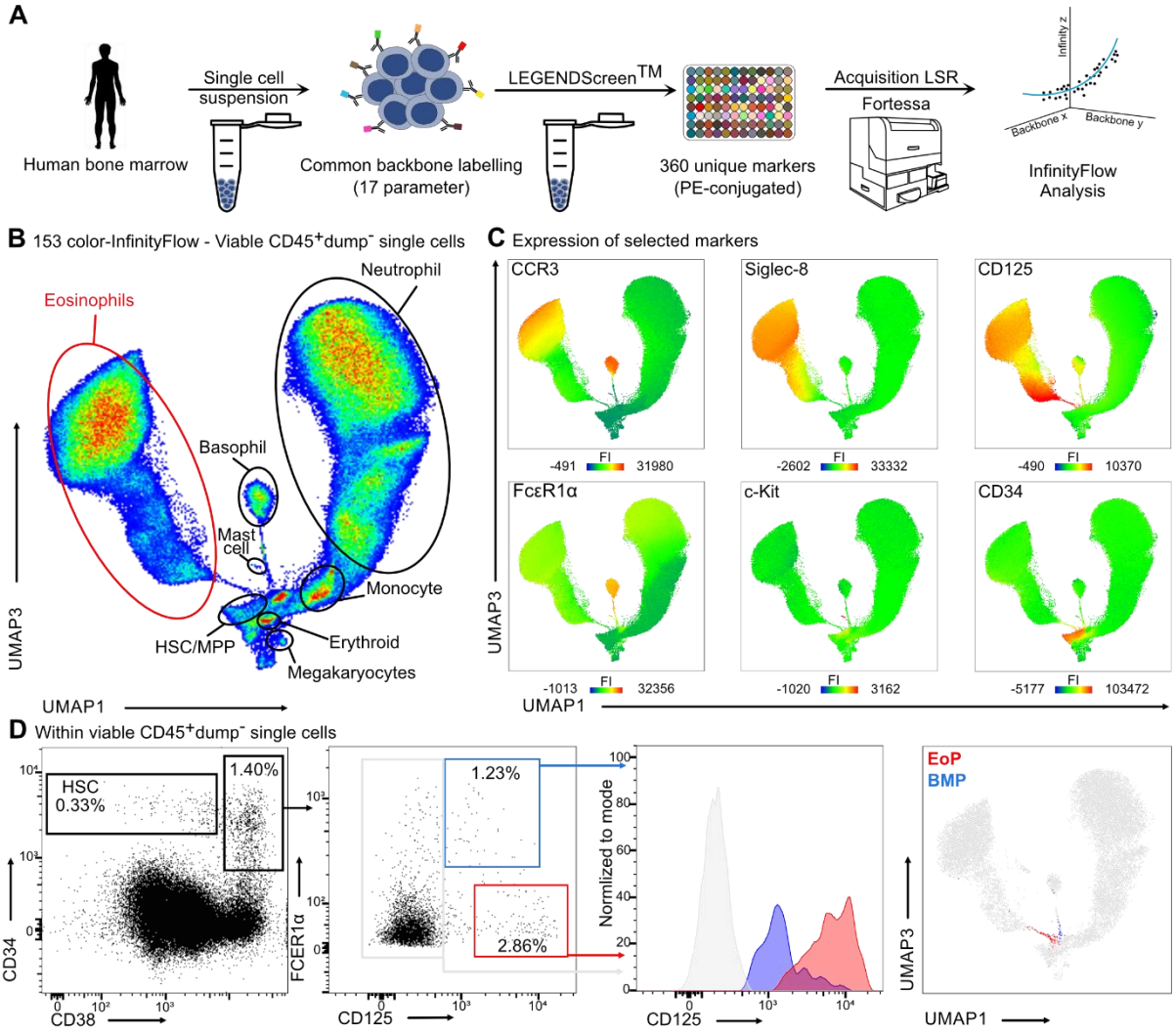


Figure 3. Resolution of human eosinophil ontogeny by high dimension flow cytometric screening.

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)

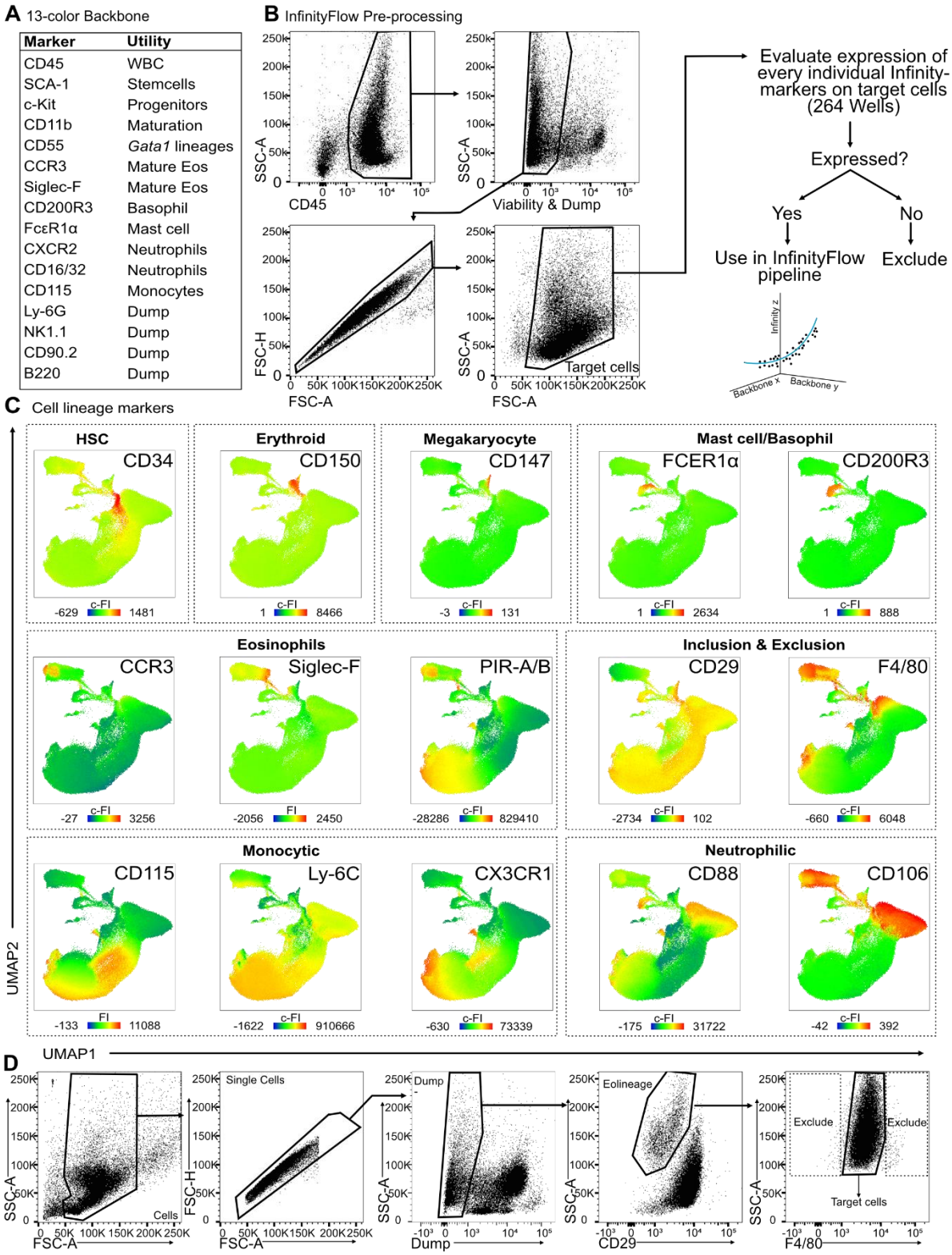
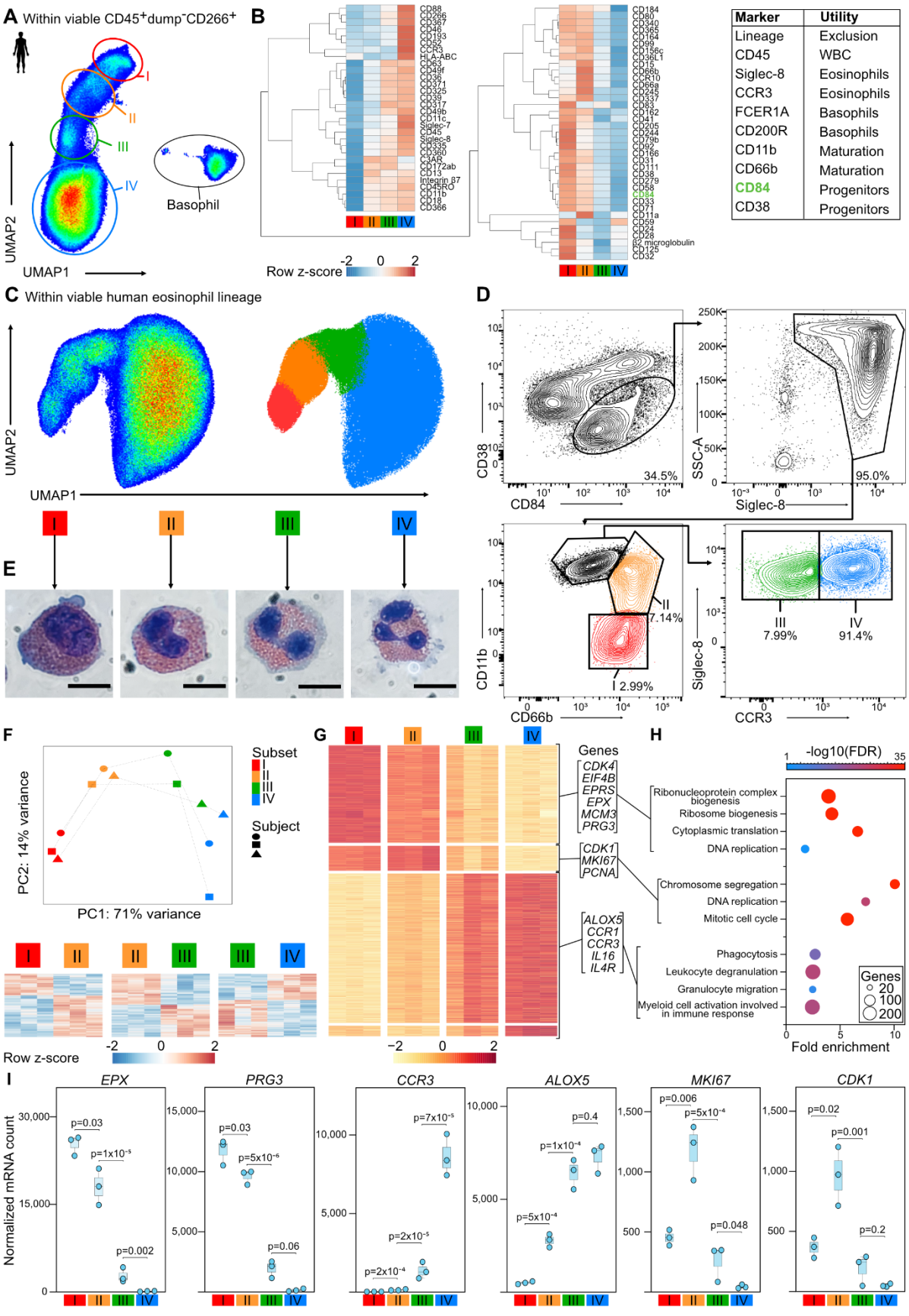

