Anti-IL5 mepolizumab minimally influences residual blood eosinophils in severe asthma

Glenn Van Hulst [1,4], Joseph Jorssen[1,4], Nathalie Jacobs[1], Monique Henket[2], Renaud Louis[2], Florence Schleich[2], Fabrice Bureau[1,3,4] and Christophe J. Desmet [1,4]

[1]Laboratory of Cellular and Molecular Immunology, GIGA Institute and Faculty of Veterinary Medicine, Liege University, Liege, Belgium. [2]Laboratory of Pneumology, GIGA Institute, Faculty of Medicine and University Hospital Center, Liege University, Liege, Belgium. [3]Walloon Excellence in Life Sciences and Biotechnology (Welbio), Wavres, Belgium. [4]Equal contributors.

Corresponding author: Christophe Desmet (christophe.desmet@uliege.be)

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Asthma patients receiving anti-IL5 therapies retain residual blood eosinophils, of which potential alterations remain unknown. This study shows that these residual eosinophils harbour largely unaltered quiescent and activated gene expression programmes. https://bit.ly/37od6QN

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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-IL5 biological therapies retain a very 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 IL5 neutralisation in patients. To tackle the effect of IL5 depletion on residual eosinophils, we used a comparative RNA-sequencing approach and compared the gene expression programme of eosinophils arising in IL5-depleted or IL5-replete human or murine hosts, at steady-state *in vivo* and following *in vitro* stimulation with the eosinophil-activating alarmin IL33. We compared blood eosinophils from patients with severe allergic eosinophilic asthma treated with anti-IL5 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 IL5. We report that restriction of IL5 availability did not elicit any detectable transcriptional response in steady-state residual eosinophils in mepolizumab-treated patients or IL5-deficient mice, and influenced only a handful of genes in their response to IL33. Together, these results support the notion that treatment with IL5 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 IL5 instigated the introduction of neutralising anti-IL5 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 very stable and interindividually consistent population of residual eosinophils persists in the blood of patients receiving anti-IL5 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 IL5 neutralisation in patients. Indeed, because of its radical effect on eosinophil amplification, IL5 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 IL5 neutralisation induces a maturational arrest of eosinophils in human bone marrow [16].

Therefore, even though the role of IL5 in eosinophil maturation has been less thoroughly studied, it is of high clinical relevance in the context of anti-IL5 biological therapies. In addition to reducing their numbers, withdrawing IL5 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 IL5 restriction *in vivo* display alterations in their gene expression programme.

Material and methods

Human subject characteristics and study design

We recruited 26 patients from the university asthma clinic of Liege (Centre Hospitalier Universitaire 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

TABLE 1 Demographic and functional characteristics of asthma patients in figure 4

	Mepolizumab	Omalizumab	p-value
Patients	10	10	
Gender (male/female)	2/8	2/8	1
Age (years)	56.1±9.45	39.5±9.6	0.0011
BMI (kg·m^{-2})	27.2±2.5	26.2±5.5	0.59
F_{eNO} (ppb)	49.11±35.0	18.7±12.2	0.019
FEV_1 (% predicted)	71.3±13.3	71.2±20.2	0.99
FEV_1 postBD (% predicted)	79.7±13.2	79.3±17.8	0.96
FVC (% predicted)	84.5±17.5	82.3±9.7	0.73
FEV_1/FVC	68.6±6.6	73.3±13.4	0.33
FEV_1/FVC postBD	74.1±9.5	76.2±12.6	0.68
RV (% predicted)	133±31.7	94.9±49.9	0.056
TLC (% predicted)	102.5±19.0	90.5±19.2	0.18
D_{LCO} (% predicted)	83.9±9.7	78.3±12.3	0.32
K_{CO} (% predicted)	96±18.3	92.8±13.9	0.71
ICS (yes/no)	10/0	10/0	1
OCS (yes/no)	0/10	0/10	1
LABA (yes/no)	10/0	10/0	1
LAMA (yes/no)	1/9	1/9	1
LTRA (yes/no)	0/10	4/6	0.00016

Data are presented as n or mean±SD, unless otherwise stated. Comparisons between patient groups were performed using unpaired t-tests for continuous variables. Chi-squared test was applied for categorical variables. BMI: body mass index; F_{eNO}: exhaled nitric oxide fraction; FEV_1: forced expiratory volume in 1 s; postBD: post-bronchodilator; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; K_{CO}: transfer coefficient of the lung for carbon monoxide; ICS: inhaled corticosteroids; OCS: oral corticosteroids; LABA: long-acting β_2-agonist; LAMA: long-acting muscarinic antagonist; SABA: short-acting β-agonist; LTRA: leukotriene receptor antagonist.

