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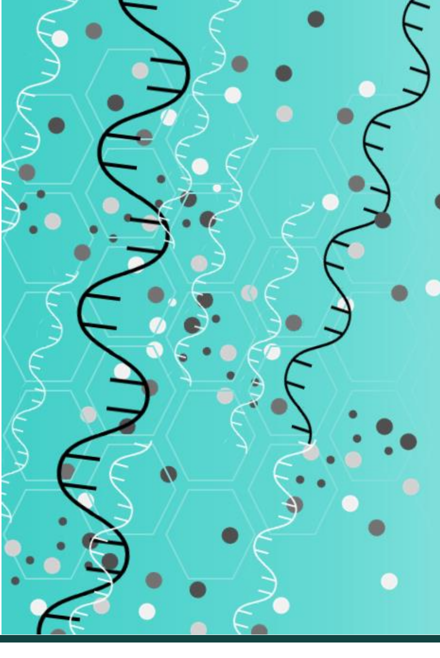

Early and late transcriptomic response of human blood eosinophils to the alarmin interleukin-33

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ABSTRACT

Eosinophils are polymorphonuclear leukocytes mainly known for their role in protection against helminthic parasites. Beyond this immune function, eosinophils have been attributed diverse roles in health and disease. On the one hand, eosinophils contribute to immune and metabolic homeostasis and participate in tissue morphogenesis and regeneration. On the other hand, eosinophils are associated with tissue lesions in a variety of human diseases such as eosinophilic asthma, in which they are thought to play a pathogenic role. Indeed, the severity of eosinophilic asthma is positively correlated with the number of blood and tissue-infiltrating eosinophils, and biological therapies that eliminate or control the number of eosinophils improve control over exacerbations of severe forms of the disease. Nonetheless, mere counting of eosinophils is not a perfect predictor of the response of individual patients to anti-eosinophil targeted therapies. This led clinicians to posit that yet-to-identify factors may determine whether eosinophils are “good”, “bad” or “bystander” cells in asthma. Indeed, eosinophils are more labile than their classical vision as terminally differentiated effector cells posits. Eosinophils are able to adapt to their tissue microenvironment, which calls in the notion of plasticity.

In this work, we address the question of whether and how the alarmin interleukin (IL)-33, a pro-inflammatory cytokine of the IL-1 family and major activator of eosinophils, could participate in the plasticity of eosinophils. Indeed, it has been shown that IL-33 induces a plastic response in macrophages, in which it first elicits a pro-inflammatory gene expression program and later reprograms macrophages into pro-resolving and pro-remodeling cells. Therefore, we studied the short- and long-term transcriptomic response of human blood eosinophils to IL-33 *ex vivo*. The results showed that IL-33 induced a short term, NF-κB-controlled pro-inflammatory program in eosinophils but no detectable long-term transcriptomic response. Thus, our results suggest that IL-33 signaling does not elicit a long-term plastic response in eosinophils.

Keywords: eosinophil, interleukin-33, transcriptomic signature

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LIST OF ABBREVIATIONS

AAMs: alternatively activated macrophages
ACQ-5 : 5-item asthma control Questionnaire
BAL: bronchoalveolar lavage
BHR: bronchial hyperresponsiveness
C/EBP-α and ε: CCAAT/enhancer-binding protein-α and -ε
CAMs: classically activated macrophages
CBM: chromatin binding motif
CCL11: C-C motif chemokine ligand 11
CCR: CC-chemokine receptor
cDNA: complementary DNA
CLCs: Charcot-Leyden crystals
CMP: common myeloid progenitor
CSF2Rβ: colony stimulating factor 2 receptor subunit β
DCs: dendritic cells
DE: differentially expressed
DGE: differential gene expression
EADs: eosinophil-associated diseases
ECP: eosinophil cationic protein
EDN: eosinophil-derived neurotoxin
EETosis: eosinophil cytolysis by eosinophil extracellular DNA trap formation
EoP: eosinophil-committed progenitor
EoPs: eosinophil lineage-committed progenitors
EPO: eosinophil peroxidase
FE_{NO}: fraction of exhaled nitric oxide
GATA-1: GATA-binding protein-1
GM-CSF: granulocyte-macrophage colony-stimulating factor
GMP: granulocyte/macrophage progenitor
HDM: house dust mite
HSCs: hematopoietic stem cells
HSPCs: hematopoietic stem and progenitor cells
ICS: inhaled corticosteroids
iEos: inflammatory eosinophils
IFN-γ: interferon-γ
Ig: immunoglobulin
IL: interleukin
IL-1RAcP: IL-1 receptor accessory protein
ILC2s: type 2 innate lymphoid cells
IL-XR: interleukin X receptor
JAK: Janus kinase
mAbs: monoclonal antibodies
MAPK: mitogen-activated protein kinase
MBP: major basic protein
MDSCs: myeloid-derived suppressor cells
MEP: megakaryocyte/erythrocyte progenitor
MHC-II: class II major histocompatibility complex
NF-κB: nuclear factor-κ B
NLS: nuclear localization sequence
OCS: oral corticosteroids
OVA: ovalbumin
PC: principal component
PCA: principal component analysis
PMN: polymorphonuclear
PU.1: E26 family transcription factor PU.1
rEos: resident eosinophils
RNAse: ribonuclease
SEA: severe eosinophilic asthma
SIGIRR: single immunoglobulin IL-1R-related
Siglec-8: sialic acid-binding immunoglobulin-like lectin 8
SSC: side scatter
sST2: soluble ST2
ST2: suppression of tumorigenicity 2
STAT: signal transducers and activators of transcription
T2-high: type 2-high phenotype
T2-low: type 2-low phenotype
TEBs: terminal end buds
TGF-β: tumor growth factor β
TGFβR: tumor growth factor β receptor
Th2: helper T type 2
TSLP: thymic stromal lymphopoietin

INTRODUCTION

1 Eosinophils

1.1 Phenotype

Mature human eosinophils are a type of polymorphonuclear (PMN) cells about 12-15 µm in size. They are recognizable in bright field microscopy by their polylobed nuclei and acidophilic granules, harboring the characteristic pink color when stained with eosin. In flow cytometry, the identification of mature eosinophils can be made on the basis of a combination of side scatter (SSC) and surface markers: eosinophils display a high SSC reflecting their high granularity and also express CC-chemokine receptor 3 (CCR3) and sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8) (1) (Fig.1).

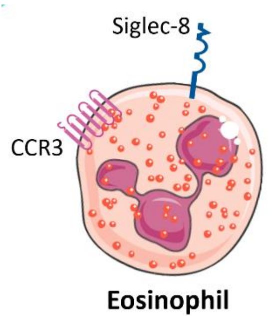

Fig.1: Eosinophil phenotype. Schematic eosinophil and its classical surface markers targeted in flow cytometry. This figure was produced using Servier Medical Art (https://smart.servier.com/).

1.2 Origin and fate

The comprehensive ontogeny of eosinophils in humans is not yet fully resolved. Eosinophils develop in the bone marrow, where they derive from hematopoietic stem and progenitor cells (HSPCs) like other blood cell types. In the current model of human eosinophil development, the first identified eosinophil-committed progenitor (EoP) arises from an IL-5Rα^{+} CD34^{+} IL-3Rα^{+} subset of common myeloid progenitors (CMP). (Fig.2a) (2). More recent studies on human (3) and mouse (4) progenitors suggest a segregation of hematopoietic lineages very early based on GATA-binding protein-1 (GATA-1) expression. In this model, eosinophils, basophils and mast cells develop from GATA-1-expressing progenitors while neutrophils and monocytes develop from GATA-1-negative progenitor (Fig.2b).

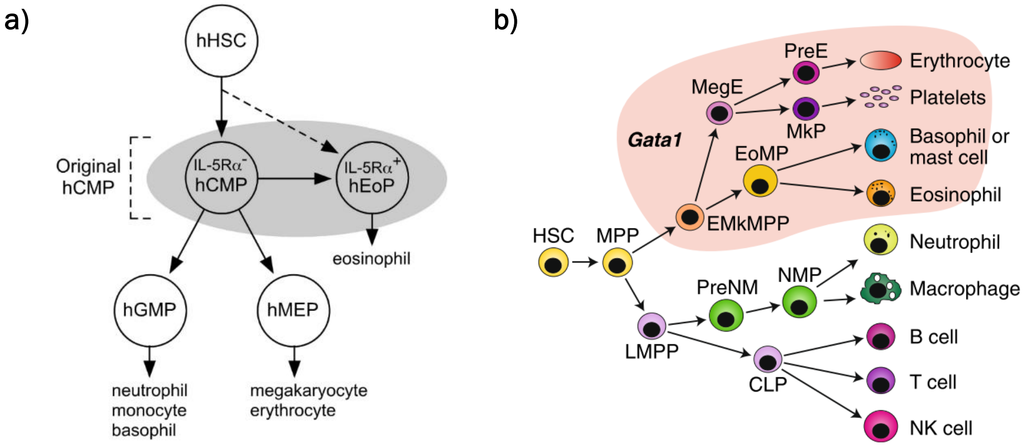

Fig.2: Models of lineage specification in hematopoiesis and position of the eosinophil lineage therein. ***(a) Myeloid progenitor segregation based on the expression of IL-5Rα.*** The human common myeloid progenitor (hCMP) is a IL-5Rα⁻ subset of original hCMP (shaded). IL-5Rα⁺ human eosinophil-committed progenitors (hEoP) develop from the hCMP or its upstream multipotent progenitor and gives rise to eosinophils (2). ***(b) GATA-1 expression-based hematopoiesis model.*** GATA-1-positive lineages generate eosinophils, mast cells or basophils whereas GATA-1-negative lineages generate neutrophils or monocytes. The shaded area encompasses GATA-1 expressing lineages (4,5) CLP, common lymphoid progenitor; EMkMPP, Erythroid–megakaryocyte-primed multipotent progenitor; EoMP, eosinophil-mast cell progenitor; hGMP, human granulocyte/macrophage progenitor; (h)HSC, (human) hematopoietic stem cell; hMEP, megakaryocyte/erythrocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; MegE, pre-megakaryocyte-erythroid progenitor; MkP, megakaryocyte progenitor; MPP, multipotent progenitor; NK, natural killer; NMP, neutrophil-monocyte progenitor; preE, pre-colony-forming erythroid progenitor; preNM, pre-neutrophil- monocyte progenitor

Eosinophilopoiesis, viz. the control of the commitment of HSPCs toward the eosinophil lineage and the subsequent maturation of eosinophils, is controlled by a network of transcription factors and influenced by surrounding cytokines. Classical cytokines described as important for eosinophil development are interleukin (IL)-5, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1). These 3 cytokines bind to dedicated receptors that share a common signal-transducing chain, the colony stimulating factor 2 receptor subunit β (CSF2Rβ) (6). Essential transcription factors in eosinophilopoiesis include GATA-1 which is the most important for eosinophil specification, as well as the E26 family transcription factor PU.1 (PU.1, also known as SPI1) and CCAAT/enhancer-binding protein-α and -ε (C/EBP-α and ε) which are essential to eosinophil maturation. All these transcription factors are implicated in complex interplays enabling the transcription of target genes including those coding for eosinophil granule proteins as well as the repression of other genes involved in non-eosinophil lineages (7).

Once mature, eosinophils leave the bone marrow to enter the blood circulation where they remain for a short time (half-life of 3-24 hours) and in low amounts as they represent 1% to 3% of peripheral blood leukocytes in healthy adults (1). At steady state, eosinophils reside in tissues such as the lung, thymus, fat tissue and mucosa of the gastrointestinal tract (except the esophagus) where they can reside for several days (8). The main chemokine involved in eosinophil recruitment to tissues in the steady state and at inflammatory sites, is eotaxin-1, also known as C-C motif chemokine ligand 11 (CCL11). Its receptors are the G protein-coupled receptors CCR3 and CCR5 (9).

1.3 Well-described protective roles

Eosinophils are well known for their roles in host protection against parasitic infections, such as helminth infection. They are also able to protect against bacterial and viral infection (9). Eosinophils own several resources to play their protective immune roles.

First, eosinophils harbor a wide variety of receptors that allow them to respond to diverse stimuli and communicate with other immune cells. For example, they express chemoattractant receptors (e.g., CCR3 and CCR5) and cytokine receptors such as IL-5Rα, IL-4R and tumor growth factor β receptor (TGFβR). Also, they express pattern recognition receptors to identify pathogens as well as Fc receptors and class II major histocompatibility complex (MHC-II) to interact with the adaptive immune system (9).

Furthermore, eosinophils are equipped with specific cytoplasmic granules containing an array of cytotoxic cationic proteins. Among the cytotoxic proteins are major basic protein (MBP), which is able to activate basophils, mast cells and neutrophils *in vitro*, eosinophil peroxidase (EPO), which catalyzes the production of reactive oxygen species, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). ECP and EDN belong to the ribonuclease (RNAse) A superfamily which has antiviral activity (10). Granule components can be released by three main secretory processes in response to diverse stimuli: *(a)* exocytosis in which granules fuse with the plasma membrane releasing granule content (1), *(b)* cytolysis during which eosinophils release intact granules that retain secretory activity (11), and *(c)* piecemeal degranulation consisting in the release of specific granule proteins through fusion of granule-derived secretory vesicles with the plasma membrane (1). Eosinophil cytolysis is also known as EETosis, or eosinophil extracellular DNA trap formation. During EETosis, the plasma membrane is disintegrated, intact granules are released, and mitochondrial DNA nets are deposited which form extracellular traps (11). These traps promote inflammation and participate to the elimination of pathogens (12). In human EETosis, galectin-10, a cytoplasmic protein which constitutes 7% to 10% of total proteins in eosinophils, is also released and forms Charcot-Leyden crystals (CLCs) (13). CLCs have been shown to enhance type 2 inflammation (14).

Finally, eosinophils can secrete cytokines such as interferon-γ (IFN-γ), tumor growth factor β (TGF-β) IL-4 and IL-5, chemokines (e.g., CCL11) and lipid mediators such as cysteinyl leukotrienes (15). Altogether, the above mediators can participate in the inflammatory response and eliminate pathogens.

1.4 Homeostatic functions

At steady state, eosinophils are found in diverse tissues where they can reside for a relatively long time as mentioned above (8). For this reason, the potential homeostatic functions they may ensure in these tissues are being increasingly investigated with the help of preclinical models.