A 13-color Backbone
Marker Utility
CD45 WBC
SCA-1 Stemcells
c-Kit Progenitors
CD11b Maturation
CD55 Gata1 lineages
CCR3 Mature Eos
Siglec-F Mature Eos
CD200R3 Basophil
FcεR1α Mast cell
CXCR2 Neutrophils
CD16/32 Neutrophils
CD115 Monocytes
Ly-6G Dump
NK1.1 Dump
CD90.2 Dump
B220 Dump
B InfinityFlow Pre-processing
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
Erythroid
CD150
Megakaryocyte
CD147
Mast cell/Basophil
FCER1α
CD200R3
Eosinophils
CCR3
Siglec-F
PIR-A/B
Inclusion & Exclusion
CD29
F4/80
Monocytic
CD115
Ly-6C
CX3CR1
Neutrophilic
CD88
CD106
c-FI
FI
UMAP2
UMAP1
D
Cells
Single Cells
Dump
Eolineage
CD29
F4/80
Exclude
Exclude
Target cells

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).



Marker	Utility
Lineage	Exclusion
CD45	WBC
Siglec-8	Eosinophils
CCR3	Eosinophils
FCER1A	Basophils
CD200R	Basophils
CD11b	Maturation
CD66b	Maturation
CD84	Progenitors
CD38	Progenitors

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).

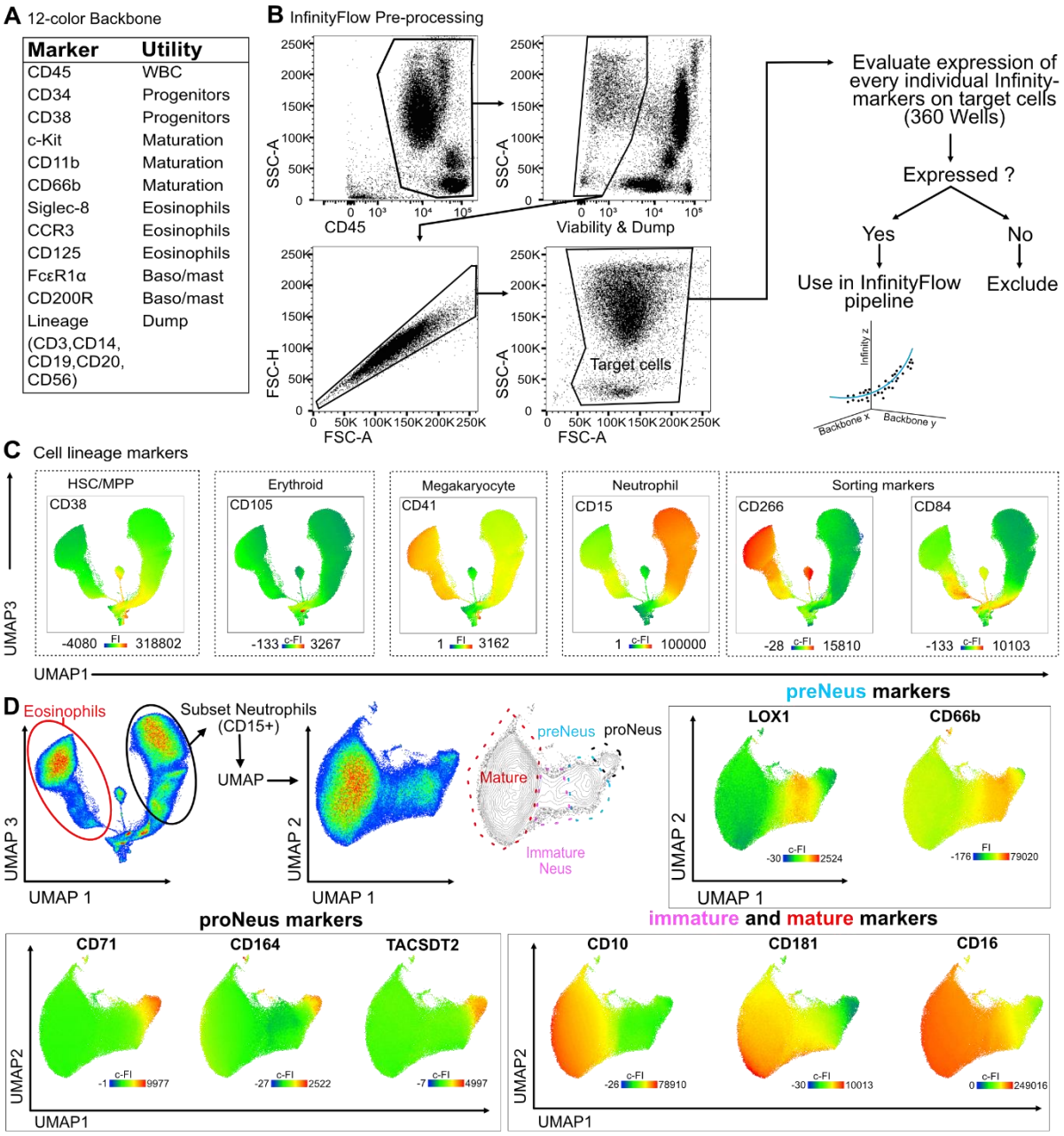

A 12-color Backbone
Marker Utility
CD45 WBC
CD34 Progenitors
CD38 Progenitors
c-Kit Maturation
CD11b Maturation
CD66b Maturation
Siglec-8 Eosinophils
CCR3 Eosinophils
CD125 Eosinophils
FcεR1α Baso/mast
CD200R Baso/mast
Lineage (CD3,CD14, CD19,CD20, CD56) Dump
B InfinityFlow Pre-processing
SSC-A
CD45
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 3
UMAP 1
UMAP 2
preNeus
proNeus
Mature
Immature Neus
preNeus markers
LOX1
CD66b
proNeus markers
CD71
CD164
TACSDT2
immature and mature markers
CD10
CD181
CD16
UMAP2

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 $Ccr3^{+}$ $Siglec\text{-}F^{+}$ 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.