First, homeostatic eosinophils contribute to immune homeostasis in different locations. For instance, in the murine gastrointestinal tract, resident eosinophils promote the development and preservation of immunoglobulin (Ig)-A-producing plasma cells as well as IgA class switching (16,17). Also, eosinophil deficiency has been associated with altered gut microbiota. Lack of intestinal eosinophils has also been correlated with decreased mucus production (16,17) accompanied by a less efficient induction of oral tolerance (17). This last observation is consistent with the hypothesis that mucus modulates oral tolerance (18). Thereby, eosinophils might contribute to intestinal homeostasis. In addition to their role in the gastrointestinal tract, eosinophils might participate in the negative selection of T cells in the thymus. Thymic eosinophils were found in the immediate vicinity of apoptotic thymocytes in mouse models of MHC-I-restricted thymic T cell deletion (19) and the absence of eosinophils reduces the clearance of apoptotic bodies in the thymus (20). In the bone marrow, eosinophils provide survival factors to plasma cells for their retention and long-term maintenance (21). In the lung, resident homeostatic eosinophils are involved in the maintenance of immune homeostasis toward inhaled antigens by preventing Th2 responses (22).

Second, it has been shown that murine eosinophils play a role in metabolic homeostasis. In murine adipose tissue, eosinophils are the main source of IL-4, which induces the polarization of macrophages toward alternatively activated macrophages (AAMs) (23). AAMs in turn play a key role in glucose homeostasis in the adipose tissue (24). Eosinophil-deficient mice fed with high fat-diet develop obesity and impaired glucose tolerance significantly more than their WT counterparts. This suggests that eosinophils are involved in protection against diet-induced obesity (23). Moreover, eosinophils have been shown to promote the biogenesis of beige fat in mice, which is involved in energy balance and thermogenesis (25).

1.5 Role in tissue morphogenesis and regeneration

In addition to their protective and homeostatic roles, another important role of eosinophils has been discovered: their involvement in tissue morphogenesis and regeneration.

First, in mice, eosinophils along with macrophages are necessary for postnatal development of the mammary gland ductal epithelium. Indeed, eosinophils were not present around terminal end buds (TEBs - the growing part of ductal tree) in eotaxin deficient mice. This was accompanied by a reduced number and branching of TEBs (26). Moreover, during postnatal lung development in mice, there is a spontaneous wave of type 2 cells including eosinophils during the alveolarization phase. Then, after this period, their number decreases. The authors have proposed that this type 2 immunity could promote tissue remodeling during lung development (27). More recently, it has been shown that recruitment of eosinophils is essential for thymus regeneration after its ablation (28).

Second, eosinophils are recruited to damaged tissues following hepatectomy or toxin-induced liver injury, as well as following muscle injury. In these models, tissue regeneration was slower in the absence of eosinophils or of signaling by IL-4/IL-13 (29,30).

1.6 Pathological roles of eosinophils: Eosinophil-associated diseases

Besides their beneficial roles presented above, eosinophils are mainly studied for their pathological roles, especially in so-called “eosinophil-associated diseases” (EADs). EADs is an umbrella term which refers to medical conditions where eosinophils are thought to participate in pathology and organ dysfunction by releasing their toxic granule contents such as MBP, EPO, ECP and EDN. EADs include eosinophilic esophagitis, eosinophilic gastritis, and eosinophilic asthma (31). In physiologic conditions, eosinophil count in blood is about 50 to 150 cells/μL, that is to say 1-3% of circulating white blood cells (32). In EADs, the number of eosinophil increases in blood and/or tissues, a phenomena known as eosinophilia (31). A classification of eosinophilia has been established according to blood eosinophilia severity, infiltration of eosinophils or deposition of eosinophil granules within tissue and tissue damage (33). In this work, we will focus on eosinophilic asthma, one of the most prevalent EADs worldwide (34).

1.6.1 Eosinophilic asthma

a) Asthma: definition and classification

In 2019, according to World Health Organization, about 262 million people, children and adults, around the world suffered from asthma (34). This disorder is a chronic inflammatory disease of the airways that leads to mucus overproduction, airway wall remodeling and bronchial hyperresponsiveness (BHR - contraction of smooth muscle cells in response to non-specific stimuli such as cold air). Consequently, asthma patients present with cough, wheeze, shortness of breath, and chest tightness (35).

Asthma is a heterogenous disease resulting from a complex interaction between genetics (e.g., atopic individuals have a genetic predisposition to the development of allergy) and exposure to environmental factors (such as pollution, airway allergens and smoking). Numerous factors can be considered when establishing asthma classification and include age of onset (early/childhood versus late/adulthood onset), the preservation of lung function, allergic status and the presence of type-2 inflammation (36). Based on this latter parameter, asthma can be divided into two main disease phenotypes: *(a)* a type 2-high (T2-high) phenotype in which T helper type 2 (Th2) cells and/or type 2 cytokines are involved accompanied by blood and lung eosinophilia and *(b)* a type 2-low (T2-low) phenotype where neither type 2 inflammation nor eosinophilia are observed (35,36). T2-high is also designated as eosinophilic asthma and T2-low asthma as non-eosinophilic asthma.

b) Eosinophils as foes in eosinophilic asthma

Eosinophils have detrimental effects in T2-high asthma as suggested by the positive correlation between the presence of eosinophils and disease severity (37,38). In addition to that, anti-eosinophils therapies (see hereinafter) have positive impacts on exacerbations (periods during which symptoms and airway obstruction worsen) in treatment of severe eosinophilic asthma (SEA). Indeed, eosinophils play a role in the pathogenesis of asthma by releasing their granule-derived proteins, cytokines and lipids mediators, which have been demonstrated to contribute to several features of asthma (39). For example, MBP may induce histamine release from mast cells and basophils (40,41), which participate in the pathophysiology of asthma through the induction of BHR (42). In addition, eosinophils may contribute to the airway wall remodeling by several secreted products such as TGF-β (43), cysteinyl leukotrienes and matrix metalloproteinases (39). Finally, it has been evidenced that CLCs can actively promote type 2 pathology (14) (Fig.3).

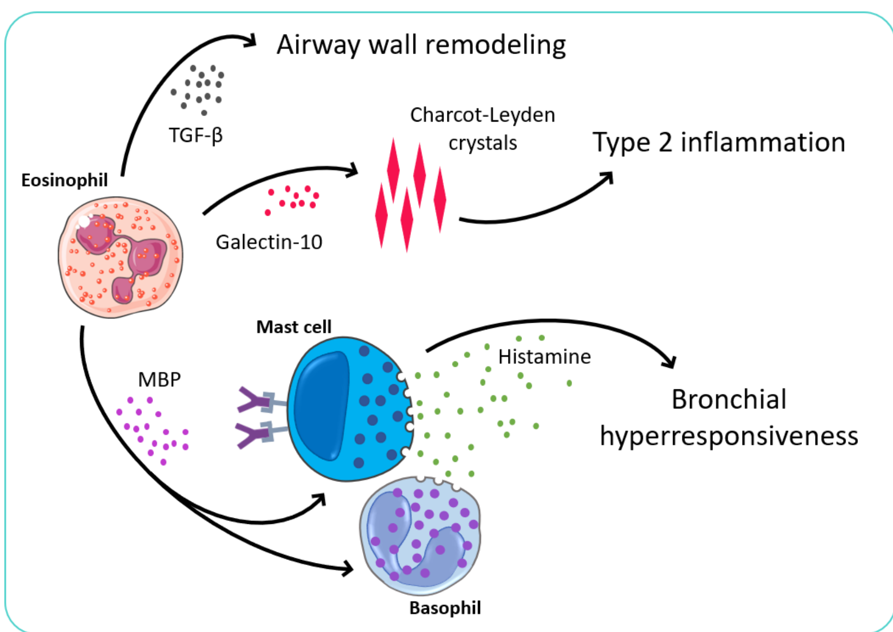

Fig.3: Examples of detrimental roles of eosinophils in eosinophilic asthma. Upon activation, eosinophils release major basic protein (MBP) which may trigger histamine release from mast cells and basophils. Histamine in turn participates in bronchial hyperresponsiveness. Eosinophils may participate in airway remodeling by releasing TGF-β. Finally, eosinophil derived-galectin-10 assemble to form Charcot-Leyden crystals which can enhance type 2 inflammation. This figure was produced using Servier Medical Art (https://smart.servier.com/).

1.6.2 Mechanisms of eosinophilia in T2-high asthma

Eosinophilia is driven by IL-5, whit is also true in T2-high asthma. IL-5 is the most well-known cytokine for eosinophil activation, survival as well for eosinophil lineage specification. It is also responsible for the expansion of eosinophil progenitors within the bone marrow (9). Binding of IL-5 to the heterodimeric IL-5Rα/CFSR2β receptor leads to activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways that regulate eosinophil proliferation and survival (6). Although IL-5 was thought to be central in eosinophil lineage commitment and maturation, IL-5-deficient mice and human patients treated with anti-IL-5 antibodies still have residual eosinophils. Furthermore, these residual cells respond to cytokine stimulation as well as eosinophils from wild type mice or from non-treated patients. These observations indicate that IL-5 mainly promotes eosinophil expansion during eosinophilopoiesis (44).

In eosinophilic asthma, both innate and adaptive mechanisms lead to IL-5 production and release. First, environmental stimuli such as allergens, pathogens (viruses, bacteria, molds, …) as well as air pollution cause damage to epithelial cells leading to release of alarmins such as IL-25, thymic stromal lymphopoietin (TSLP) and IL-33. These epithelial cell-derived cytokines activate type 2 immune cells like Th2 cells and type 2 innate lymphoid cells (ILC2s), which consequently produce and release IL-5. Also, IL-33 acts on dendritic cells (DCs) and induce them to become pro-Th2 cells which activates Th2 cells. Second, at re-exposition to an allergen, DCs capture allergen in the lumen airways and, after maturation and migration to lymph nodes, present it to Th2 and memory T cells specific to the allergen. Consequently, they produce type 2 cytokines including IL-4, IL-13 and IL-5 (45). Such amount of IL-5 leads to extensive eosinophil expansion in bone marrow resulting in systemic eosinophilia (Fig.4).

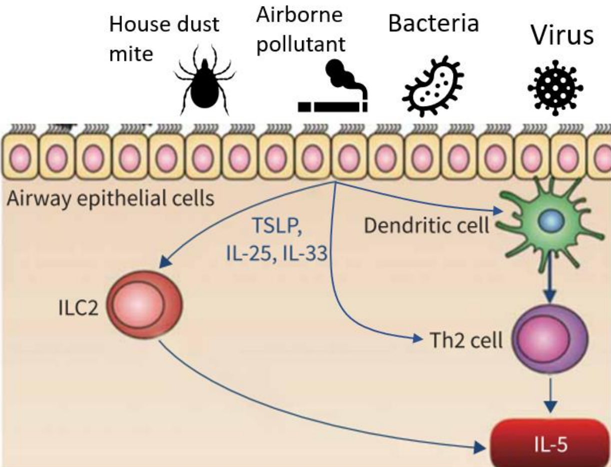

Fig.4: Sources of interleukin (IL)-5 in eosinophilic asthma. On the one hand, epithelial cells damaged by inhaled allergens, respiratory pathogens or airborne pollutant release the alarmins IL-33, IL-25 and thymic stromal lymphopoietin (TSLP). In response, group 2 innate lymphoid cells (ILC2s) and T helper type 2 (Th2) cells product and release IL-5. Dendritic cell becomes pro-Th2 cells under IL-33 control. On the other hand, dendritic cell captures allergens in the lumen airways and activates specific Th2 cells which produce IL-5. *Adapted from* (46) and produced with free icone provided by PowerPoint Microsoft.

1.6.3 Current and tested treatments of eosinophilic asthma

In asthma management, the main goals are enhancing quality of life by reducing the inflammation and associated symptoms and limiting the risk of exacerbation. Asthma control can be assessed at visits with questionnaires such as 5-Item Asthma Control Questionnaire (ACQ-5). A more objective manner to evaluate asthma is to measure the forced expiratory volume in one second that reflects the lung function. Along with asthma control, the level of pro-inflammatory markers can also be measured: blood eosinophil counts, total serum IgE levels and fraction of exhaled nitric oxide (FE_{NO}) (47).

The basic treatments for asthma are bronchodilators (long- or short-acting β_2-agonist) which increase air flow in lungs by dilating bronchi. Corticosteroids are used in the management of asthma in children and adults for their anti-inflammatory effects (48). Particularly, inhaled corticosteroids (ICS) are administrated in case of persistent asthma while oral corticosteroids (OCS) are supplied to control exacerbations. ICS are added to bronchodilator and this combination results in a reduced ICS dosage, better control of symptoms and decreased exacerbation risk (49).

In most patients, asthma is well-controlled following these treatment guidelines. However about 10% of patients see their asthma insufficiently controlled by standard therapies and suffer from severe symptoms, a medical condition called severe asthma (45). In addition, steroids are not efficient at controlling T2-low asthma and can cause local and systemic adverse effects (48), especially when used in high-dose and over prolonged periods. For these reasons, biologics targeting more specific mechanisms involved in the pathology of severe asthma have been developed or are in clinical trials. The first targeted therapy introduced was omalizumab, an anti-IgE monoclonal antibody, for the management of severe allergic asthma (50). Later followed biological therapies targeting eosinophils, as well as dupilumab, which targets type 2 immune signaling (51). Upcoming targeted therapies aiming at controlling all asthma phenotypes irrespective of their eosinophilic or non-eosinophilic profile by targeting alarmin signaling, should arrive on the market shortly (52) .

In the context of this work, I only discuss anti-eosinophilic therapies. Because of their identification as contributors to asthma exacerbations, eosinophils have become the targets of biological therapies for the precision management of severe eosinophilic asthma (SEA). These biological therapies exploit the dependency of eosinophils on IL-5 through 2 distinct strategies. The first strategy relies on humanized monoclonal antibodies (mAbs) that neutralize circulating IL-5. Mepolizumab, one of these neutralizing antibodies, has been shown to significantly decrease blood and sputum eosinophilia as well as the rate of exacerbations in patients suffering from SEA (53–55). Moreover, mepolizumab allows a reduction of the dose of oral

corticosteroids administered to these patients (54–57). Another anti-IL-5 antibody, reslizumab, has also been shown to reduce the rate of asthma exacerbations in SEA (58,59). The second approach is based on a humanized mAb, benralizumab, directed against IL-5Rα. Its binding elicits antibody-dependent cellular cytotoxicity leading to the complete depletion of blood eosinophils and IL-5Rα-expressing progenitors in the bone marrow (60). Like anti-IL-5 biologicals, benralizumab reduces asthma exacerbations and allows corticosteroid-sparing in SEA (61).

1.7 Eosinophil diversity

Eosinophils should not be considered as terminally differentiated, invariant innate effector immune cells. Indeed, eosinophils display a wide variety of activities and phenotypes matching their function to their surrounding tissular context (62). Eosinophil diversity can in this regard be approached through the diverse functions attributed to eosinophils in health and disease as discussed above. Next to tissue location, timing may also be an additional factor to consider. In this line of thought, Schetters and Schuijs have proposed that eosinophil functions evolve throughout the lung allergic inflammation, regarding the time and their localization [reviewed in (63)]. For instance, during the acute phase of allergic inflammation within lung tissue, eosinophils stimulate mast cells to release histamine which increases vascular permeability, mucus production and constriction of small airways. In contrast, during the adaptative phase which takes place days to weeks after the allergen exposure, eosinophils migrate to lymph nodes and could sustain the Th2 inflammation by participating in DC recruitment and allergen-specific T cells proliferation in lymph nodes (63).

Besides, several “subtypes” of eosinophils that can coexist in tissues have been characterized in humans and mice. First, in the early 1980s, 2 types of eosinophils in human were described based on cellular density profile: “classical” normodense eosinophils found in healthy individuals and additional hypodense eosinophils reported in blood, bronchoalveolar lavage (BAL) and lung tissue of SEA patients (64). These hypodense eosinophils have been shown to have an increased response to activating stimuli compared to normodense ones (65). Nonetheless, more studies are needed to determine the clinical impact of hypodense eosinophils in SEA. Second, 2 subtypes of eosinophils have been evidenced based on the expression of IL-3R and CD62L. Indeed, sputum eosinophils from asthmatic patients have shown a higher expression of IL-3R and a lower expression of CD62L compared to eosinophils from normal lung tissue (22). Third, in a murine model of airway allergy induced by intranasal administration of extracts of house dust mite (HDM), 2 distinct types of eosinophils have been described. Lung resident eosinophils (rEos), found in both steady state mice and HDM-challenged mice, harbor a ring-shaped nucleus, display a $CD62L^{+}$ $CD125^{+}$ Siglec-F^{int} $CD101^{low}$ phenotype, and are exclusively found in the lung parenchyma. In addition, inflammatory

eosinophils (iEos) with a segmented nucleus and a CD62L^{low} CD125^{int} Siglec-F^{high} CD101^{high} phenotype get recruited in response to allergen challenge and localize in the peribronchial and perivascular areas, typical of bronchial eosinophilic infiltration in asthma. Gene expression analysis revealed that rEos have an immunosuppressive profile and iEos, a proinflammatory profile (22). This dichotomy of murine lung eosinophils has also been reported since in other studies (66).

Collectively, these clues show that lung eosinophils may be heterogeneous, but the origins of such heterogeneity need to be clarified. The observed diversity of eosinophils brings the question of its origin. Two non-mutually exclusive mechanisms have been suggested in the context of SEA [reviewed in (67)].

Eosinophil "endotyping" is a first proposed mechanism, in which distinct subsets of eosinophils would emerge from eosinophilopoiesis. It posits that environmental and tissular clues would "instruct" eosinophil progenitors and elicit distinct functional programs in their mature progeny (67). This mechanism would resemble the process by which myeloid progenitors, under influence of GM-CSF, IL-6 and IL-10, give rise to myeloid-derived suppressor cells (MDSCs) (68). Eosinophil endotyping could explain the presence of hypodense eosinophils in human (65) or iEos in mouse (22) (Fig.5).

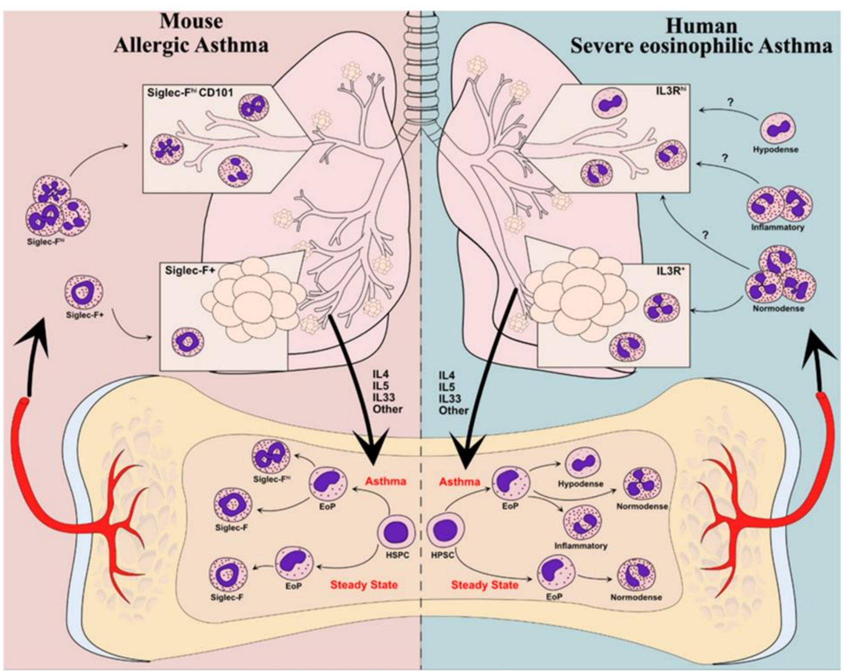

Fig.5: Endotyping model to explain eosinophil diversity in mice and humans. In murine models of allergic asthma **(left)**, type-2 cytokines induce the differentiation of eosinophil progenitors (EoPs) into "inflammatory" Siglec-F^{high} eosinophils. In humans **(right)**, inflammatory type-2 cytokines in severe eosinophilic asthma alter the development of EoPs, giving the hypodense eosinophils (67). HSPC, hematopoietic stem and progenitor cell

The second mechanism implies a local plasticity of eosinophils. Like blood monocytes which infiltrate lungs and are able to differentiate into interstitial or alveolar macrophages depending on surrounding environment (69), eosinophils would be instructed by the specific local context to activate distinct effector programs (67) (Fig.6). This master thesis investigates more directly this scenario by assessing the long-term consequences of the activation of eosinophils by the alarmin IL-33 which seems to be an important cytokine in eosinophilia and its associated disorders (70).

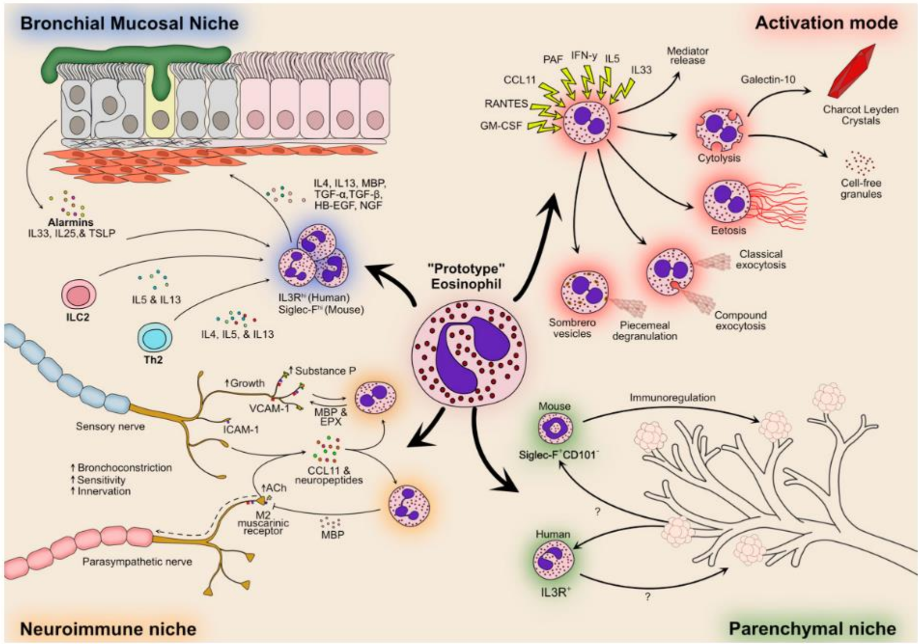

Fig.6: Plasticity of eosinophils in asthma. In lungs, three "niches" may influence the final functions of eosinophils upon its arrival. Thus, blood eosinophils are considered as "prototype" cells which may be tailored by its surrounding environment. In bronchial mucosal niche, type-2 cytokines and alarmins may induce eosinophils to produce and release mediators which play a role airway remodeling. Eosinophils have a tropism towards nerves, integrating the neuroimmune niche where they can influence innervation. In mouse, eosinophils found at steady state exert immunoregulation in parenchyma niche. Such regulatory eosinophils in humans need to be determined. The activation mode that eosinophils take in response to the specific "niche" will influence their mediator release (67).

2 Interleukin-33

2.1 Generalities

IL-33 is a member of the IL-1 family and is constitutively expressed by various cell types including epithelial cells of mucosa exposed to the external environment (e.g., skin, gut, and lung) (71). Following an insult, necrotic epithelial cells passively release IL-33 which initiates and amplifies type 1 and type 2 immune responses (70).

Many cells can respond to IL-33, including immune cells participating in type 2 immunity, namely Th2 cells, ILC2s, eosinophils, basophils, and mast cells. To respond to IL-33, these cells express the suppression of tumorigenicity 2 (ST2) receptor encoded by the *Il1rl1* gene. ST2 belongs to the IL-1R superfamily. Upon the IL-33 binding to ST2, the co-receptor IL-1 receptor accessory protein (IL-1RAcP) is recruited leading to coupling of Myd88 and subsequent activation of the mitogen-activated protein kinase (MAPK) and nuclear factor-κ B (NF-κB) pathways (72).

Although IL-33 is part of the same family as IL-1β and IL-18, cleavage of a “pro” sequence is not required to activate it upon release. Indeed, it has been shown that full-length IL-33 has biological activity. Full-length IL-33 consists in *(a)* an amino-terminal domain containing a nuclear localization sequence (NLS) and a chromatin binding motif (CBM), *(b)* a central linkage domain and *(c)* a carboxy-terminal domain responsible for binding to ST2. One could however add that IL-33 can be cleaved within its linkage domain by different proteases. This generates fragments of IL-33 with higher biological activity depending on the cleavage site (73).

Because IL-33 is a potent pro-inflammatory cytokine that is constitutively expressed and may activate a lot of immune cells, a fine regulation of its activity is required. First, in homeostasis, IL-33 is retained in the nucleus through its NLS and CBM domains (74). Second, an alternative mRNA splicing of *Il1rl1* gene produces a soluble ST2 (sST2) which is a truncated form of the receptor that acts as a “decoy” to sequester IL-33 in the extracellular medium. When a rise in IL-33 occurs after a trauma, a subsequent increase in sST2 expression ensues to compensate the potential deleterious effects of IL-33 (75). Third, rapid oxidation (within 2 hours) of released IL-33 leads to the formation of disulfide bridges in its C-terminus, disabling ST2 binding (76). Fourth, during apoptosis, caspase-3 and -7 cleave and inactivate IL-33. This process enables the programmed cell death to occur without triggering any inflammation (77). Finally, a negative regulation can occur upon IL-33 binding to ST2: preformed IL-1R8, also known as single immunoglobulin IL-1R-related (SIGIRR) can form a complex with ST2 that interferes with the recruitment of IL-1RAcP and impairs downstream signaling (78).

2.2 IL-33 and asthma

Several studies have demonstrated the implication of IL-33 in asthma in mice and human.

First, exogenously administered IL-33 induces lung eosinophilia, type 2 cytokine production, AHR and goblet cell metaplasia, hallmarks of asthma (71,79). Also, in an OVA-induced asthma model, an additional administration of IL-33 has been shown to aggravate airway inflammation, AHR and remodeling when compared to antigen challenge alone (80).

Second, in humans, it has been shown that bronchial epithelial cells from asthma patients express elevated levels of IL-33 transcripts compared to healthy subjects. In addition, levels of IL-33 are more elevated in BAL, serum and induced sputum of asthmatic patients (81,82). This may suggest that epithelial cells in asthma secrete more IL-33 or/and are exposed to environmental clues favoring IL-33 release (81). Furthermore, IL-33 levels (mRNA and protein) have been correlated with asthma severity (82,83). Last, genome-wide association studies indicate that variants of the *IL33* gene causing an increase IL-33 expression are associated with asthma risk and eosinophilia (84). Reinforcing this association between elevated IL-33 expression and asthma risk, de Kleer and colleagues have demonstrated that IL-33 release during postnatal lung development promotes type 2 immunity, which could explain why and how some polymorphisms in *IL33* are associated with asthma development in childhood (27). Finally, a mutation causing *IL33* loss-of-function has been shown to decrease blood eosinophil counts and protect from asthma development (85).

Numerous clinical trials have been or are being conducted on IL-33 and other alarmins, cytokines that alert the immune system of the tissue damage, as potential therapeutic targets for the control of allergic as well as non-allergic asthma (52). Itepekimab (SAR440340/REGN3500) is an IL-33 neutralizing monoclonal antibody (mAb). Results from the phase-2 trial in patients with moderate-to-severe asthma have shown that IL-33 blockade enhances asthma control and lung function. Interestingly, blood eosinophil counts were reduced. This could be due to the blockade of IL-33 effect on eosinophils directly or on ILC2s which are a source of IL-5 (86). In addition to antibodies targeting IL-33 directly, antibodies directed against its receptor called IL-1 Receptor-Like 1 (IL-1RL1) (more commonly known as ST2) are also being developed. In a phase-2 study in severe asthma patients, astegolimab (MSTT1041A/RG 6149) significantly reduces the asthma exacerbation rate as in patients with high blood eosinophil counts (at least 300 eosinophils per µL) as in patients with low blood eosinophil counts (less than 150 eosinophils per µL) (87).

2.3 Effect on eosinophils

IL-33 has an indirect effect on eosinophils by its activating effect on Th2 cells and ILC2s, which in turn secrete IL-5 (Fig.4). But IL33 can also directly activate eosinophils, which express ST2. Indeed, *in vitro* studies on human blood eosinophils have demonstrated that IL-33 increases eosinophil adhesion as well as expression of cell surface markers such as CD11b. Moreover, eosinophil survival is enhanced with IL-33 stimulation, although the effect is weaker than that of IL-5 (88–90). In addition, IL-33 has been shown to trigger superoxide and IL-8 production, as well as degranulation (89,90). However, eosinophil degranulation was not observed by Suzukawa et al. following IL-33 stimulation (88).

The transcriptomic response of eosinophils to IL-33 was assessed in a study on murine eosinophils where cells were cultured for 4 hours with IL-33. This study revealed that IL-33 activates 2 major responses in eosinophils. First, and foremost, IL-33 activates the NF-κB pathway, as expected from ST2 activation, and leads to the secretion of IL-6 and IL-13 (91). Second, IL-33 induces the release of IL-4 that auto-stimulates eosinophils via STAT-6 phosphorylation. Our laboratory also analyzed the short-term transcriptomic response of murine but also human eosinophils to IL-33 (44).

Together, these studies have demonstrated that IL-33 is a potent activator of eosinophils and elicits, within a few hours, a pro-inflammatory gene expression program driven by the NF-κB pathway.

2.4 A 2-phase response to IL-33 in macrophages

Macrophages encompass a heterogeneous population of phagocytic cells with many distinct phenotypes. Macrophages play a critical role in homeostasis, tissue remodeling and host defense. In a very simplified view, tissue macrophages can be polarized into two opposing subsets depending on the microenvironment: classically activated macrophages (CAMs) and AAMs. CAMs differentiate in response to type 1 cytokines such as IFN-γ and contribute to the clearance of extra- and intracellular pathogens. Conversely, AAMs are induced by type IL-4 and IL-13 and are involved in anti-inflammatory responses, clearance of dying cells and tissue remodeling and repair (92).