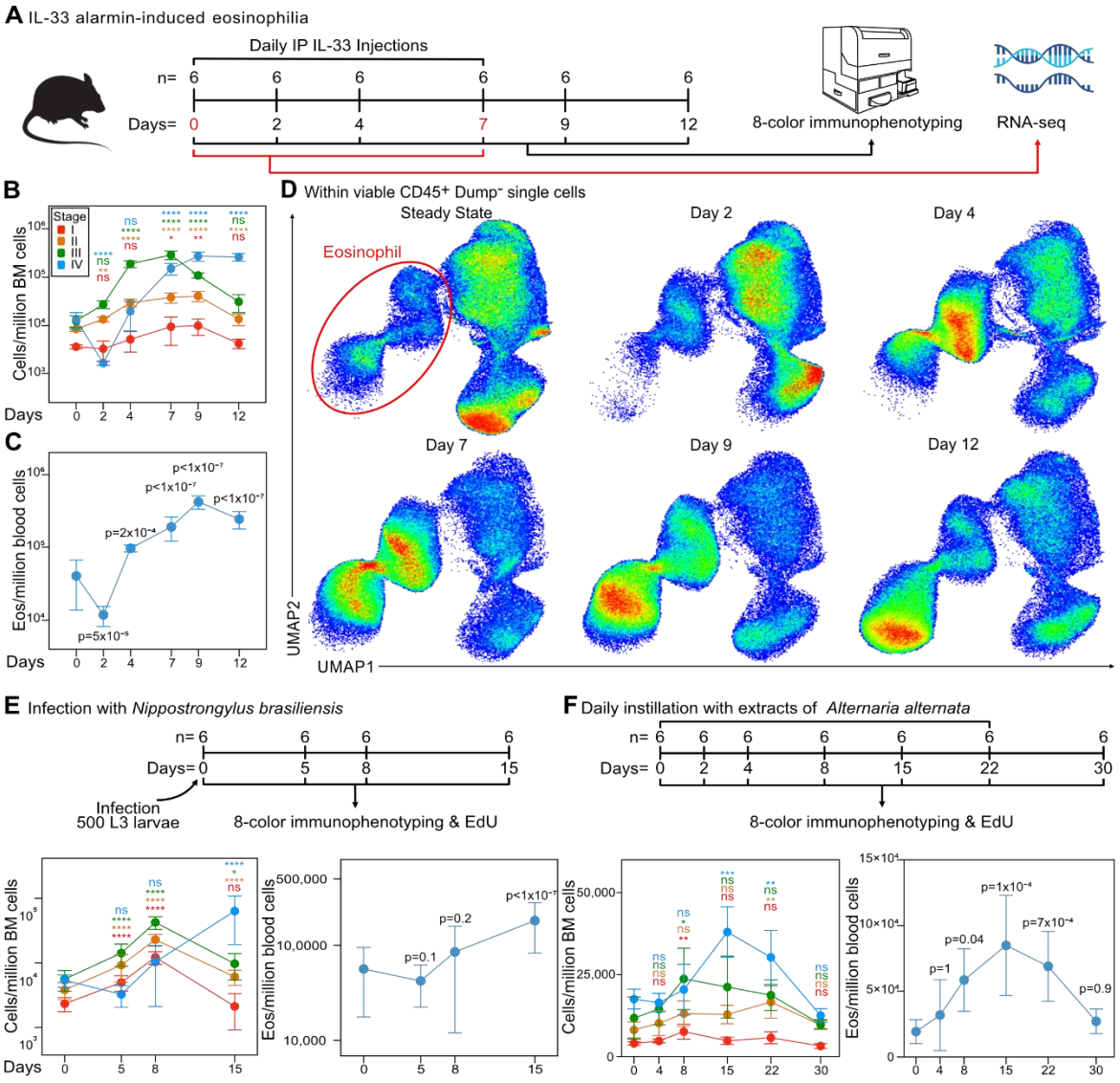


Figure 5. Eosinophil lineage amplification in models of eosinophilic conditions.

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

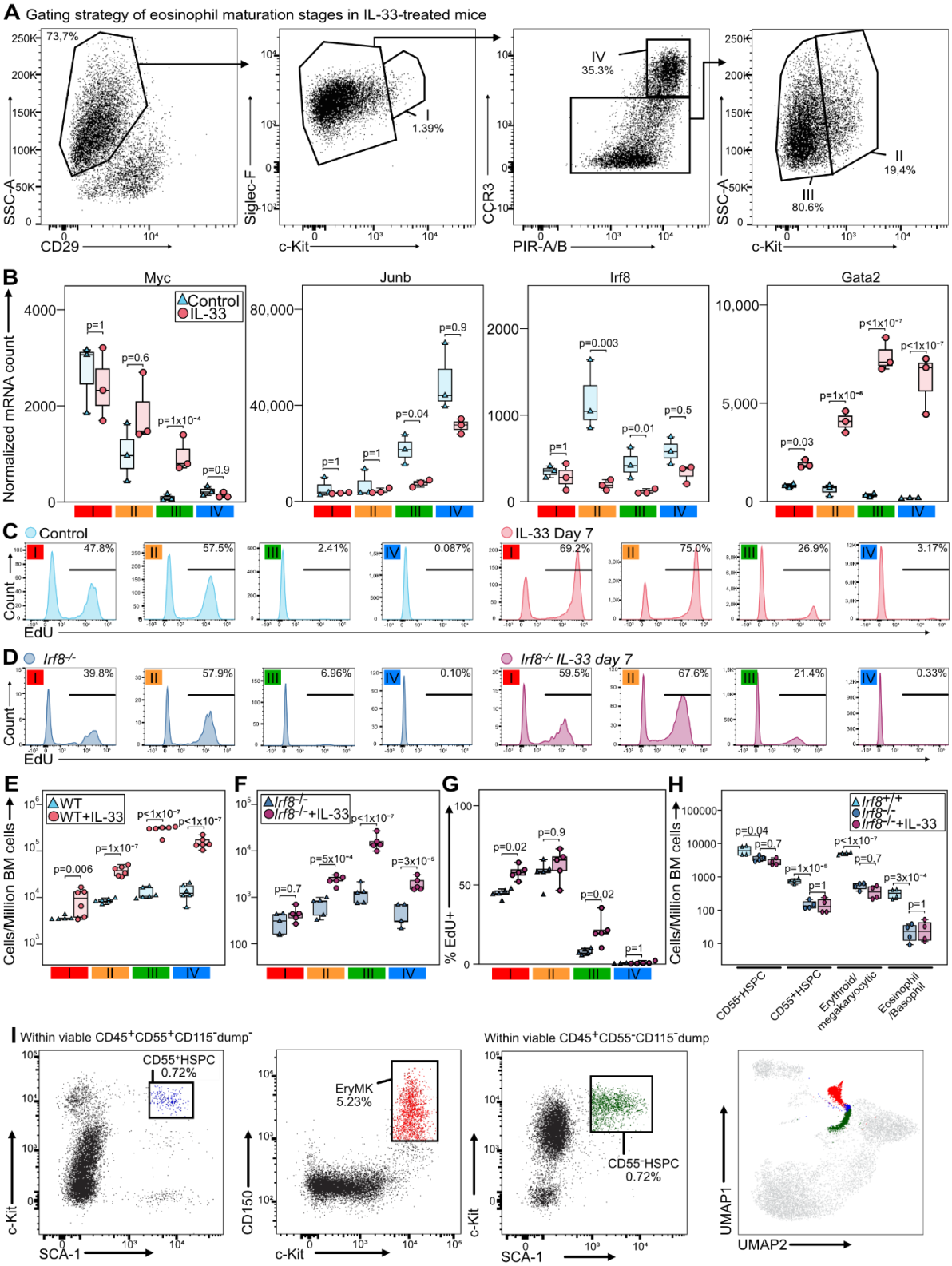

A Gating strategy of eosinophil maturation stages in IL-33-treated mice
73,7%
SSC-A
CD29
Siglec-F
c-Kit
I
1.39%
CCR3
PIR-A/B
IV
35.3%
II
19,4%
III
80.6%
B
Myc
Junb
Irf8
Gata2
Normalized mRNA count
Control
IL-33
C
Control
IL-33 Day 7
Count
EdU
47.8%
57.5%
2.41%
0.087%
69.2%
75.0%
26.9%
3.17%
D
Irf8-/-
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)