IL-33 favors macrophage polarization towards the AAM phenotype *in* vitro when combined with IL-4 or IL-13. Furthermore, intranasal injection of IL-33 results in ST2-dependent polarization of lung macrophages into AAMs (93). At the transcriptional level, the response of macrophages to IL-33 was recently shown to comprise 2 distinct phases. Within the first hours of stimulation, IL-33 induces the expression of pro-inflammatory genes via activation of NF-κB pathway. Days later yet, IL-33-stimulated macrophages switch to an anti-inflammatory AAM-like program, making them able to digest and clear necrotic cells, and, thereby to contribute to the resolution of inflammation (Fig.7) (94).

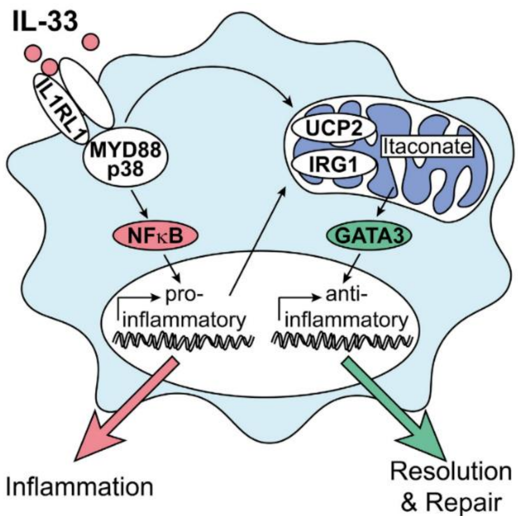

Fig.7: 2-phase action of IL-33 on macrophages. Upon tissue injury, IL-33 first induces pro-inflammatory phenotype in macrophages via activation of Nf-κB pathway (**left**). Days later, IL-33-stimulated macrophages rewire their mitochondrial metabolism, consequently activating the transcription factor GATA-3, which reroutes the transcriptional program towards a pro-resolution and repair phenotype (**right**) (94).

HYPOTHESIS, OBJECTIVE, AND STRATEGY

Eosinophils display some level of plasticity that could tailor their protective or pathogenic functions in asthma. The alarmin IL-33, a type 2 immune signal important in asthma, is a prime candidate regulator of eosinophil plastic responses. Especially, IL-33 has been shown to elicit a 2-phase plastic response in macrophages shifting them from an inflammatory phenotype towards pro-resolving and pro-remodeling activities. This raises the possibility that a similar late reprogramming of eosinophils could occur in asthma under the effect of IL-33, tailoring lung eosinophil functions over time.

The objective of this master's thesis is thus to characterize the "early" and "late" gene expression programs of eosinophils in response to IL-33, as well as to identify key signaling pathways involved.

A specific strategy was established to achieve this aim (Fig.8). First, eosinophils were purified from the blood of voluntary donors and stimulated *ex vivo* with IL-33 and IL-5 for 6 or 96 hours in order to capture their early and late responses, respectively. Two control conditions were included. First, a fraction of the purified eosinophils was analyzed directly, providing a baseline condition of "naïve" blood eosinophils. Second, a culture with IL-5 alone served as a control condition. IL-5 had to be provided in the experimental group treated with IL-33 and in this control condition in order to allow eosinophil survival *in vitro* (95). RNA was subsequently extracted, and its quality was assessed. cDNAs were synthetized and underwent quality control. Then, cDNAs were sequenced, and differential gene expression (DGE) was calculated. Subsequently, several software presented in section 4.3.2 were used to infer activated or inhibited pathways.

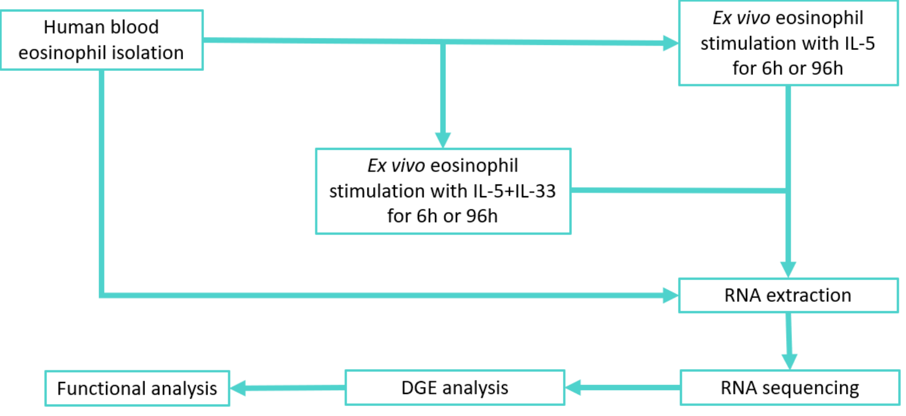

Fig.8: Experimental outline.

1 Blood sample origin

This study was undertaken in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained from all study participants.

Donor	Sex	Allergic status	Eosinophil count/µL
1	Man	Allergic	500
2	Woman	Allergic	/
3	Man	Healthy	<50

Table 1: demographic information of donors.

2 Human blood eosinophil isolation

2.1 Polymorphonuclear cell isolation by double-layer density gradient centrifugation

To isolate eosinophils, we first isolated polymorphonuclear (PMN) cells using double-layer density gradient centrifugation. 3 mL Histopaque®-1119 (1.119 g/mL), 3 mL Histopaque®-1077 (1.077 g/mL) (Sigma-Aldrich, Missouri, USA), and 5 mL whole blood were carefully layered. The samples were centrifuged at 600 x g for 20 minutes at 20 °C and without active deceleration. The top PBMC layer was first discarded and the second PMN cell layer was subsequently collected (Fig.9).

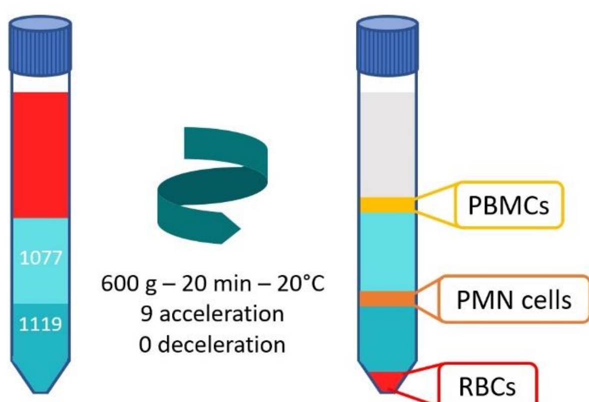

Fig.9: Fractionation on a density gradient. Eosinophils segregate with PMN cells away from peripheral blood mononuclear cells (PBMC) and red blood cells (RBC) in classical density gradients. During the centrifugation, the cells migrate until they are no longer able to pass through a higher density layer than their own cellular density. Hence, different cell rings are found after centrifugation. (personal illustration)

2.2 Immunomagnetic negative selection of blood eosinophils

Eosinophils were purified from PMN cells by immunomagnetic negative selection using EasySep™ Human Eosinophil Isolation kits and EasySep™ magnets (STEMCELL™ Technologies, Vancouver, Canada) following the manufacturer's instructions (Fig.10).

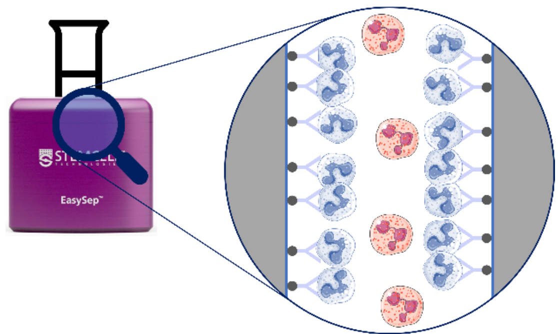

Fig.10: Immunomagnetic negative selection of blood eosinophils. Magnetic bead-coupled antibodies target all cells other than eosinophils. Placed in a magnetic field, marked cells are retained in the so-called positive fraction, allowing the collection of a negative fraction containing more than 95 per cent of eosinophils. This figure was produced using Servier Medical Art (https://smart.servier.com/).

3 Eosinophil stimulation with IL-33

The setup of *ex vivo* eosinophil stimulation was as follows. First, the number of eosinophils recovered from each patient was assessed after isolation using a Scepter™ 2.0 Handheld Automated Cell Counter and associated Scepter™ Sensors – 60 µm (Merck, Darmstadt, Germany). Second, for the baseline condition, 25,000 to 100,000 eosinophils were directly placed in 1 mL TRIzol™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the samples were vortexed and stored at -80°. Then, 25,000 to 100,000 eosinophils were put in culture with recombinant human IL-5 (PeproTech) at 50 ng/mL alone (negative control) or with recombinant human IL-33 (R&D Systems, Minneapolis, Minnesota, USA) at 100 ng/mL. The culture medium was prepared using the components in Table 2. After 6 or 96 hours of culture (at 37°C with 5% of CO_2), the eosinophils were placed in 1 mL TRIzol™. All the TRIzol™ tubes were stored at -80°C for downstream RNA applications.

Component	Quantity
IMDM, no phenol red (Fisher Scientific)	38.5 mL
Fetal Bovine Serum (FBS) (Fisher Scientific)	10 mL
Penicillin-Streptomycin (5,000 U/mL) (Fisher Scientific)	1 mL
MEM Non-Essential Amino Acids Solution (100X) (Fisher Scientific)	500 µL
2-Mercaptoethanol 50 µM (Fisher Scientific)	50 µL

Table 2: Culture medium components.

4 RNA applications

4.1 RNA extraction

High-quality RNA was obtained from eosinophils as follows. Before starting the RNA extraction, benches, hoods, and all small equipment (pipets, tip boxes, gloves…) were passed at RNAse AWAY™ (Thermo Fisher Scientific) solution to eliminate all the RNAse and to avoid RNA degradation. The TRIzol™ tubes (non-stimulated eosinophils, after 6 or 96 hours of stimulation) were thawed at room temperature (RT). 200 µL of chloroform (Merck) were added to each TRIzol™ tube which were then vortexed for 20 seconds and incubated for 2 minutes at RT. After centrifugation at 10 000 x g for 15 minutes at 4 °C, RNA was found in the upper phase which was collected and to which 2 µL GlycoBlue™ (Thermo Fisher Scientific) and 475 µL isopropanol (Thermo Fisher Scientific) were subsequently added. Then, the samples were incubated for 10 minutes at RT. After another centrifugation at 10 000 x g for 15 minutes at 4 °C, the supernatant was carefully removed without disturbing the blue pellet. The pellet was washed with 500 µL ethanol 75% and the tubes were centrifuged at 10 000 x g for 1 minute at 4 °C. Next, 430 µL of supernatant were removed and the tubes were centrifuged at 10 000 x g for 30 seconds at 4 °C. The rest of the supernatant was carefully removed. Then, the dried pellet was resuspended in 40 µL UltraPure™ DNase/RNase free water (Thermo Fisher Scientific). Any remaining DNA was eliminated using the DNase included in the RNA Clean and Concentrator kit (ZYMO RESEARCH) and the samples were passed on purification columns according to the manufacturer's instructions. RNA integrity and quantity were assessed (Annex 1) using the RNA 6000 Pico kit and the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, California, USA) following the producer's directives. RNAs were processed even

4.2 Library preparation and sequencing

RNAs were reverse transcribed using SMART-Seq® v4 Ultra® Low Input RNA kit (Takara Bio, Kusatsu, Shiga's prefecture, Japan), then resulting cDNAs were isolated with AMPure XP beads (Beckman Coulter, Brea, California, USA) following the manufacturer's instructions. The samples were stored at -80 °C. Integrity and quantity of purified cDNAs were assessed (Annex 2) using High Sensitivity DNA kits (Agilent Technologies). The cDNA libraries were prepared using Nextera® XT DNA library preparation kit (Illumina, San Diego, California, USA) and sequencing was performed with NovaSeq™ 6000 sequencing system (Illumina). The sequenced reads were first aligned to the human genome (HG19) with RNA-seq Alignment (2.0.2) using STAR aligner (2.6.1a) on BaseSpace (https://basespace.illumina.com). For each sample, a report on the quality of the alignment was provided with "Insert Length Distribution", "Alignment Distribution" and "Transcript Coverage". A table with the number of reads per genes (read counts) for each condition was retrieved from BaseSpace.

4.3 *In silico* analysis

4.3.1 Differential gene expression analysis

We first aimed to identify genes which are significantly differentially expressed (DE) between different conditions using the DESeq2 R package. The principle of DESeq2 is based on the comparison of relative RNA abundance (depicted by normalized read counts) between experimental and control conditions. The result consists of an equal, up- or down-expression of genes compared to the control or to another test condition (96).

DGE was calculated using the DESeq2 package (1.34.0) in R (4.1.0). The genes with a sum of read counts through all samples of less than 150 were excluded from the analysis to eliminate background noise. Then, a design formula needed to be defined. This enables to indicate to the statistical software the known sources of variation to be controlled, as well as the factor of interest to be tested in the differential expression tests. Here, donors were the source of variation known, which we specified in the design formula through donor identification ("Donor_ID"). The factors of interest were the treatment and its duration which were grouped into one variable ("group"). Thus, the design was written as `~ Donor_ID + group`. The hypothesis test used was the likelihood ratio test (LRT). This test compares a *full* model to a *reduced* one and the returned p-values of the whole model are determined solely by the difference in deviance between the *full* and *reduced* model formula. The reduced formula was `~ Donor_ID` in order to identify the actual statistic effect of the "group" variable.

After the DGE analysis, a principal component analysis (PCA) was performed using ggplot2 package (3.3.5) in R. Details are provided section 1 of Results chapter.

Next, the `contrast` argument in the `results()` function of DESeq2 was used to specify the comparison of interest to be extracted from the entire model. The code we used was in the format `results(dds, contrast=c(”condition”,”level_to_compare”, “base_level”))`. After this, log fold change (LFC) shrinkage is performed to correct for random variation in lowly expressed genes and obtain more accurate estimates of LFC when extracting the comparisons. Here, we used the ashr package (97) that enables to use contrast argument when specify the comparison of interest. The function we entered was `lfcShrink(dds,contrast=c(”condition”,”level_to_compare”,“base_level”).type="ashr")`. We used MA plot which present LFC in function of average of counts normalized for all genes to estimate the effect of the shrinkage. The plotMA associated to comparisons of interest are presented in Annex 3.

Finally, DE genes from paired comparisons were retrieved after LFC shrinkage and the criteria were: baseMean >50, absolute log_2 fold change (LFC) >1, and the associated adjusted p-value <0.05. BaseMean is the mean of normalized counts of all samples, normalized for sequencing depth. We put the threshold at >50 to eliminate background noise of low expressed genes.

4.3.2 Functional analysis

Activated or inhibited pathways were inferred from list of DE genes using several softwares presented below.

First, Ingenuity Pathway Analysis (IPA) developed by Qiagen is a knowledge-based pathway analysis application. Enrichment pathway analysis can be performed. Its algorithms compare the input gene list data to data originating from the literature and then, return the most significant activated or inhibited pathways. IPA also allows to identify upstream regulators which could induce the observed transcriptomic response. In addition, it provides the overrepresented diseases and functions in the input data set which could be the consequences of such up- or downregulation of specific gene expression (98). Briefly, outcomes from DESeq2 analysis (Log2 Fold Change and adjusted p-value) were uploaded into Qiagen's IPA system for core analysis.

Second, Ranked Gene Set Enrichment Analysis (GSEAR) is also a knowledge-based approach for identifying activated or inhibited pathways. The algorithm confronts the DE gene set to a control data set containing genes involved in a pathway of interest. Thereby, it gives an enrichment score (ES) that is related to the level of enrichment in genes implicated in the pathway considering the LFCs associated of these genes. The algorithm does this process for several pathways and thus, provides several ES with associated p-value and false discovery rate (FDR) (99). GSEAR analyses were performed on pre-ranked list of DE genes ordered according to their LFC. Online GSEAR v7.2.1 (genepattern.broadinstitute.org/gp/pages/index.jsf) was used with the "h.all. v7.2.symbols" (Hallmarks) gene sets and default parameters were used, except for a "classic" scoring scheme and minimal gene set size of 5.

ShinyGO is an online tool performing enrichment analysis. This web application compares the input list of significant DE genes to lists of genes correlating to pathways. At the end of the process, ShinyGO returns the pathways for which the input data set is enriched with associated fold enrichment, FDR and number of corresponding genes retrieved in the input list (100). Lists of DE genes had to be built from each condition and the up-regulated genes needed to be separated from the down-regulated ones. These lists were then entered in the online ShinyGO v0.75 (http://bioinformatics.sdstate.edu/go75/).

Finally, Rcis Target is an R package that identifies transcription factor (TF) binding motifs enriched in a given gene list based on databases containing motifs with genome-wide rankings. It infers potentially activated transcriptional regions and thereby, predicts the activation/inhibition of transcription factors (101). The same lists of genes built for ShinyGO were used for Rcis Target analysis (1.16.0) and the Gene-motif rankings database was hg19-500bp-upstream-10species.mc9nr (accessed on 17 May 2022).

RESULTS

1 Principal component analysis

Blood eosinophils were isolated from 3 healthy donors using first, a density gradient centrifugation and then, performing an immunomagnetic negative selection. Eosinophils were then stimulated with IL-33 and IL-5 for 6 or 96 hours in order to capture the early and late response, respectively. In addition to that, a fraction of the purified eosinophils was analyzed directly, providing a baseline condition of “naïve” blood eosinophils (“unstimulated” condition). Finally, another fraction was stimulated *ex vivo* with IL-5 alone for 6 or 96 hours, these cultures served as a control condition. RNA was extracted from the unstimulated condition and from the cultures of 6 and 96 hours. RNA quality was assessed, and majority of RNA integrity numbers (RIN) were >7 (Annex 1). cDNAs were synthetized and their quality was assessed (Annex 2). Then, cDNAs were amplified before being sequenced and resulting reads were aligned to the human genome. The quality of alignment was checked for each sample. A table with read counts per gene for each condition was retrieved. DGE was performed on these read counts with DESeq2 package on R using LRT test.

To get a first idea of the distribution of samples with respect to each other, we performed a principal component analysis (PCA) on the normalized reads obtained after DGE analysis. This method allows a reduction of the dimensionality of large datasets to make them interpretable while minimizing the loss of statistical information (loss of variability) caused by this reduction.

Briefly, each sample is placed virtually in a space that has as many dimensions as there are variables. In this case, the variables are the first 500 genes that have the most variability between samples, that is, whose expression (represented by the number of reads) is the most variable. Thus, a 500-dimensional space is created in which each sample is placed according to the expression level of the 500 genes. The PCA method will then reduce this number of dimensions to make them visualizable and interpretable. New variables, the principal components (PCs), are calculated and constructed from linear combinations of the initial variables (the 500 genes). These combinations are made in such a way that the PCs are not correlated with each other and that most of the information of the initial variables is compressed in the first components. The first PC will therefore contain the most information (explain the biggest fraction of the variance), the second a little less and so on. The PCA thus provides the dominant directions (depicted by PCs) of the highest variability in our dataset. A 2-dimensional graph whose axes are the first PCs can be created to investigate how samples cluster.

In figure 11, the resulting PCA on our 15 samples is presented. First, the 3 unstimulated samples cluster whether in PC1 or PC2 (circle in Figure 11a). This shows that isolated eosinophils are really closed to each other at baseline. Second, cultured and stimulated samples are distributed along PC1 and PC2. On the one hand, samples shift along the PC1 axis to the right after 6h and even more so after 96h of culture. At 6h, the 3 patients' samples clustered in PC1. Yet at 96h, donors 1 and 2 shift more to the right than donor 3 (arrows in Figure 11a). Therefore, PC1, which captures 47% of the variance, separates samples on the basis of culture time from left to right. On the other hand, samples stimulated with IL33+IL5 locate lower along the PC2 axis, which captures 21% of the variance. This is especially true at 6h of culture where samples stimulated with IL-33+IL5 are shifted downwards (dashed lines in Figure 11a). This shift is yet reduced at 96h of culture. To facilitate visualization of the distribution of the different samples of coming from each of the 3 patients, we also provide individual PCA plots in Figure 11b, c and d. This helps illustrate that the general distribution of samples is similar across patients, even though the amplitude of the changes at 96h is variable between patients. Altogether, our dataset is of good quality even though there is a donor-related heterogeneity in the response of eosinophils to culture.

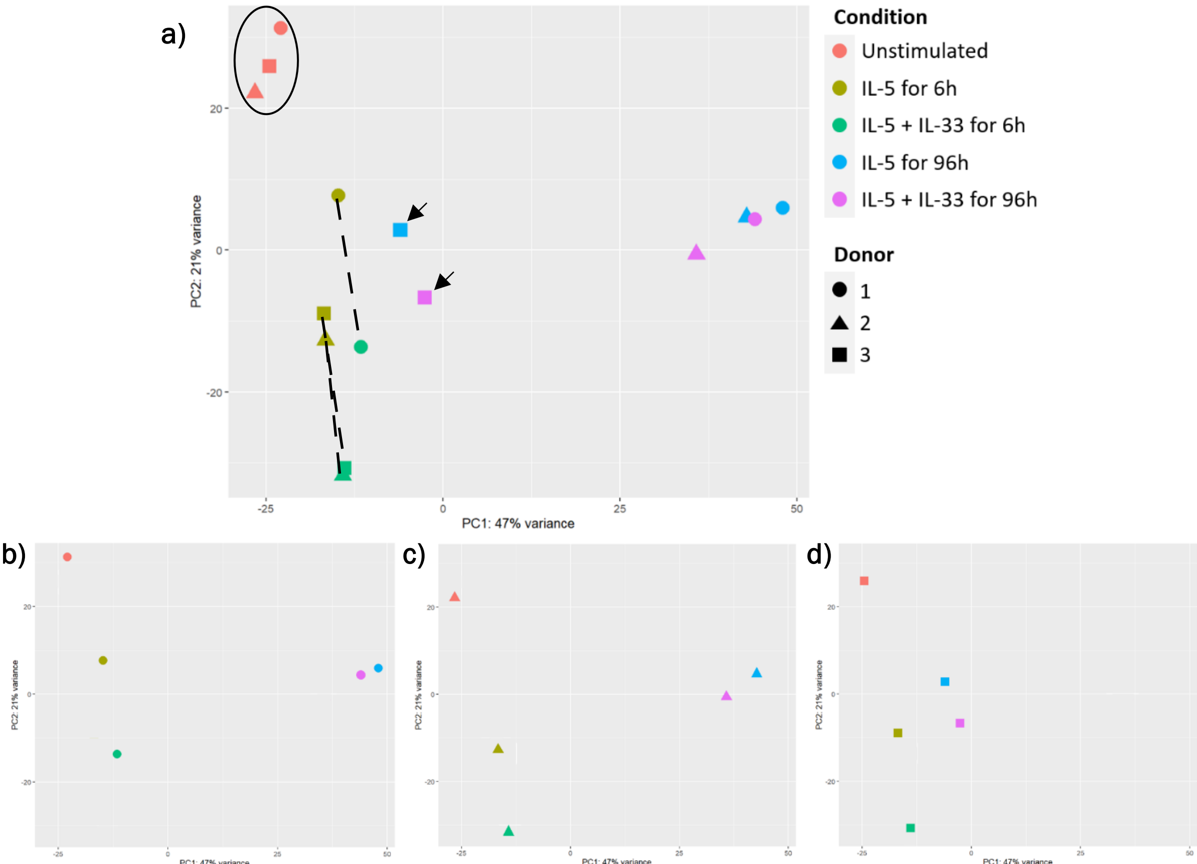

Fig.11: **Principal component (PC) analysis. (a)** Global PCA plot. **(b)** PCA plot of patient 1. **(c)** PCA plot of patient 2. **(d)** PCA plot of patient 3. Percentages on each axis represents the percentages of variation explained by the PC.

2 Transcriptomic response of human eosinophils to IL-33

2.1 Short-term transcriptomic response

Our PCA suggested that IL-33 elicits, as expected, a short-term transcriptomic response in human eosinophils. To single-out the short-term transcriptomic response to IL-33, we needed to compare eosinophils from the same donors stimulated for 6h with IL-5+IL-33 to those stimulated with IL-5 alone (Fig.12a). Thus, we extracted this comparison from the global DGE analysis as explained in section 4.3.1 of Material and Methods chapter.

Analysis for differential gene expression returned 136 DE genes (adjusted $p<0.05$, $|\log2(\text{fold change})|>1$, baseMean>50) (Fig.12b). Among these genes, 103 ones were upregulated (including *IL1A)* and 33 ones were downregulated such as *IL5RA*. A heatmap is also provided showing the pattern of differential expression of these genes across samples (Fig.12c). We thereby have captured the early transcriptional response to IL-33 in eosinophils.

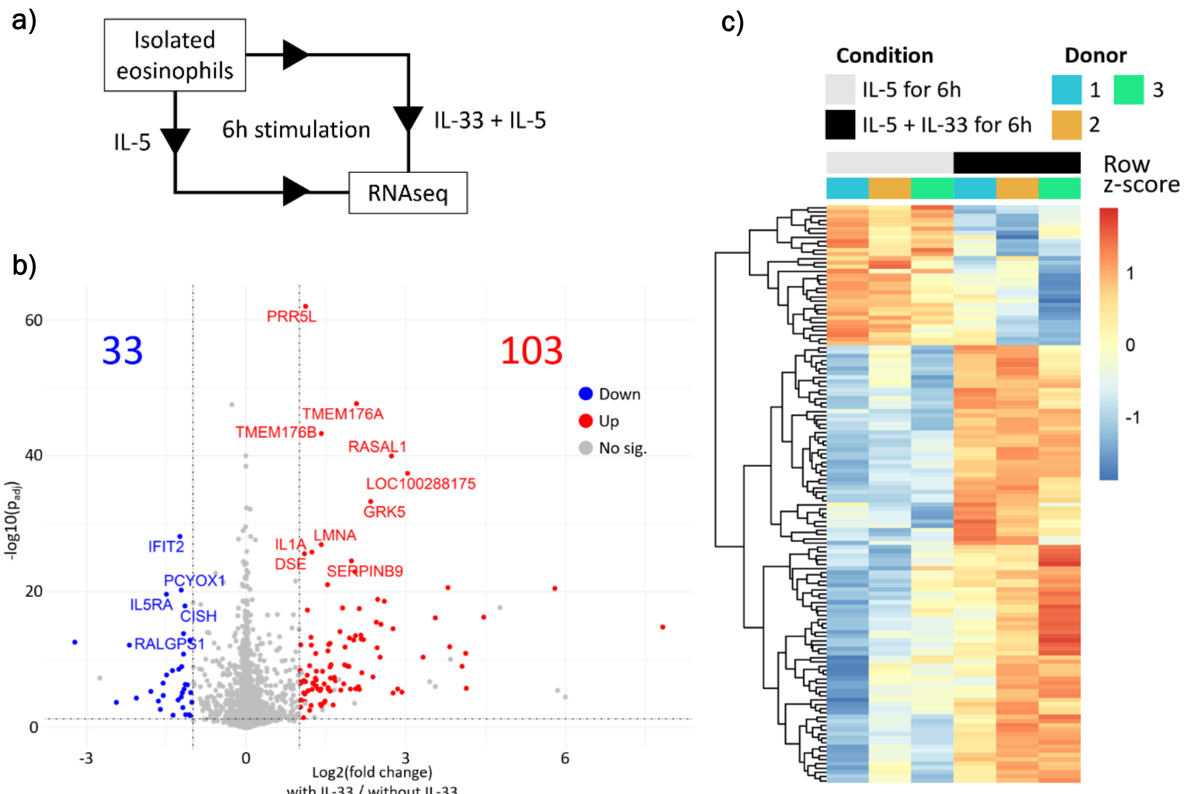

Fig 12: Short-term transcriptomic response of eosinophils to IL-33 stimulation. (a) Experimental layout. **(b)** Volcano plot of changes in gene expression of eosinophils in response to IL-33 stimulation (thresholds for DE genes: adjusted $p<0.05$, $|\log2(\text{fold change})|>1$, baseMean>50). **(c)** Heatmap of changes in expression of DE genes (scaling by row).

We next interrogated the biological pathways affected by IL-33 stimulation in eosinophis.

The 136 DE genes retrieved from DGE analysis were input in software of enrichment analysis such as IPA, ShinyGO and GSEAR.

First, the list of DE genes with their associated Log_2 Fold Change and adjusted p-values were entered in IPA. Tables of enriched biological process with associated z-score were returned: a z-score > 2 indicates that the process is activated while a negative z-score below -2 indicates a down-regulated process. We put the threshold at |z-score|>2.5 to filter the returned processes (Table 3). Among relevant processes with our context (cells in culture), “cell survival”, “cell movement” and “quantity of progenitor cells” were predicted to be activated. IPA also returned a list of enriched canonical pathways. In the top15 of pathways highly predicted as activated (Fig.13a), majority of pathways relied on an activation of a pro-inflammatory response. Among those, NF-κB pathway was also predicted as activated.

Second, we used ShinyGO, which returns gene ontology biological processes that are enriched based on user-provided gene list. In the first place, a list with the 103 upregulated genes was provided to identify predicted up-regulated pathways. We asked ShinyGO to return the first most significant 30 biological processes (with FDR < 0.05). Among these, NF-κB signaling was the most enriched geneset (Fig.13b), followed by processes related to response to TNF and to “molecule of bacterial origin". The graphical network returned by ShinyGO gathers processes based on the number of shared genes (thickness of connections), clustering them to easily have an overview on global path. Processes with the most significant FDR p-value (the darkest nodes) rely on cell survival (Fig.13c). In the second place, the 33 downregulated genes were entered in ShinyGO but no enriched process was returned.