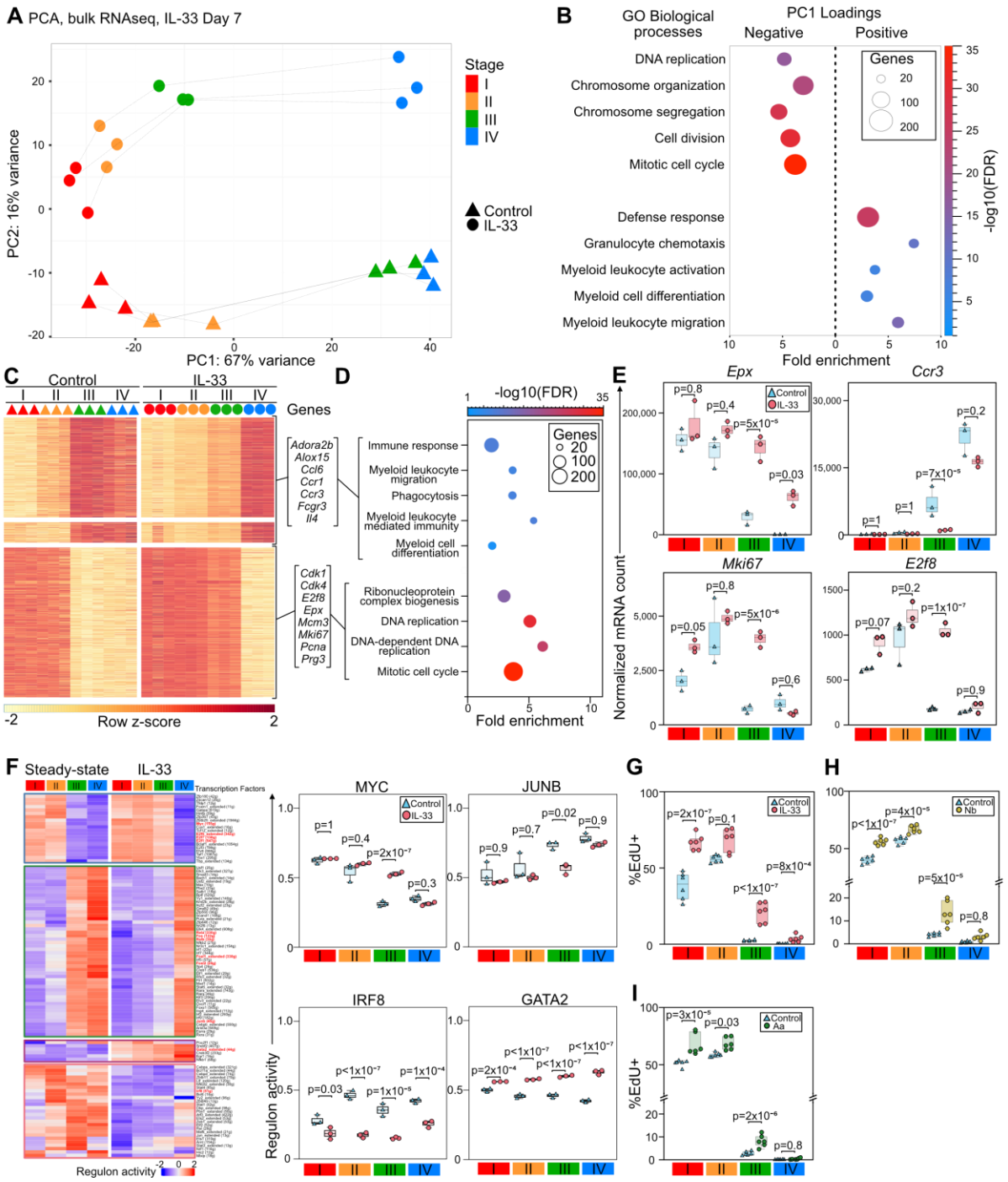

A PCA, bulk RNAseq, IL-33 Day 7
PC2: 16% variance
PC1: 67% variance
Stage
I
II
III
IV
Control
IL-33
B
GO Biological processes
PC1 Loadings
Negative
Positive
DNA replication
Chromosome organization
Chromosome segregation
Cell division
Mitotic cell cycle
Defense response
Granulocyte chemotaxis
Myeloid leukocyte activation
Myeloid cell differentiation
Myeloid leukocyte migration
Genes
-log10(FDR)
Fold enrichment
C
Control
IL-33
Genes
Row z-score
Adora2b
Alox15
Ccl6
Ccr1
Ccr3
Fcgr3
Il4
Cdk1
Cdk4
E2f8
Epx
Mcm3
Mki67
Pcna
Prg3
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
D
-log10(FDR)
Genes
Fold enrichment
E
Epx
Ccr3
Mki67
E2f8
Normalized mRNA count
Control
IL-33
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.

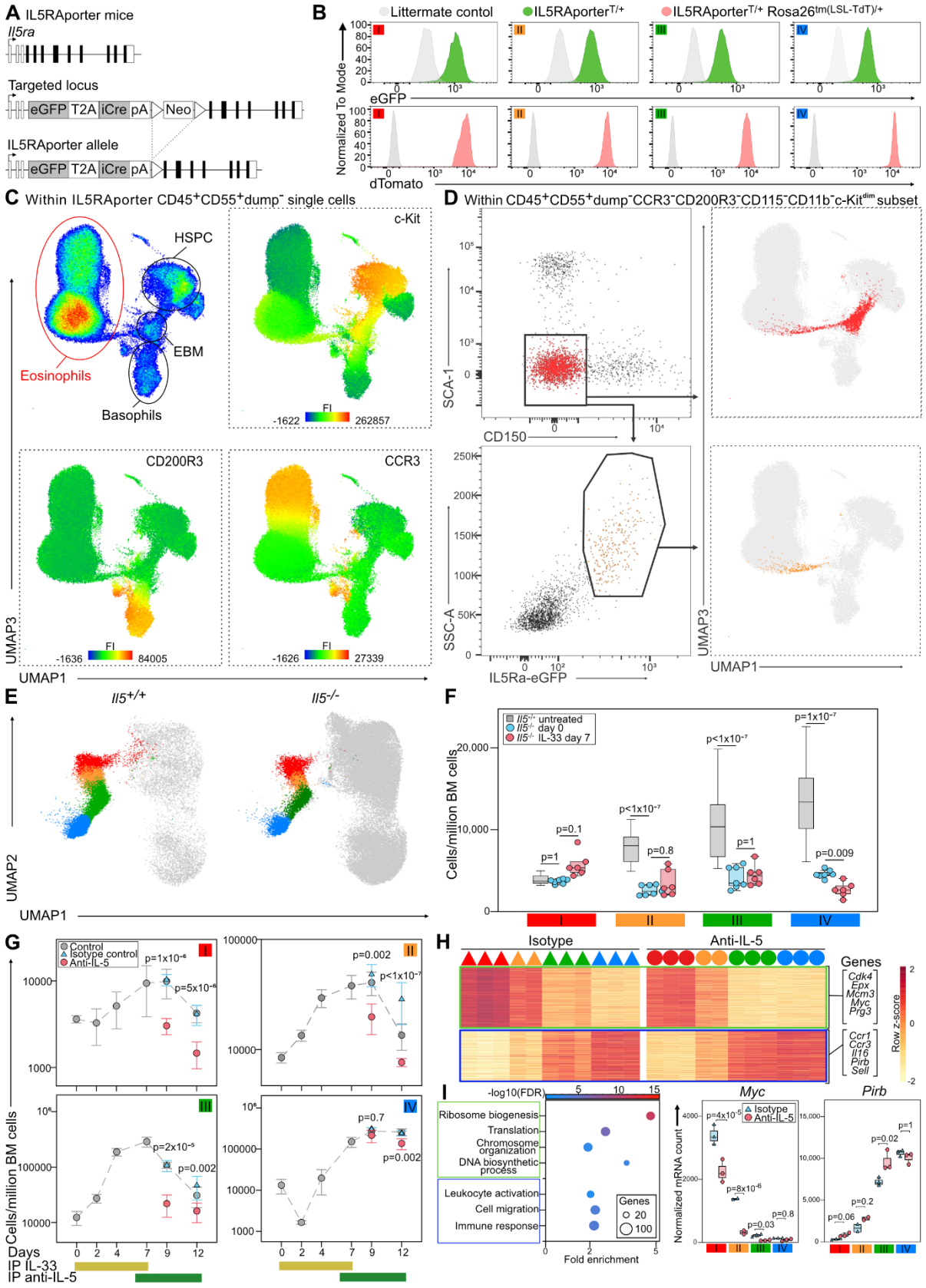

A IL5RAporter mice
Il5ra
Targeted locus
eGFP T2A iCre pA
Neo
IL5RAporter allele
B
Littermate contol
IL5RAporterT/+
IL5RAporterT/+ Rosa26tm(LSL-TdT)/+
Normalized To Mode
eGFP
dTomato
C Within IL5RAporter CD45+CD55+dump- single cells
HSPC
EBM
Eosinophils
Basophils
c-Kit
CD200R3
CCR3
UMAP3
UMAP1
D Within 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
Genes
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).