Then, a pre-ranked list of DE genes ordered according to their LFC was input in GSEAR. No significant up- or downregulated pathway was identified.

Finally, results from ShinyGO and IPA were crossed to identify the redundant and potentially actually activated or inhibited pathways. Among the up-regulated processes, it reveals that NF-κB pathway and cell survival process were commonly returned (blue shaded Fig.13c). There is an overlap of genes between NF-κB pathway and regulation of apoptotic process (hypergeometric test p-value<0.001) (Fig.13 d & e). Indeed, NF-κB signature includes positive (*TNFAIP3*) and negative (*BIRC3*) regulators of apoptosis.

In conclusion, based on enriched pathways returned by enrichment analysis, IL-33 triggers the activation of NF-κB pathway in eosinophils as early response.

Categories	Diseases or Functions Annotation	p-value	Predicted Activation State	z-score
Organismal Survival	Organismal death	2.12E-08	Decreased	-3.101
Infectious Diseases	Infection of mammalia	1.10E-06	Decreased	-2.645
Developmental Disorder	Aplasia or hypoplasia	0.00012	Decreased	-2.503
Developmental Disorder	Hypoplasia	0.000196	Decreased	-2.503
Cell Death and Survival	Cell viability	1.23E-06	Increased	2.543
Cellular Movement	Cell movement	1.19E-08	Increased	2.572
Cellular Function and Maintenance	Cellular homeostasis	1.51E-05	Increased	2.632
Cell Death and Survival	Cell survival	4.14E-07	Increased	2.698
Cellular Movement	Cell movement of tumor cell lines	6.76E-05	Increased	2.754
Hematological System Development and Function,Hematopoiesis,Tissue Morphology	Quantity of hematopoietic progenitor cells	4.45E-06	Increased	3.151
Organismal Development	Size of body	5.55E-06	Increased	3.242
Tissue Morphology	Quantity of progenitor cells	4.27E-07	Increased	3.414

Table 3 : Biological processes returned by IPA as significantly activated or inhibited

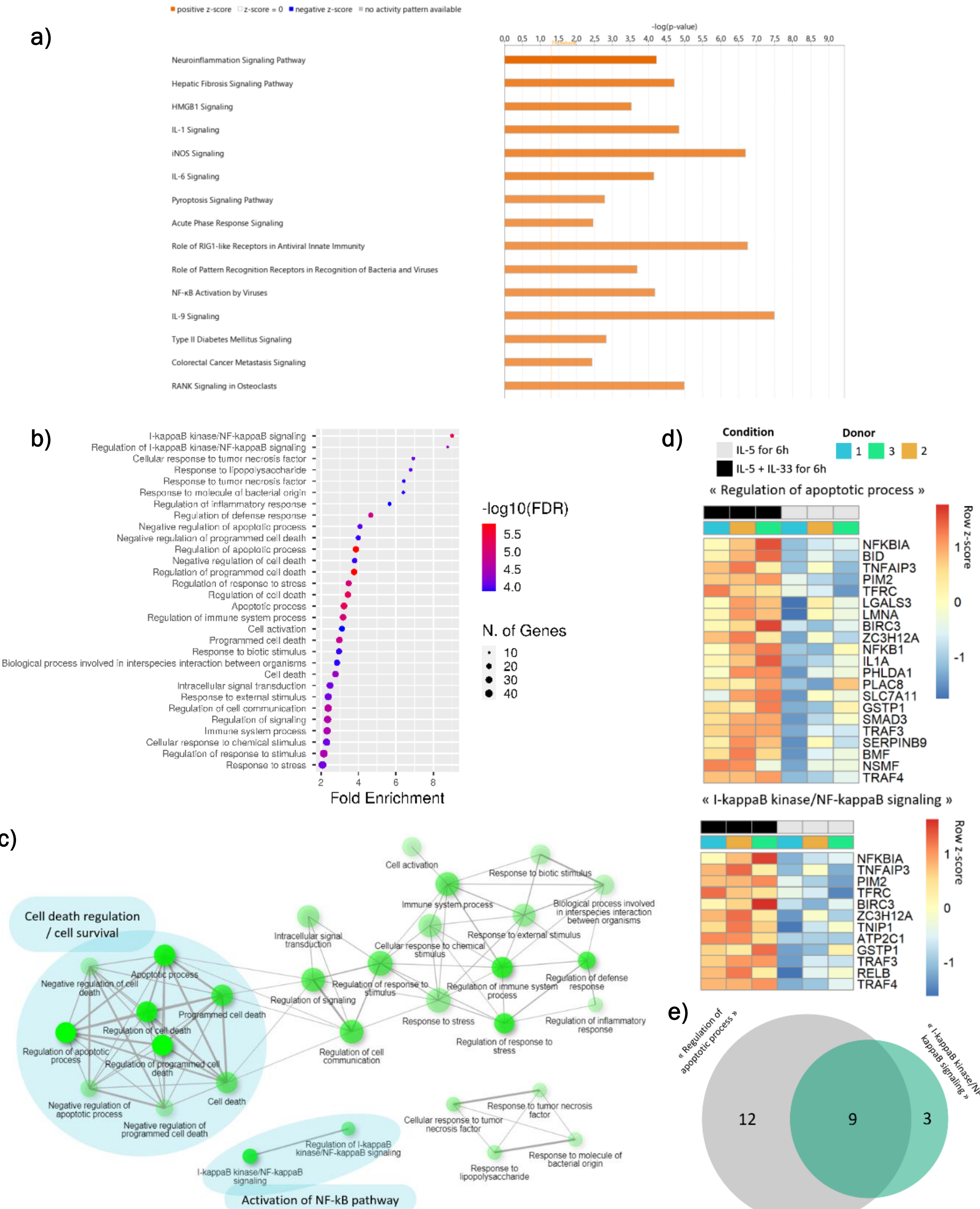

Fig.13: Enriched molecular pathways in human blood eosinophils in response to IL-33 stimulation for 6h. (a) Canonical pathways predicted as activated by IPA. **(b)** ShinyGO chart plot of top 30 enriched pathways. Circle size represent number of genes in the pathway that are included in the input gene list. Circle color reflects the significance of enriched pathways with red = the most significant. Fold enrichment of a term is designated as overrepresented compared to the background. **(c)** ShinyGO graphical network of top 30 enriched pathways. Two pathways (nodes) are connected if they share 50% or more genes (edge cut-off = 0.5). Darker nodes are more significantly enriched gene sets (FDR p-value cut-off = 0.05). Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes between pathways. Pathways that were commonly returned by IPA as significantly enriched (p-value<0.05 & |z-score|>2) are highlighted with light blue shades. **(d)** Heatmap of changes in expression of genes from most significant enriched pathway returned by ShinyGO and shared with IPA (scaling by row).**(e)** Venn diagram showing the overlap between the genes of the enriched pathways NF-kB and regulation of apoptotic process. Hypergeometric test p-value < 0.001.

The 103 up-regulated genes retrieved from DGE analysis were processed with RCis Target. It infers potentially activated transcriptional regions and thereby, predicts the transcription factors (TF) that are activated.

Rcis target returned 235 over-represented transcription factor (TF)-binding motifs. The top 10 binding motifs with the highest normalized enrichment score (NES) correspond to recognition motifs of RELA, REL, NFKB1 and BCL3, all subunits of TF NF-κB (Table 4). Thus, RCis Targets predicts the activation of NF-κB pathway. Common genes predicted target genes of the top 10 motifs are highlighted in bold. Among these, there are regulators of apoptosis (*BIRC3, SMAD3, TNFAIP3*).

Motif - Logo	NES	AUC	TF	Number of predicted target genes	Genes
cisbp__M4491	8.17	0.133	RELA	22	**BIRC3**;BMF;C3;**EGR2**;ENO3;HIVEP2;IFIH1;IL2RG;LMNA;NANOS3;**NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;PRR5L;RCAN1;**RELB;SMAD3;TMEM176A;TMEM176B;TNFAIP3**
factorbook__NFKB1	7.85	0.128	BCL3, NFKB1	20	**BIRC3**;BMF;C3;**EGR2**;HIVEP2;IFIH1;IL2RG;LMNA;NANOS3;**NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;PRR5L;**RELB;SMAD3;TMEM176A;TMEM176B;TNFAIP3**
cisbp__M1924	7.79	0.127	REL	14	**BIRC3;EGR2;NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;RCAN1;**RELB**;SLC39A8;**SMAD3;TMEM176A;TMEM176B;TNFAIP3**
hocomoco__TF65_HUMAN.H11MO.0.A	7.76	0.127	RELA	21	**BIRC3**;BMF;**EGR2**;HIVEP2;IFIH1;IL2RG;LMNA;**NFKB1;NFKB2;NFKBIA;NFKBIZ**;PIM2;**POU2F2**;PRR5L;RAB5A;RCAN1;**RELB;SMAD3;TMEM176A;TMEM176B;TNFAIP3**
cisbp__M3091	7.73	0.127	REL	14	**BIRC3;EGR2;NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;RCAN1;**RELB**;SLC39A8;**SMAD3;TMEM176A;TMEM176B;TNFAIP3**
transfac_public__M00053	7.47	0.123	REL	14	**BIRC3;EGR2;NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;RCAN1;**RELB**;SLC39A8;**SMAD3;TMEM176A;TMEM176B;TNFAIP3**
cisbp__M4498	7.45	0.123	RELA	23	**BIRC3**;BMF;C3;**EGR2**;ENO3;HIVEP2;IFIH1;IL2RG;LMNA;NANOS3;**NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;PRR5L;RAB5A;RCAN1;**RELB;SMAD3;TMEM176A;TMEM176B;TNFAIP3**
cisbp__M4497	7.43	0.123	RELA	19	**BIRC3;EGR2**;HIVEP2;IFIH1;IL2RG;NANOS3;**NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;PRR5L;RAB5A;RCAN1;**RELB;SMAD3;TMEM176A;TMEM176B;TNFAIP3**
jaspar__MA0101.1	7.38	0.122	REL	14	**BIRC3;EGR2;NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;RCAN1;**RELB**;SLC39A8;**SMAD3;TMEM176A;TMEM176B;TNFAIP3**
cisbp__M4444	7.38	0.122	RELA	20	**BIRC3**;BMF;**EGR2**;ENO3;HIVEP2;IFIH1;IL2RG;LMNA;NANOS3;**NFKB1;NFKB2;NFKBIA;NFKBIZ;POU2F2**;PRR5L;**RELB;SMAD3;TMEM176A;TMEM176B;TNFAIP3**

Table 4: Top 10 of over-represented transcription factor-binding motifs returned by Rcis Target. Common genes predicted target genes of the top 10 motifs are highlighted in bold. AUC, area under the curve; NES, normalized enrichment score; TF, transcription factor.

2.2 Comparison with previous data of the laboratory

My host laboratory has already studied the early transcriptomic response of human eosinophil to IL-33 stimulation *ex vivo* (44). They first aimed to determine whether reactivity of residual eosinophil from patients treated with mAb neutralizing IL-5 were impacted by this IL-5 depletion. For this purpose, three group of patients were constituted: healthy donors, SEA patients receiving omalizumab (an anti-IgE antibody) and SEA patients treated with mepolizumab (an anti-IL-5 antibody). They isolated blood eosinophil and a fraction was directly processed for RNA extraction, providing the baseline transcriptomic signature ("unstimulated"condition). The other fraction of purified eosinophils was stimulated *ex vivo* for 6h with IL-33 only. Then, they compared the transcriptomic response elicited by *ex vivo* stimulation between omalizumab- and mepolizumab-treated groups. Patients did not segregate in function of treatment groups showing that all patients responded to IL-33 stimulation in the very similar way. Subsequently, to get an idea about the gene expression programs elicited by IL-33, they compare the stimulated eosinophils with non-stimulated counterparts. They have shown that NF-κB pathway was upregulated upon stimulation that is consistent with what we have shown in this study (44).

To compare this dataset with ours, the table of read counts was retrieve from Basespace and was merged with our own read count table. To determine whether technically these data are comparable to our data, we performed a DGE analysis (with a similar design formula as that of our previous analyses) and inspected data distribution in a PCA plot (Fig.14). We observed that all unstimulated samples clustered tightly together in PC1. Then, along PC1, samples stimulated with IL-33 alone for 6h were shifted to the right like our samples stimulated for 6h (with IL-5 alone or with IL-5+IL-33). This made us confident we could compare samples even though they were not analyzed synchronously. Nevertheless, all samples stimulated for 6h do not position at the same place, which may be excepted since they underwent 3 different treatments (IL-33 or IL-5 alone or IL-5+IL-33).Thus, our data set can be compare to the data set where eosinophils were stimulated with IL-33 alone.

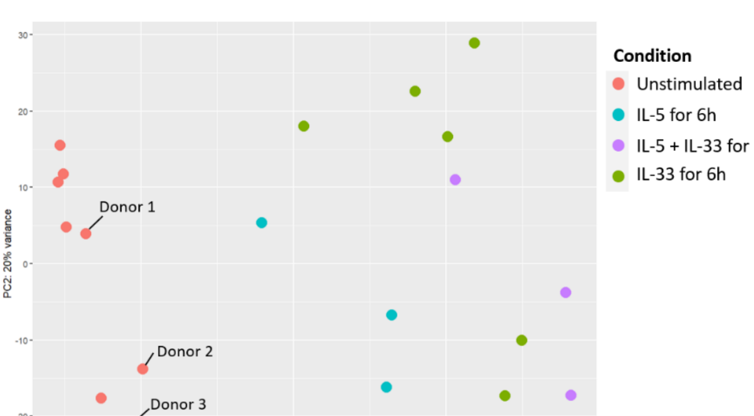

Fig.14 : Principal component (PC) analysis of merged data. Percentages on each axis represents the percentages of variation explained by the PC. In unstimulated condition, donors enrolled in the present study are annotated.

To compare the two datasets, we need to perform a new DGE analysis only with IL-33 alone data with the same LRT test than used in this study. This DGE analysis compared stimulated eosinophils with IL-33 to unstimulated eosinophils (Fig.15a). It results in 1,323 DE genes (Fig15b) that is much more than what was found comparing IL-33+IL-5 condition to IL-5 (136 DE genes). This is explained by the fact that comparison directly between IL-33 stimulated eosinophils with unstimulated ones comprises the effect of culture itself, which profoundly impacts on eosinophils (102). Then, a pre-ranked list of DE genes ordered according to their LFC was input in GSEAR. the default parameters, except for a "classic" scoring scheme. The most significantly upregulated hallmark process identified was "hallmark_TNFA_signaling_via_NFKB" (Fig.15c) consistent with what was previously published (44), and consistent with our own findings above.

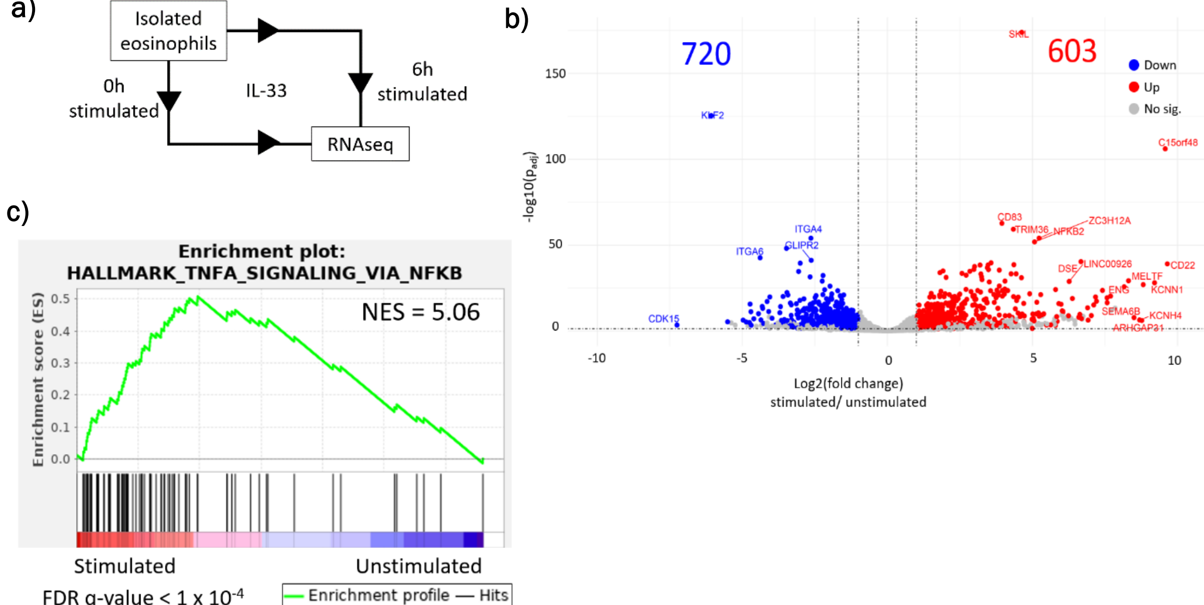

Fig.15: Transcriptomic response of blood eosinophils cultured with IL-33 alone for 6h. (a) Experimental layout. **(b)** Volcano plot of changes in gene expression of eosinophils in response to IL-33 stimulation (thresholds for DE genes: adjusted p<0.05, |log2(fold change)|>1, baseMean>50). **(c)** Ranked gene set enrichment analysis (GSEAR) plot for the indicated Molecular Signatures Database hallmark gene set. FDR, false discovery rate; NES, normalized enrichment score.

We could now better single-out the actual transcriptomic response induced specifically by IL-33 by comparing our list of 136 DE genes (comparing eosinophils cultured with IL-5 versus IL-5+IL-33) with the previously identified 1,323 DE genes (comparing freshly isolated blood eosinophils with eosinophils cultured with IL-33). A Venn diagram returned 108 common genes between the two comparisons (hypergeometric test p-value<0.001) (Fig.16a), that correspond to almost the entire set of DE genes in our study. By redoing a ShinyGO analysis with the overlapping genes, we found the specific IL-33 signature including the NF-kB pathway (Fig16.b)

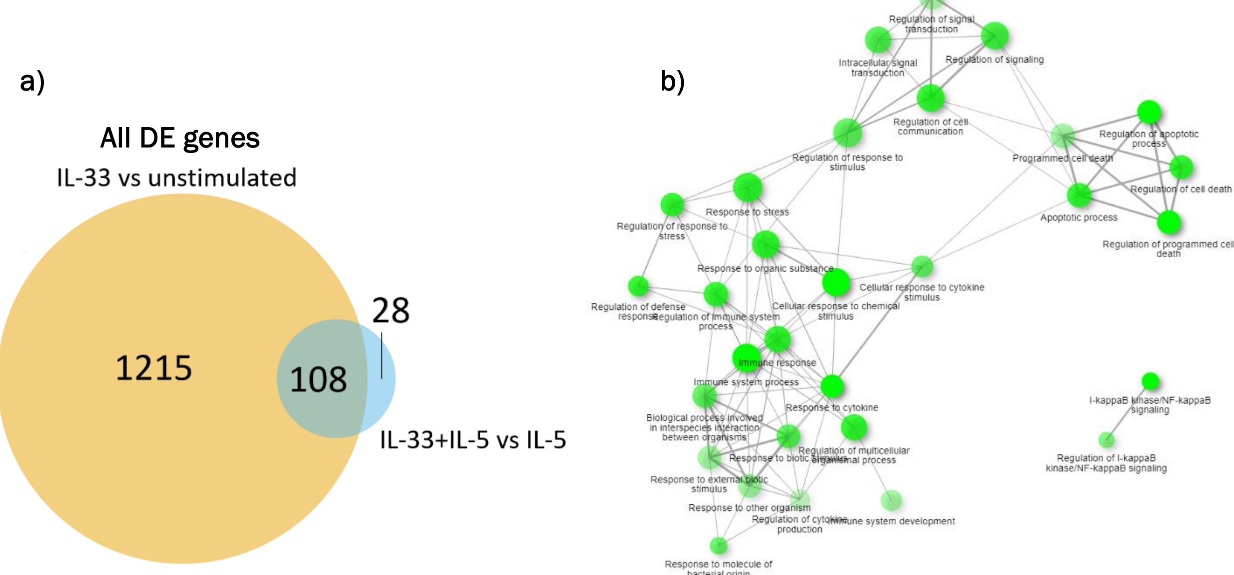

Fig.16: The specific response of IL-33. (a) Venn diagram showing the overlapped genes between the 136 DE genes (comparing eosinophils cultured with IL-5 versus IL-5+IL-33) and the previously identified 1,323 DE genes (comparing freshly isolated blood eosinophils with eosinophils cultured with IL-33). Hypergeometric test p-value < 0.001. **(b)** ShinyGO graphical network of top 30 enriched pathways when inputting the 108 overlapped genes in ShinyGO. Two pathways (nodes) are connected if they share 50% or more genes (edge cut-off = 0.5). Darker nodes are more significantly enriched gene sets (FDR p-value cut-off = 0.05). Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes between pathways.

2.3 Long-term response to IL-33

Finally, we aimed to assess the longer-term response of eosinophils to IL-33. If we return to our initial PCA, we can observe that there is no major response of the 3 donors to IL-33 at 96h, as samples treated with IL-5 or IL-5+IL-33 cluster tightly together for each individual patient (dashed lines in Fig.17a). Thus, it seemed that there is no identifiable IL-33-specific response after long-term stimulation.

This was confirmed by DGE analysis comparing eosinophils stimulated for 96h with IL-5+IL-33 to those stimulated with IL-5 alone (Fig.17b). Only one gene (*HLA-DRA*) was returned as differentially expressed (Fig.17c).

In conclusion, IL-33 does not seem to elicit a specific delayed response in eosinophils

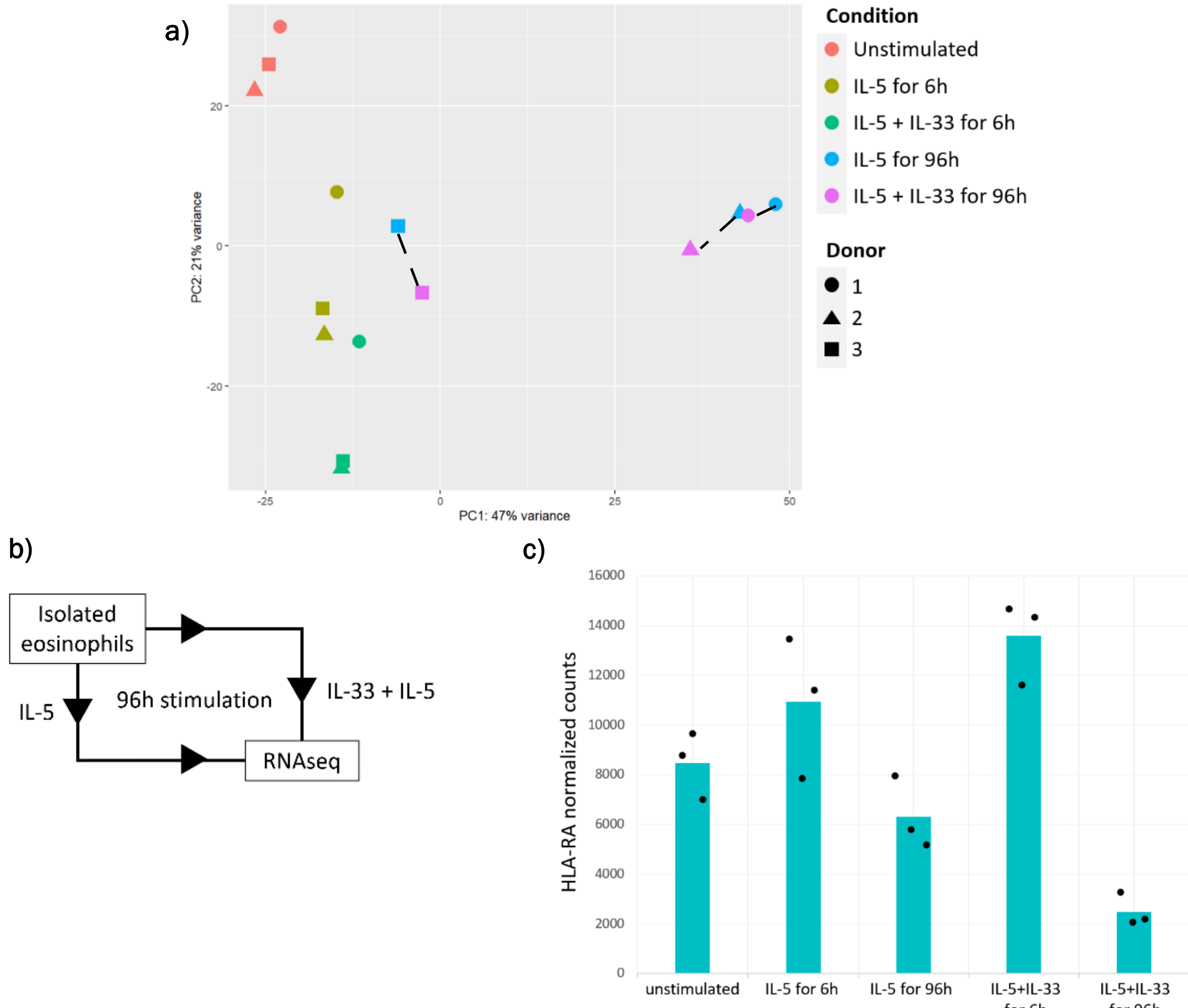

Fig.17: Long-term transcriptomic response of eosinophils to IL-33 stimulation. (a) Principal component (PC) analysis. Percentages on each axis represents the percentages of variation explained by the PC. **(b)** Experimental layout. **(c)** Bar plots of *HLA-DRA* gene normalized counts among all samples.

DISCUSSION

In this master's thesis, we studied the short and long-term transcriptomic response of human eosinophils to IL-33.

Our data indicate that human blood eosinophils respond early to IL-33 since the PCA showed segregation of samples considering the addition of IL-33 after 6h of culture. This response is predicted by pathway analysis to rely on the activation of the NF-kB pathway. Indeed, all functional analysis software tested, with the exception of GSEAR, returned NF-kB signaling as activated. The low performance of GSEAR could be explained by the number of input DE genes which was only about 136. The comparison with previous short-time *in vitro* study on human (44) from the laboratory confirms that NF- kB is activated in our data set. Therefore, IL-33 likely triggers the activation of NF-kB pathway to induce the expression of pro-inflammatory cytokines such as IL-1a. This observation is consistent with previous study in human (103) and murine (44,91) eosinophils that have demonstrated that IL-33 elicits a pro-inflammatory expression program involving NF-kB signaling within few hours of culture. Moreover, it is known that IL-1RL1/IL-33 axis activates this pathway in other cell types (71). The survival biological process was also promoted that probably results from activation of NF-kB, as genes between the two processes overlapped. Thereby, IL-33 might promote survival of eosinophils by eliciting the NF-κB pathway.

No pathway was identified as negatively regulated in the early response to IL-33. Indeed, ShinyGO and GSEAR did not return any pathway and biological processes returned by IPA were not relevant in relation to eosinophils. Nevertheless, one could note that the gene coding for IL-5Rα was down-regulated. This is in apparent contradiction with a previous study that reported that IL-33 up-regulates IL-5Rα mRNA in eosinophil progenitors, thereby promoting their responsiveness to IL-5 (104). This discrepancy may be explained by the different maturation stage as early eosinophil progenitors were considered in this study, compared with mature eosinophils in ours. Recent data suggest that IL-5 mostly promotes eosinophil expansion, rather than maturation, during eosinophilopoiesis (44). One could hence argue that eosinophil precursors need to express IL-5Rα in order to proliferate. In contrast, mature eosinophils that encounter IL-33 become activated and arguably, should see their lifespan controlled to eventually allow resolution of eosinophilic inflammation. Downregulation of IL-5Rα, which controls eosinophil survival in response to IL-5, might hence contribute to a feedback control of inflammation. Arguably yet, we did not observe reduced survival of eosinophils cultured with IL-33+IL-5 compared to those cultured with IL-5 alone after 4 days in culture. It also remains to be determined whether reduced *IL5RA* gene expression translates into downregulated surface expression of IL-5Rα in IL-33-stimulated eosinophils.

On the longer term, stimulation with IL-33 did not seem to alter the gene expression program of eosinophils. Several explanations for this lack of response can be proposed.

Firstly, the long duration of culture exhausts the eosinophils which no longer respond to the surrounding cytokines but just try to survive in a harmful environment (oxidative stress, toxins). Another possibility is that neither IL-5 nor IL-33 are available in the culture anymore because they have been consumed by the cells or because they are no longer stable. In this regard, IL-33 is easily oxidized leading to a conformational change and deactivation of the biological activity of IL-33 (76).

Another possibility would be that a negative feedback loop would inactivate IL-33-induced signaling. However, we could not detect such regulation in our RNAseq experiments since it does not involve *de novo* RNA synthesis. Rather, it relies on the activation of already existing regulatory proteins such as SIGIRR that interfere with IL-33 binding to ST2 (78). The ST2 receptor can also be internalized or protein inhibitors such as IKKα and IKKβ can block its signaling (105). A limitation of this study is that only the expression of messenger RNAs is taken into account, so we omit all the regulatory phenomena outside of gene expression that may be involved. Proteomic studies would then be useful to complement the characterization of the response to IL-33.