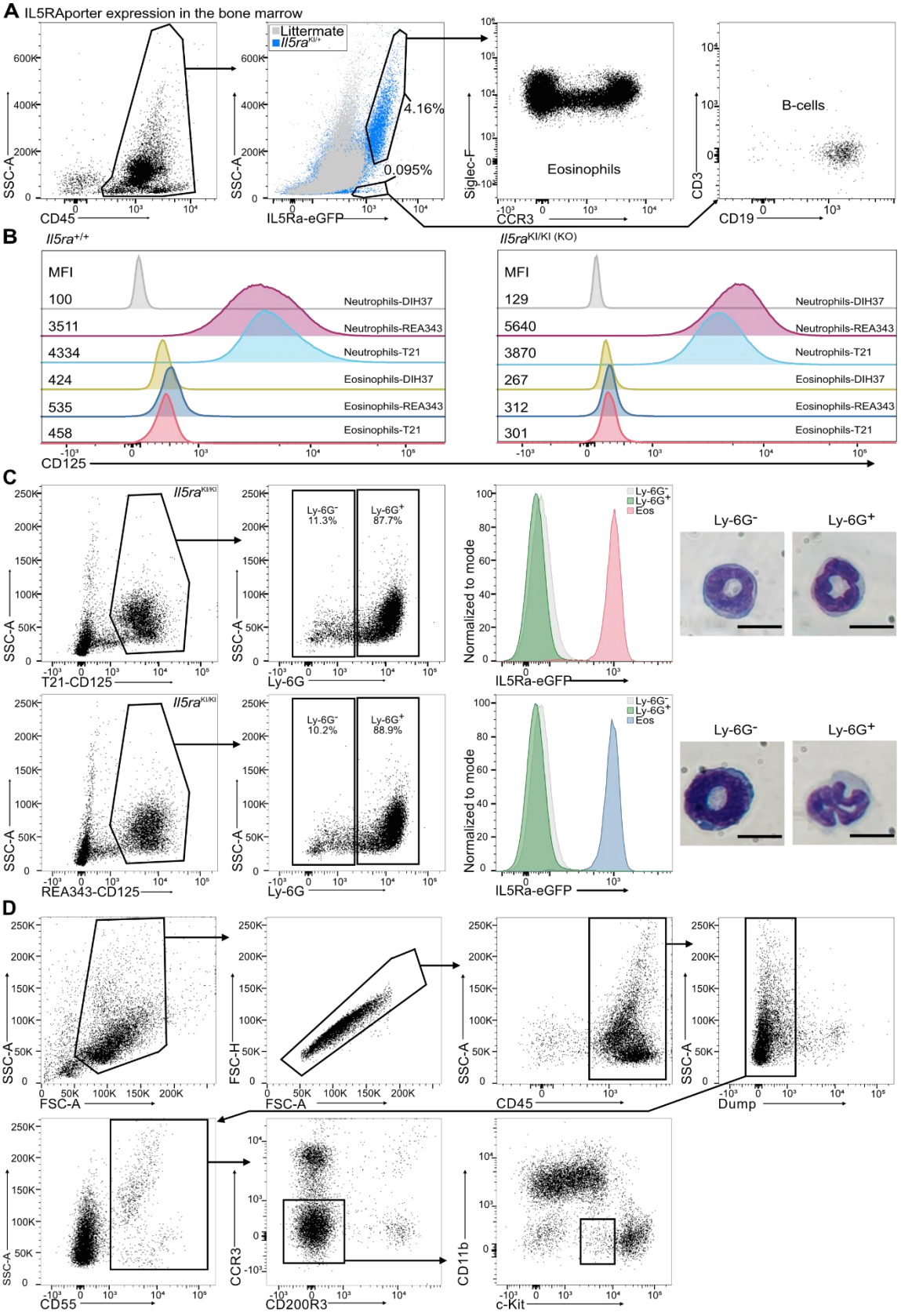

A IL5RAporter expression in the bone marrow
Littermate
Il5ra KI/+
4.16%
0.095%
SSC-A
CD45
IL5Ra-eGFP
Siglec-F
CCR3
Eosinophils
CD3
CD19
B-cells
B
Il5ra+/+
Il5raKI/KI (KO)
MFI
100
Neutrophils-DIH37
3511
Neutrophils-REA343
4334
Neutrophils-T21
424
Eosinophils-DIH37
535
Eosinophils-REA343
458
Eosinophils-T21
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
Eos
REA343-CD125
10.2%
88.9%
D
FSC-A
FSC-H
Dump
CD55
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).

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.

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Additional figure Study 1

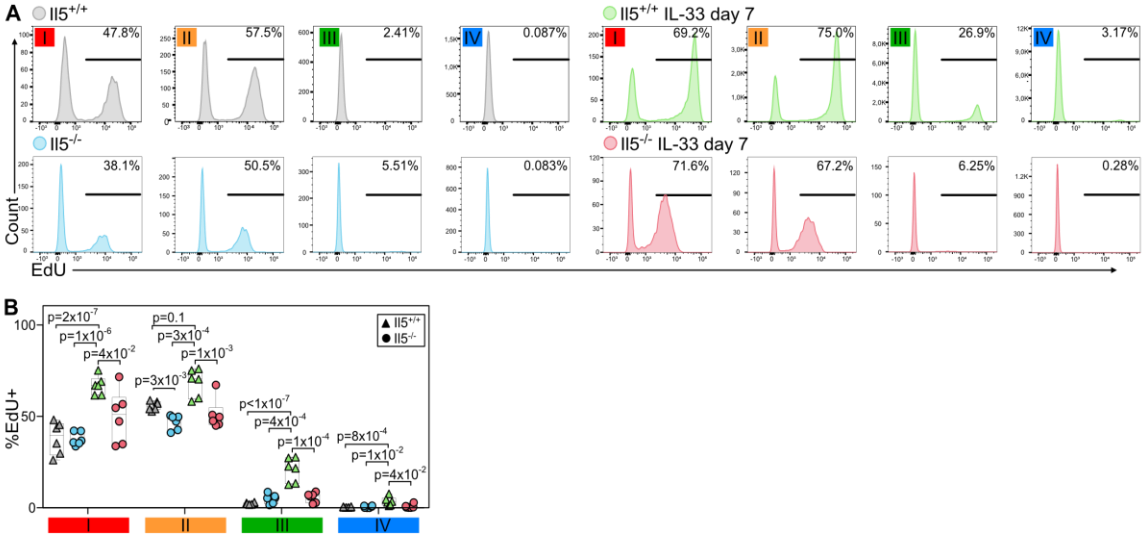

Figure AS1. Eosinophil progenitors maintain proliferative capacity independent of IL-5

A Representative flow cytometric histograms of 5-ethynyl-2'-deoxyuridine (EdU) incorporation after a 1h pulse in stage I-IV progenitors of $Il5^{+/+}$ and $Il5^{-/-}$ mice, at steady state and following 7 day intra peritoneal injection with recombinant IL-33. **B** Comparison of percentage proliferating cells within stage I-IV in $Il5^{+/+}$- and $Il5^{-/-}$ mice at steady state or stimulated with recombinant IL-33.

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

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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.

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.

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 ⩾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.

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.

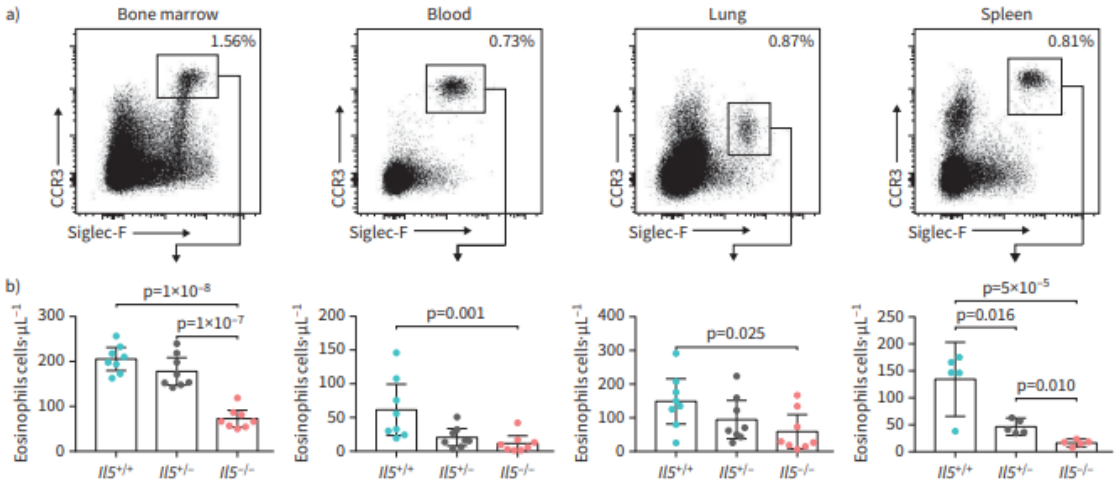

Figure 1. IL-5 deficient mice retain residual eosinophils. a) Representative plots of flow cytometric gating strategy with percentage of eosinophils in indicated organs. **b)** Quantification of eosinophils in specified organs of Il5$^{+/+}$, Il5$^{+/-}$ and Il5$^{-/-}$ mice as in a). Data were pooled 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.