Finally, an explanation that depends on the biology of eosinophils could be given: the cells cultured are from peripheral blood. It is then possible that blood eosinophils are not sufficiently reactive to IL-33 because they have not received a signal allowing them to be more labile. Indeed, the plasticity of eosinophils has been mainly demonstrated for tissue eosinophils (22,66). Thus, the influence of surrounding cytokines could play on the reactivity of eosinophils and "prime" them, allowing them to change their phenotype. It is true that "hypodense" blood eosinophils that are more reactive to stimuli and more labile have been identified (64). However, these eosinophils are not found in the PMN fraction, from where the eosinophils are isolated in this study, but they are with PBMCs since their density is lower than classical eosinophils. It would then be interesting to evaluate the long-term response of these hypodense eosinophils.

Interestingly, even if IL-33 does not trigger any specific pathway after 96 hours of stimulation, eosinophils change their transcriptomic signature over time. This could be due to the effect of long-term culture, as explained above. Nevertheless, another possibility should not be overlooked: IL-5 might induce a long-term response that shifts the eosinophil response. Indeed, the presence of IL-5 is the common point between the treatment of cultures. To test this hypothesis, the best control would be to culture eosinophils without IL-5. However, this is not possible because eosinophils die by apoptosis if they are not supplemented with a survival factor (95).

We observed substantial variability in the response to long term culture depending on the donor. On the one hand, a technical cause may explain this observation. In fact, the number of eosinophils obtained from the three donors was very variable. We were able to culture about 100,00 cells per condition for the first two donors while a minimum of cells was isolated from the third donor allowing to have 25,000 cells per condition. Thus, the cell concentration during *in vitro* stimulation was different between the first two patients and the third. In all likelihood, the cultures with a high cell concentration would consume the provided cytokines quicker, activating a deprivation response sooner. Conversely, cultures with few cells would not deplete the available stock of cytokines which could still exert their effect. Moreover, a high concentration of cells induces more oxidative stress since more waste products are produced. Considering the difference in availability of cytokines as well as the oxidative stress induced, eosinophils that were cultured at low concentration would thus express a different transcriptional signature of those cultured at high concentration.

An alternative hypothesis is that differences in response to long term stimulation and culture could have a biological origin and depend on the medical condition of the donor. Indeed, samples from allergic donors clustered and samples from the donor 3, who is non-allergic, responded differently. Also, this discrepancy might relate to the eosinophilic status as the third donor had a low count in eosinophils. However, we did not assess the eosinophil counts of the second donor, thus, we cannot then determine whether this difference in behavior relies on eosinophilia. In any case, the very low number of patients is not enough to draw any conclusion. To do so, another study should be conducted with more donors who would be grouped considering their allergic status and eosinophil counts.

A limitation of this study is the culture of eosinophils. Indeed, the cells are deprived of their "natural" environment and one of the hypotheses of the origin of eosinophil diversity is based on the dependence of eosinophils on the surrounding cellular niche to change their phenotype (69). This niche is lost during culture and therefore the behavior of eosinophils in vitro is not the same as in vivo. It would therefore be interesting to study the short- and long-term transcriptomic response of pulmonary eosinophils after intranasal injections of IL-33 in mice. Another limitation could be the number of donors that does not enable us to establish any conclusion about donor-related effect regarding response heterogeneity to culturing However, this low number was chosen as a first pilot considering the long, delicate and costly procedures involved. Moreover, all samples responded homogeneously to IL-33 stimulation. It seems therefore that the number of donors is appropriate for our main question.

In conclusion, IL-33 induces an early pro-inflammatory program involving the NF-kB pathway in human blood eosinophils. IL-33 does not appear to change the phenotype of eosinophils after a prolonged stimulation as observed in macrophages. Finally, a heterogeneity of the eosinophil response to culture was observed that could deserve further scrutiny.

We are here in an acute model where IL-33 is given only once. It can then be considered that the effect of IL-33 has disappeared due to oxidation, deprivation or negative feedback from the NF-kB pathway itself. However, *in vivo*, eosinophil can remain in the presence of IL-33 for prolonged periods of time, undergoing repeated stimulation of their ST2 receptor. It would then be interesting to study the effect of a more chronic stimulation by adding IL-33 several times during the culture to determine the transcriptomic response after several rounds of activation.

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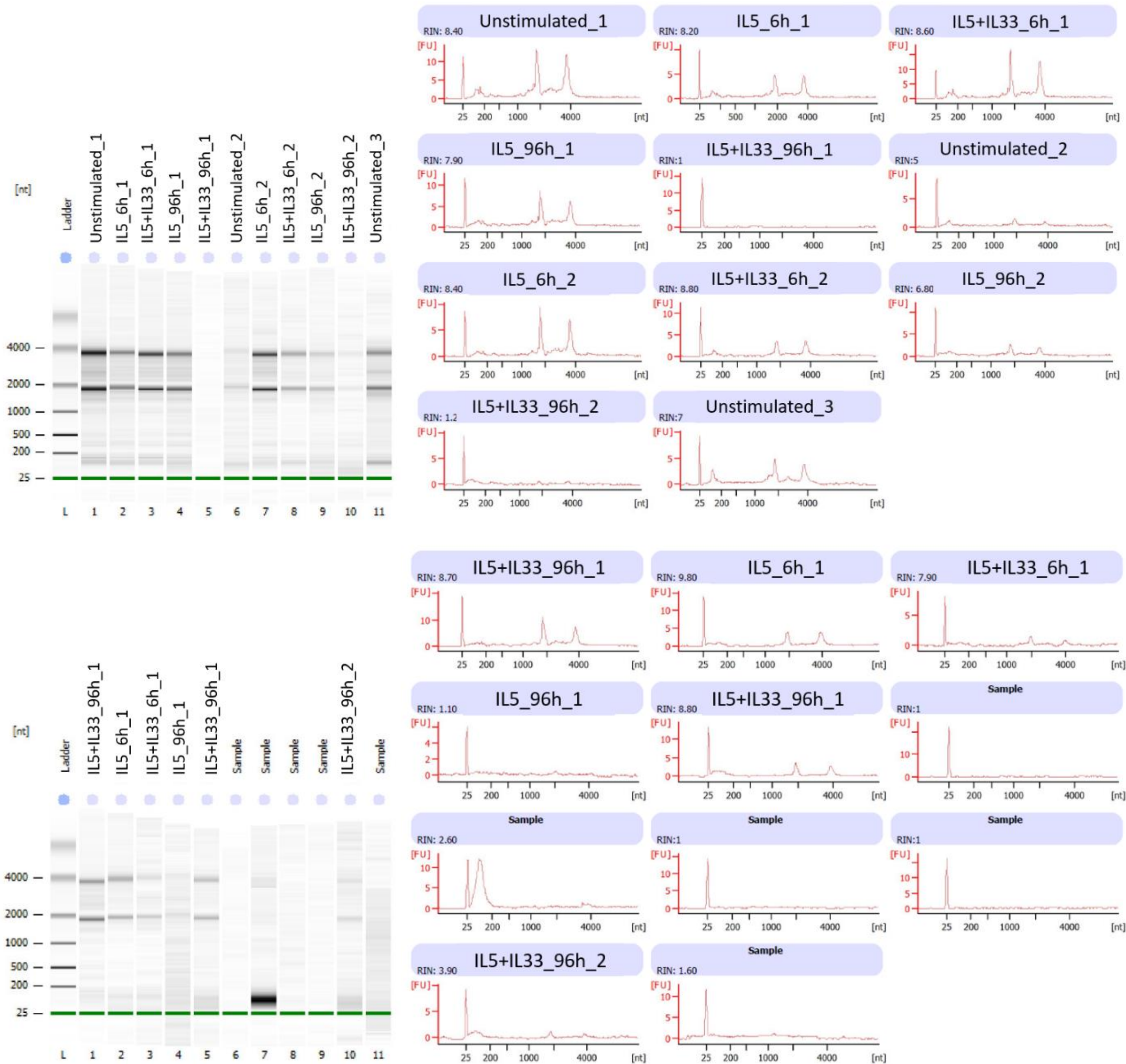
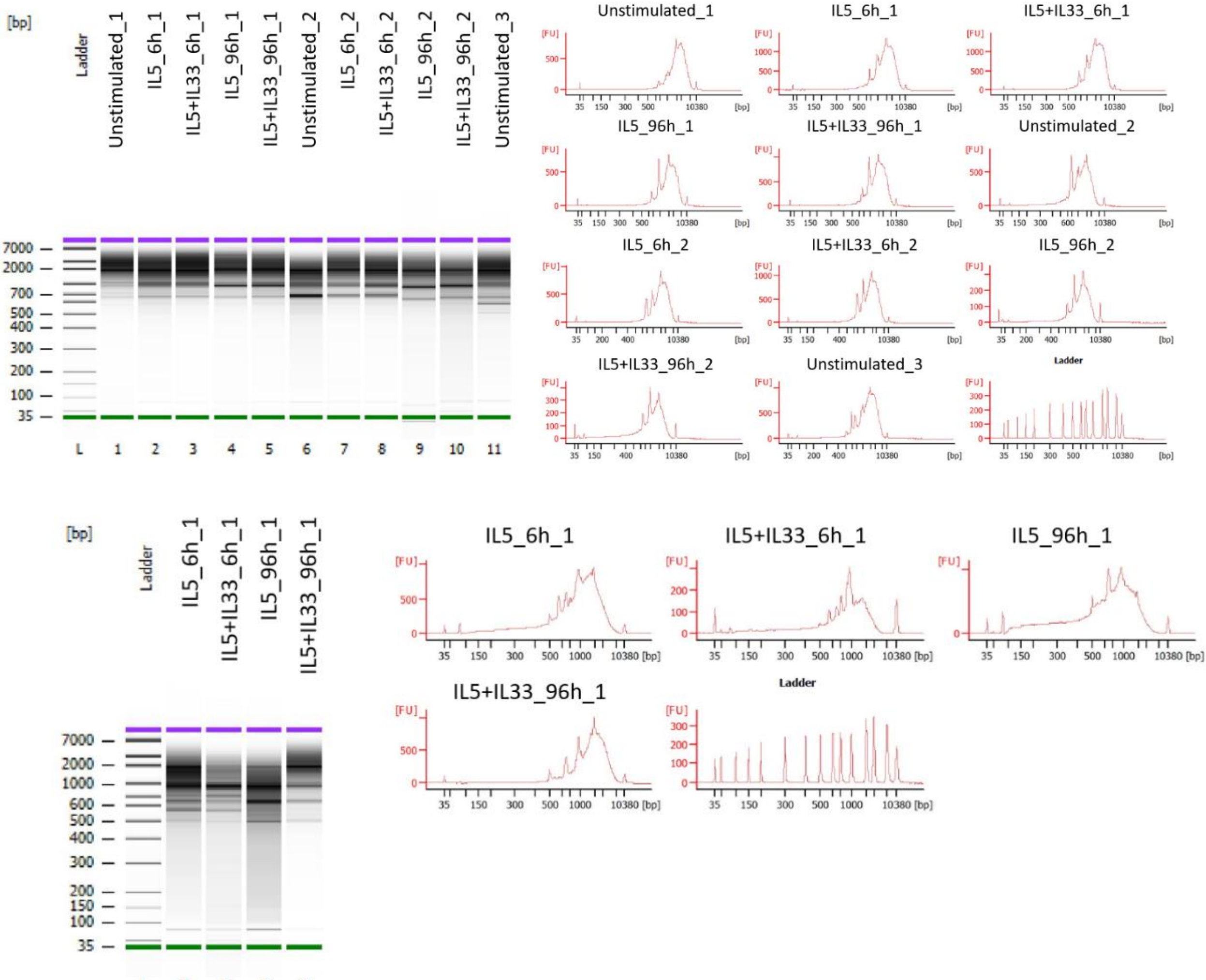
ANNEX

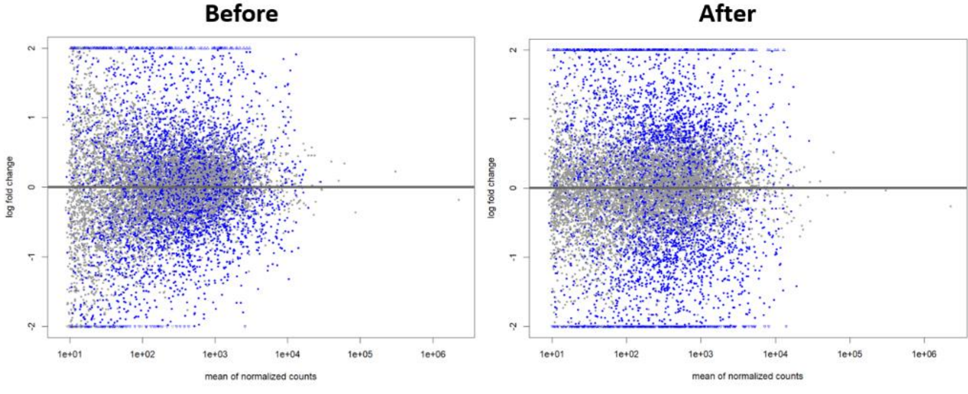
Annex 1: Quality control of extracted RNAs. RNA integrity and quantity were assessed using the RNA 6000 Pico kit and the Agilent 2100 Bioanalyzer System (Agilent Technologies).

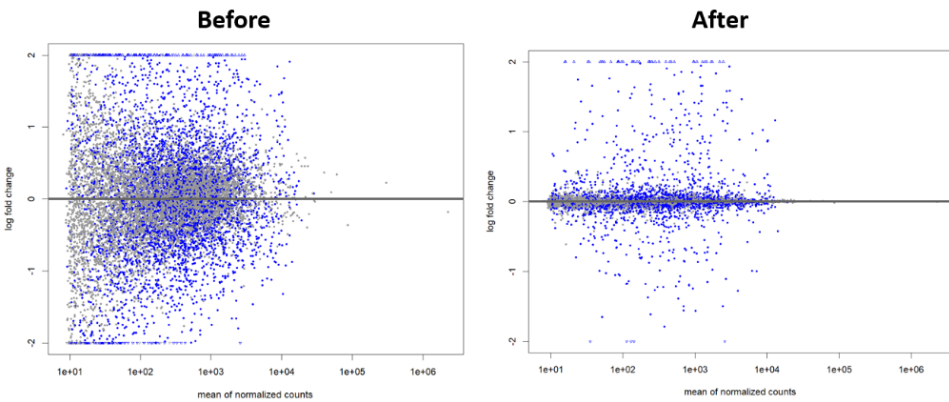


Annex 2: Quality control of synthetized cDNA. Integrity and quantity of purified cDNAs were assessed using High Sensitivity DNA kits (Agilent Technologies).



Annex 3: plot MA of DGE results before and after log2 fold change shrinkage for comparisons of interest. LFC shrinkage was made using ashr package (97).





Comparison: IL-33 for 6h vs unstimulated