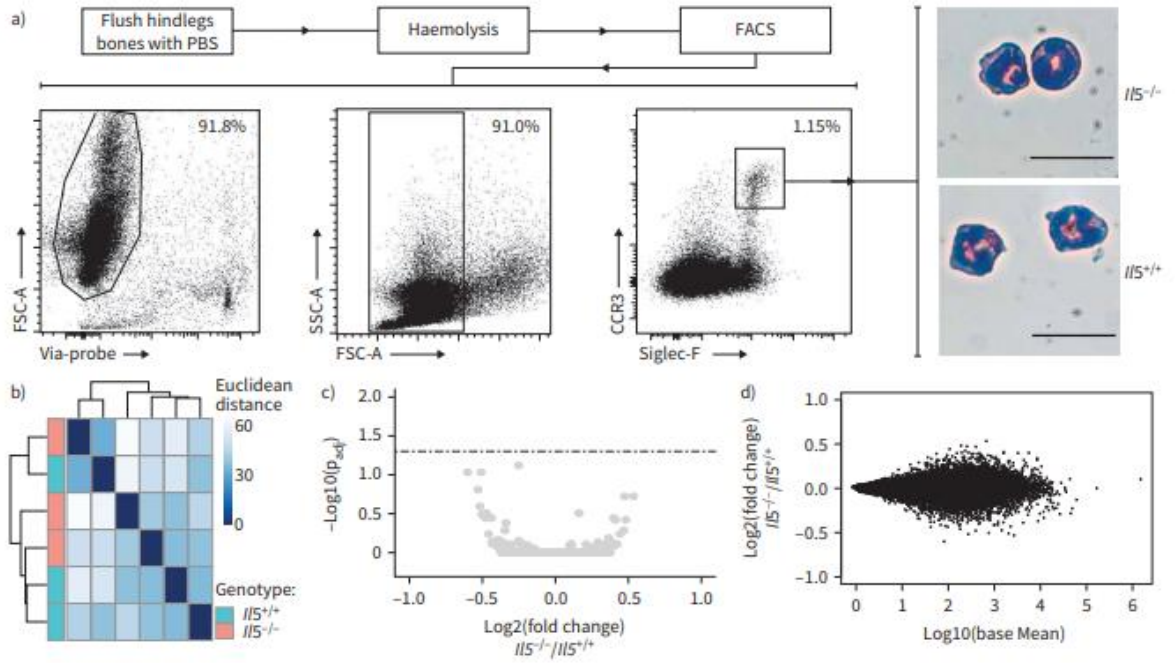

Figure 2. Genetic deficiency in *IL-5* has no detectable impact on mouse residual eosinophils. a) Isolation strategy of IL-5$^{+/+}$ and IL-5$^{-/-}$ mouse bone marrow eosinophils and representative post-sort light microscopy picture. Scale bars=15µm. **b)** Sample clustering, **c)** volcano plot and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

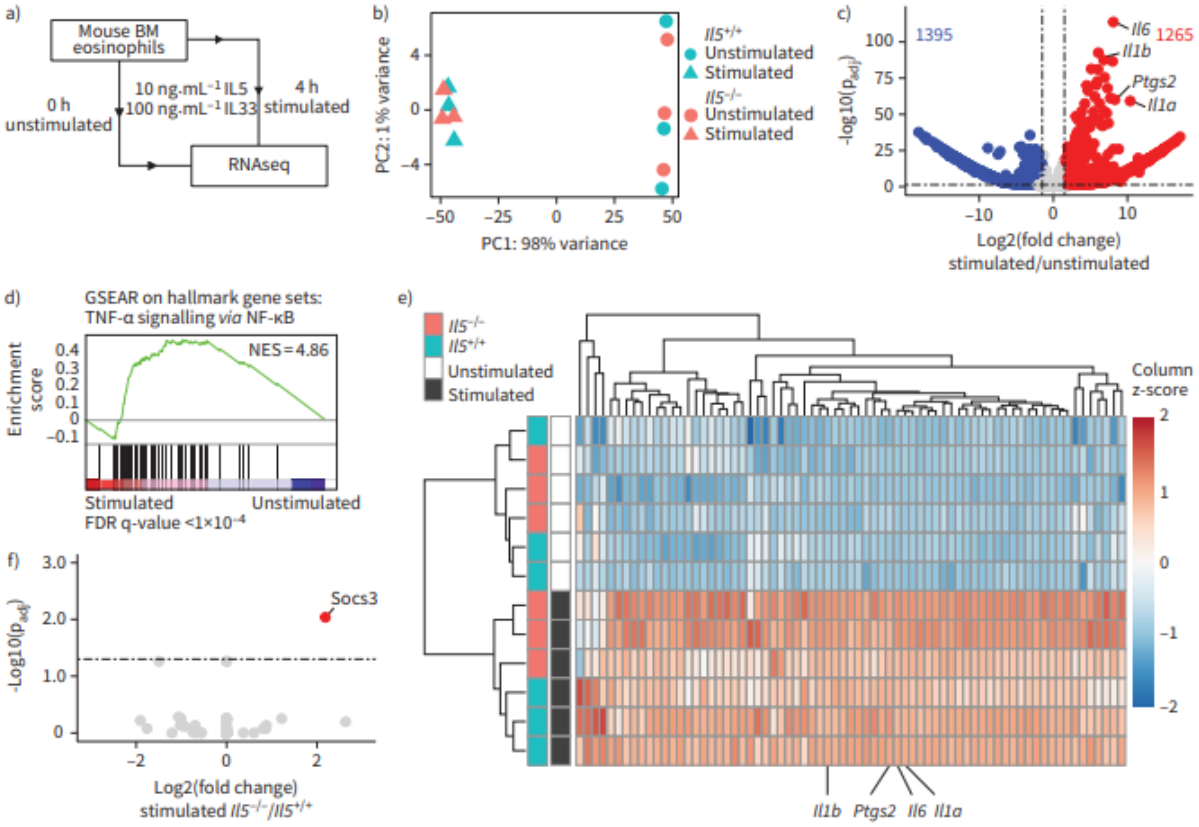

Figure 3. Response to stimulation of IL-5$^{+/+}$ and IL-5$^{-/-}$ bone marrow (BM) eosinophils. a) Experimental outline; **b)** principal component (PC) analysis; **c)** changes in gene expression of IL-5$^{+/+}$ and IL-5$^{-/-}$ eosinophils in response to stimulation; **d)** ranked gene set enrichment 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.

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.

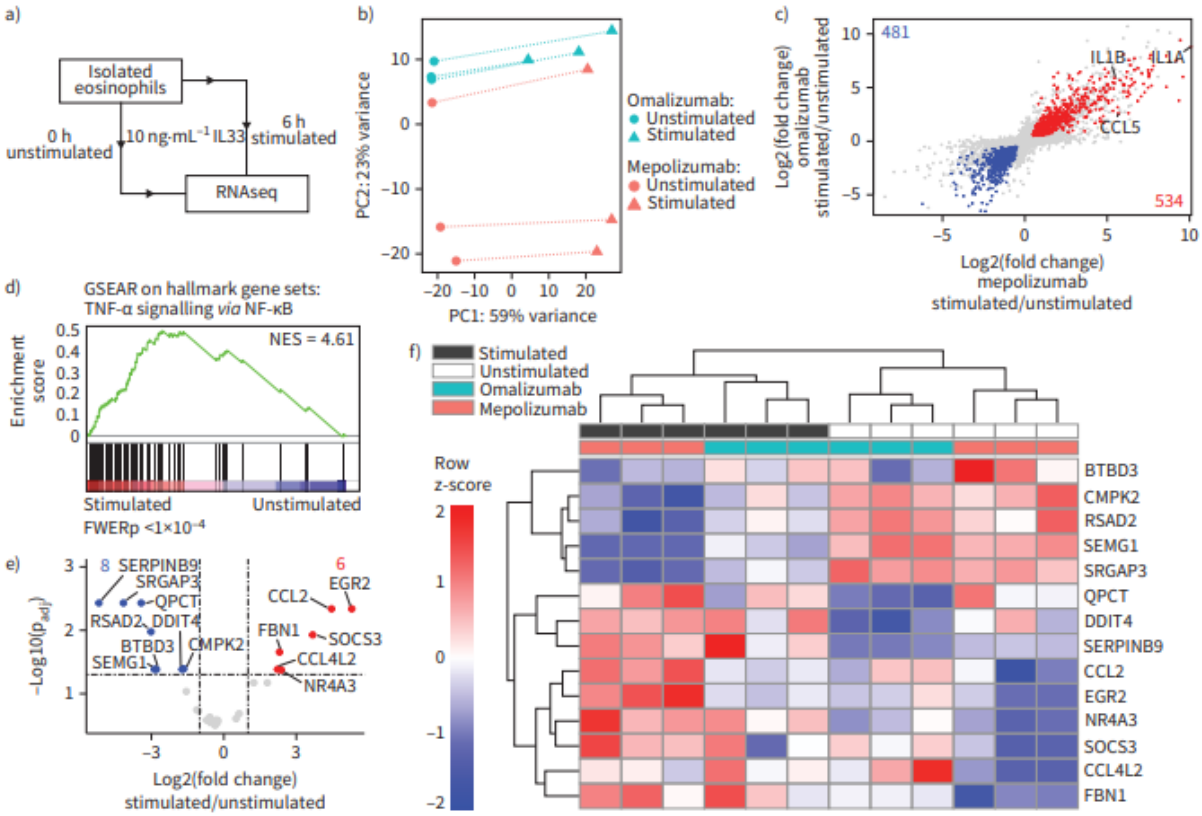

Figure 5. Transcriptomic response to stimulation of blood eosinophils from mepolizumab- or omalizumab-treated patients. **a)** Experimental outline; **b)** principal component (PC) analysis; **c)** correlation of changes in gene expression in response to IL-33 in eosinophils from both patient groups; **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.

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.

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Thesis discussion

This study aimed to unravel the mechanisms by which IL-5 neutralization impacts on eosinophil development in the bone marrow, a question of significant clinical relevance given the widespread use of biological therapies targeting IL-5 or its receptor in eosinophilic asthma. Despite the recognition of IL-5 as a master regulator cytokine in eosinophil biology, its exact roles in regulating eosinophilopoiesis and eosinophilia have remained incompletely understood.

Eosinophil commitment and responsiveness to IL-5

At the initiation of this work, we noticed several limitations in previous efforts to pinpoint the earliest stages of eosinophil development. Firstly, the inconsistent use of different phenotyping strategies for identifying early eosinophil progenitors has hindered a unified view of -and consistent data on- eosinophil lineage development. Secondly, we observed that the commonly used anti-murine Il5ra/CD125 antibodies, used as the discriminating marker for detecting eosinophil progenitors among GMPs in mice, showed high off-target staining of neutrophil lineage cells. We therefore developed an Il5ra reporter mouse strain which allowed for a more accurate identification of Il5ra expressing cells (objective 3).

Although studies using murine helminth models show decreased eosinophil production in IL-5- and Il5ra deficient mice, these mice still produce a basal number of eosinophils at steady state (M. Kopf et al., 1996; Yoshida et al., 1996). We and others also observed a basal production of eosinophils in human patients that are treated with mepolizumab (Ortega et al., 2014; Van Hulst et al., 2022a). These findings suggest that factors other than IL-5 may be involved in or compensate for the commitment of progenitor cells to the eosinophil lineage. It has been proposed that eosinophil, basophil, and mast cell fates diverge early within the GATA1 arm of myelopoiesis (S.-W. Wang et al., 2022), and that basophils and bone marrow-derived mast cells arise from a shared progenitor pool (Dahlin et al., 2018). However, to date, no in vivo physiological mechanism has been identified that governs the upstream commitment of hematopoietic stem and progenitor cells (HSPCs) toward the

eosinophil, basophil, or mast cell (EBM) lineages. Our findings demonstrate that, at least in mice, IL-5 is exclusively expressed in committed eosinophil progenitors. This observation restricts IL-5's role to eosinophil lineage development and excludes it from influencing the commitment decision between eosinophil and basophil/mast cell lineages.

A key difference between human and murine eosinopoiesis is the expression pattern of IL5RA. In humans, IL5RA is expressed not only on committed eosinophil progenitors but also on basophils, albeit at lower levels than on eosinophils. Consequently, anti-IL-5 therapy may potentially impact basophil populations alongside eosinophils and these findings may also explain why basophils are comparatively less reduced than eosinophils in patients treated with benralizumab (Lommatzsch, 2020). Because of IL5RA expression in basophil/eosinophil progenitors, our data cannot rule out a putative involvement of IL-5 in eosinophil lineage choice in humans. We, however, deem unlikely that IL-5 plays a role in regulating the balance between basophil and eosinophil fates since eosinophils are still produced in IL-5 depleted conditions, albeit at reduced numbers (Ortega et al., 2014; Van Hulst et al., 2022a).

Impact of IL-5 on eosinophilopoiesis and progenitor dynamics

In the current literature, IL-5 is widely recognized for its diverse and critical roles in regulating the eosinophil lineage (objective 2). It is proposed that eosinophils depend on IL-5 during their maturation into fully functional eosinophils and that IL-5 promotes the proliferation of eosinophil progenitor cells in the bone marrow (Menzies-Gow et al., 2003; Yamaguchi et al., 1988). Our results show that all stages of eosinophil were present even in IL-5$^{-/-}$ mice, albeit in decreased numbers. This observation indicates that eosinophils may progress through all maturation stages independent of IL-5, at least in mice. Furthermore, by utilizing IL-5$^{-/-}$ mice and those treated with anti-IL-5, our findings suggest that its role may not be as indispensable as previously thought. Using IL-5$^{-/-}$ mice, we observed that early eosinophil stages (1 and 2) still proliferate, indicating that eosinophil progenitor cells can maintain their proliferative capacity independent of IL-5 (Additional figure study 1 A-B). However, upon stimulation with IL-33, the lack of IL-5 impaired the retention of proliferative

capacity into stage 3 eosinophils and disrupted enhanced eosinophil lineage transit amplification (Additional figure study 1B).

Based on studies of erythropoiesis, it is proposed that transit amplification of committed progenitors in haematopoiesis is regulated by a balance between pro-proliferative gene expression programs, which inhibit terminal maturation, and anti-proliferative programs promoting terminal maturation (Li et al., 2019). Our transcriptomic and functional analyses of the eosinophil lineage reveal a comparable regulation of transit amplification, with IL-5 functioning as an essential enhancer of this process. These findings support a model in which IL-5 bioavailability enhances progenitor transit amplification, serving as a potent regulatory mechanism for eosinophil output from the bone marrow in both steady-state conditions and during eosinophilia.

Impact of IL-5 on circulating eosinophils

We investigated the impact of IL-5 depletion on circulating mature eosinophils (objective 4). Contrary to earlier assumptions that IL-5 is involved in the maturation of eosinophils, we did not observe any cellular or transcriptional evidence of maturational arrest in eosinophils following IL-5 depletion. Additionally, residual eosinophils in IL-5$^{-/-}$ mice and in asthmatic patients treated with anti-IL-5 therapy do not exhibit any signs of disrupted development at the transcriptional level at steady state and following *ex vivo* stimulation (Van Hulst et al., 2022a).

Of note, Socs3 was the sole differentially expressed gene in stimulated murine eosinophils under IL-5 depleted condition and the human counterpart SOCS3 was similarly induced in stimulated eosinophils from mepolizumab-treated patients. SOCS3 is short for suppressor of cytokine signalling 3. SOCS3 is a protein that negatively regulates the signalling of different cytokines and growth factors. An example of a cytokine whose signalling is downregulated by SOCS3 is IL-12, which functions as a key regulator of auxiliary T-cell polarisation toward Th1 (Carow & Rottenberg, 2014). SOCS3 gene expression in peripheral T cells correlates with asthma severity and transgenic mice in which the Socs3 gene is overexpressed in T

cells develop more robust Th2 responses and associated airway allergy (Seki et al., 2003). Speculatively, increased SOCS3 gene responsiveness to IL-33 in IL-5-deprived eosinophils could make them more prone to recruitment or activation and therefore contribute to asthma exacerbations (Couillard, 2022). However, SOCS3 is mostly suspected to affect asthma risk through the specific impact it has on T cell polarization into Th2 cells and there is no experimental evidence yet published that SOCS3-overexpressing eosinophils are more prone to activation (Van Hulst et al., 2022b).

Contribution to our understanding of anti-IL-5 therapy

By characterizing the stages of eosinophil development and the expression of Il5ra, we rejected previous beliefs that IL-5 may be involved in eosinophil lineage commitment and that IL-5 is necessary for eosinophil maturation. IL-5 deficiency does not cause intrinsic differences in eosinophils developed under IL-5-depleted conditions on a cellular and transcriptional level. 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.

Our studies provide insights into the role of IL-5 in eosinophilopoiesis, especially highlighting IL-5 as a key regulatory cytokine controlling transit amplification of eosinophil progenitors in the bone marrow. We expect that neutralizing IL-5 in patients with severe eosinophilic asthma might reduce the cycling activity of committed eosinophil progenitors in the bone marrow similar to what we observed in mice causing the previously reported ‘’left shift’’ of the eosinophil lineage.

Taken together, at steady-state, committed eosinophil progenitors progress through four characterized stages in a continuum of maturation, with stages 1 and 2 responsible for proliferation and thus maintaining homeostatic eosinophil numbers. During eosinophilia, eosinophil progenitors retain their proliferative capacity in stage 3 through enhanced transit amplification. Increased transit amplification results from increased and prolonged retention of cycling capacity in eosinophil progenitors, leading to a rapid and exponential rise in eosinophil production from the bone marrow. This transit amplification mechanism is IL-5 dependent, and we propose its inhibition is the primary mechanism of action of anti-IL-5 neutralizing therapies on eosinophilia in asthma and other EADs (Figure 6).

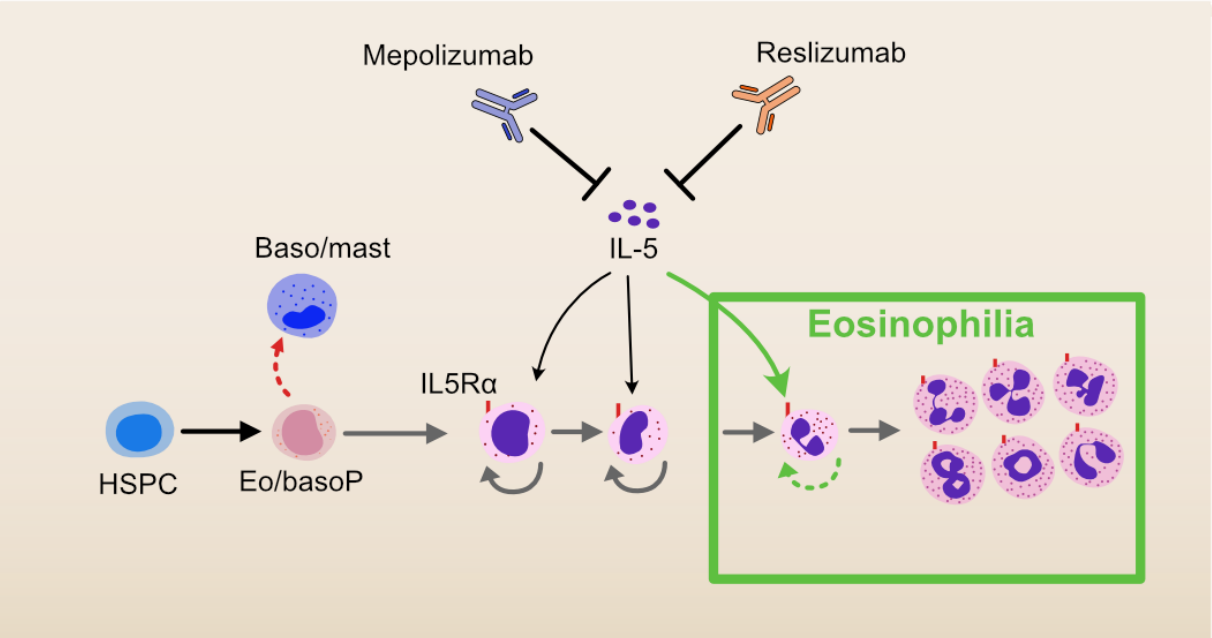

Figure 6. Interleukin-5 neutralizing antibodies may prevent the enhanced transit amplification mechanism of eosinophil production in the bone marrow.

Limitations and future perspectives

Our work presents with some limitations. In our first study, we have not analyzed human bone marrow in eosinophilia-promoting conditions. It is still unclear whether eosinophil lineage expansion in patients with EADs is a sustained process or whether it involves dynamic, time resolved pulses of progenitor expansion, similar to what we have observed in mice. It would therefore be worthwhile to expand on the current study and investigate the dynamics in the bone marrow of human patients during exacerbation and following anti-IL-5 therapy. Hypothetically, eosinophilic patients with absence of eosinophil progenitor expansion in the bone marrow might have lower responses to anti-IL-5 therapy. The timing of intervention could be also critical in optimizing therapeutic outcome similar to what was seen in mice. New anti-IL-5 mAbs are currently in development and are specifically engineered for high affinity and long-acting suppression of IL-5 function (Pavord et al., 2024).

In both studies we have focused on bone marrow and blood eosinophils. A dynamic assessment of the entire eosinophil compartment from the bone marrow 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. A recent study also showed that residual eosinophils can still be

recruited to the lung of mepolizumab treated patients (Wilson et al., 2024). It could be hypothesized that, contrary to previous beliefs, eosinophils are recruited to the lungs independently of IL-5. Our Il5ra reporter mouse is well suited to investigate this thoroughly since homozygotes are also Il5ra deficient.

Human EBMs 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, remains to be thoroughly explored *in vivo*. In this line of thought, a recent study showed that IL-3 promotes mast cell progenitor proliferation, whereas IL-5 mostly supports mast cell progenitor survival *in vitro* (Wu et al., 2022). These data strongly suggest that mast cells do, at the very least, express a functional IL5RA receptor.

Finally, the question remains open whether anti-IL-5 or anti-IL5RA primarily and only work through their effects on eosinophils. IL5RA is known and proposed to be expressed by non-eosinophil cells as well. For instance, on human basophils but also in a primitive B-1 B cell subset (W. Wang et al., 2022; Yoshida et al., 1996). However, expression and potential impact of IL-5 on these cells at steady state or in the context of respiratory diseases has seldom been evaluated. Intriguingly, anti-IL-5 therapy leads to increased IgA production following rhinovirus infection in asthmatic patients through yet unknown mechanisms (Sabogal Piñeros et al., 2019). In addition, nasal polyps in patients with aspirin-exacerbated respiratory disease harbor IL-5-responsive IL5RA expressing plasma cells (Buchheit et al., 2020). Other cells such as neutrophils, epithelial cells and fibroblasts have also been proposed. However, these claims are based on very limited and/or low confidence evidence to date. In our study, we have shown that neutrophils deficient for Il5ra stain with commonly used readily available murine monoclonal CD125 antibodies but do not express the *Il5ra* reporter transgene. Our newly developed Il5ra reporter mouse strain has opened up new research opportunities to further investigate and track the cells expressing Il5ra in murine models of EADs. Crossing our Il5ra mouse strain to Rosa26-LSL-tdTomato (tdT) mice will identify cells expressing tdT in all cells that actively express, have expressed or descend from cells that expressed Il5ra. This project may find additional cells that potentially engage in IL-5-responsive activities in preclinical models of human respiratory diseases and may add to our understanding of the impact of anti-IL-5 biological therapies.

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Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
ADCC	Antibody-dependent cellular cytotoxicity
BHR	Bronchial hyperreactivity
BM	Bone marrow
CCR3	C-C chemokine receptor 3
CMP	Common myeloid progenitor
COPD	Chronic obstructive pulmonary disease
CRS	Chronic rhinosinusitis
CXCR	Chemokine C-X-C motif receptor
CRSwNP	Chronic rhinosinusitis with nasal polyps
C/EBP	CCAAT/enhancer-binding protein
EADs	Eosinophil-associated disorders
EBM	Eosinophil, basophil, or mast cell
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EdU	5-ethynyl-2'-deoxyuridine
EETs	Eosinophil extracellular traps
EGPA	Eosinophilic granulomatosis with polyangiitis
EoPs	Eosinophil progenitors
EPX	Eosinophil peroxidase
ES	Embryonic stem
FcεRI	High-affinity IgE Receptor
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity
GMPs	Granulocyte/monocyte progenitors
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled corticosteroids
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate lymphoid cells
ILC2s	Type 2 innate lymphoid cells
IL-5Rα`	IL-5 specific receptor
IRF8	Interferon-response factor-8
IV	Intravenous
LABA	Long-acting beta-agonist
LAMA	Long-acting muscarinic agonist
Lin	Lineage
L.m.	Listeria monocytogenes
LTRA	Leukotriene receptor agonist
mAbs	Monoclonal antibodies
MBP	Major basic protein
MDI	Metered dose inhaler
mRNA	Messenger ribonucleic acid
OCS	Oral corticosteroids
OVA	Ovalbumin
RNA	Ribonucleic acid
RNase	Ribonuclease

RSV	Respiratory syncytial virus
RV	Rhinovirus
SABA	Short-acting beta-agonist
scRNAseq	Single cell RNA sequencing
SIGLEC	Sialic acid-binding Ig-like lectin
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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