TABLE 2 Demographic and functional characteristics of asthma patients in figure 5

	Mepolizumab	Omalizumab	p-value
Patients	3	3	
Gender (male/female)	0/3	1/2	1
Age (years)	34±13.9	52.3±27.5	0.36
BMI (kg·m^{-2})	23.9±1.2	26.3±1.5	0.092
F_{eNO} (ppb)	35.3±37.1	30.3±9.5	0.83
FEV_1 (% predicted)	85±11.5	70.7±2.1	0.10
FEV_1 postBD (% predicted)	88±11.5	79.3±14.5	0.45
FVC (% predicted)	87.3±17.6	84±14.6	0.81
FEV_1/FVC	83±12.5	69.3±14.8	0.29
FEV_1/FVC postBD	80.7±7.1	75.3±18.2	0.66
RV (% predicted)	103.0±23.5	116.7 ±14.6	0.52
TLC (% predicted)	96.0±6.2	82±18.2	0.072
D_{LCO} (% predicted)	79.3±12.1	75.3±24.1	0.73
K_{CO} (% predicted)	85.3±3.2	100±7.8	0.39
ICS (yes/no)	3/0	3/0	1
OCS (yes/no)	0/3	0/3	1
LABA (yes/no)	3/0	3/0	1
LAMA (yes/no)	0/3	0/3	1
LTRA (yes/no)	2/1	0/3	0.1258

Data are presented as n or mean±SD, unless otherwise stated. Comparisons between patient groups were performed using unpaired t-tests for continuous variables. Chi-squared test was applied for categorical variables. BMI: body mass index; F_{eNO}: exhaled nitric oxide fraction; FEV_1: forced expiratory volume in 1 s; postBD: post-bronchodilator; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; K_{CO}: transfer coefficient of the lung for carbon monoxide; ICS: inhaled corticosteroids; OCS: oral corticosteroids; LABA: long-acting β_2-agonist; LAMA: long-acting muscarinic antagonist; SABA: short-acting β-agonist; LTRA: leukotriene receptor antagonist.

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 <80% forced expiratory volume in 1 s (FEV_1), blood eosinophil counts >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 100 mg 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% 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 ($CCR3^{+}SiglecF^{+}$) were sorted (purity ⩾95%) into TRIzol® (ThermoFisher) and stored at −80°C for downstream RNA applications.

Ex vivo *activation of eosinophils*

Mouse bone marrow eosinophils were stimulated for 4 h at 37°C and 5% carbon dioxide (CO_2) in culture medium and 100 $ng \cdot mL^{-1}$ purified IL33 (BioLegend), and 10 $ng \cdot mL^{-1}$ purified IL5 (Peprotech). Human blood eosinophils were stimulated for 6 h at 37°C and 5% CO_2 in culture medium and 100 $ng \cdot mL^{-1}$ purified IL33 (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 $\log_2$ 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 the *Il5* gene ($Il5^{-/-}$ 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

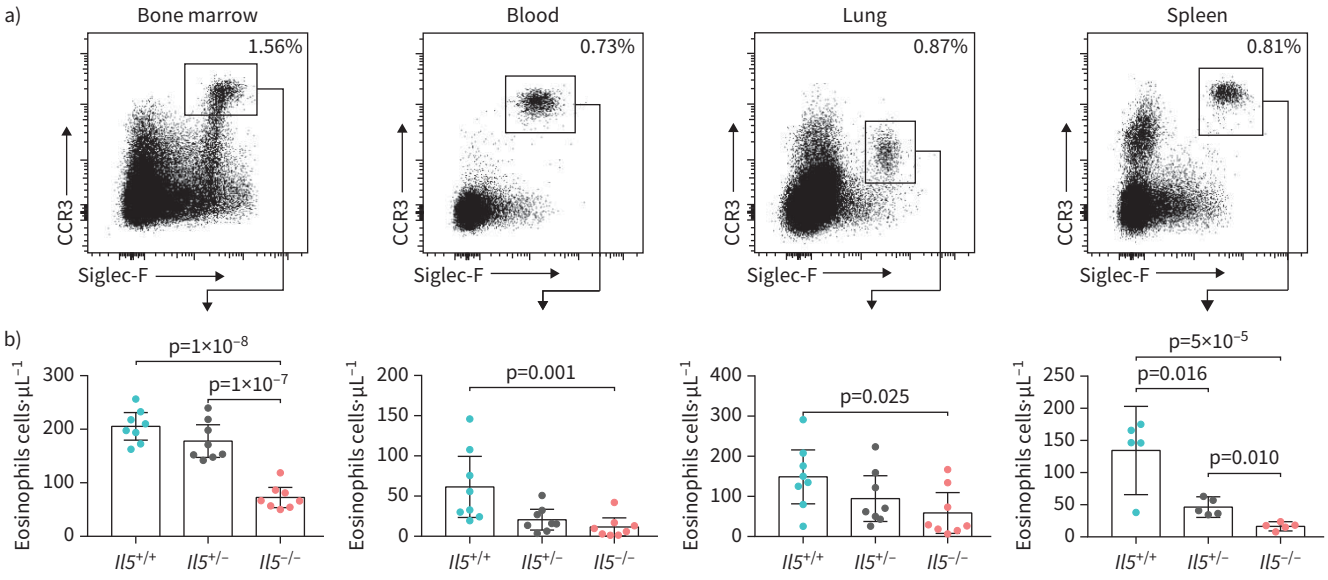

FIGURE 1 Interleukin (*Il*)5-deficient mice retain residual eosinophils. a) Representative plots of flow cytometric gating strategy with percentage of eosinophils in indicated organs. b) Quantification of eosinophils in specified organs of $Il5^{+/+}$, $Il5^{+/-}$ and $Il5^{-/-}$ mice as in a). Data were pooled from two to three independent experiments, presented as mean (95% 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.

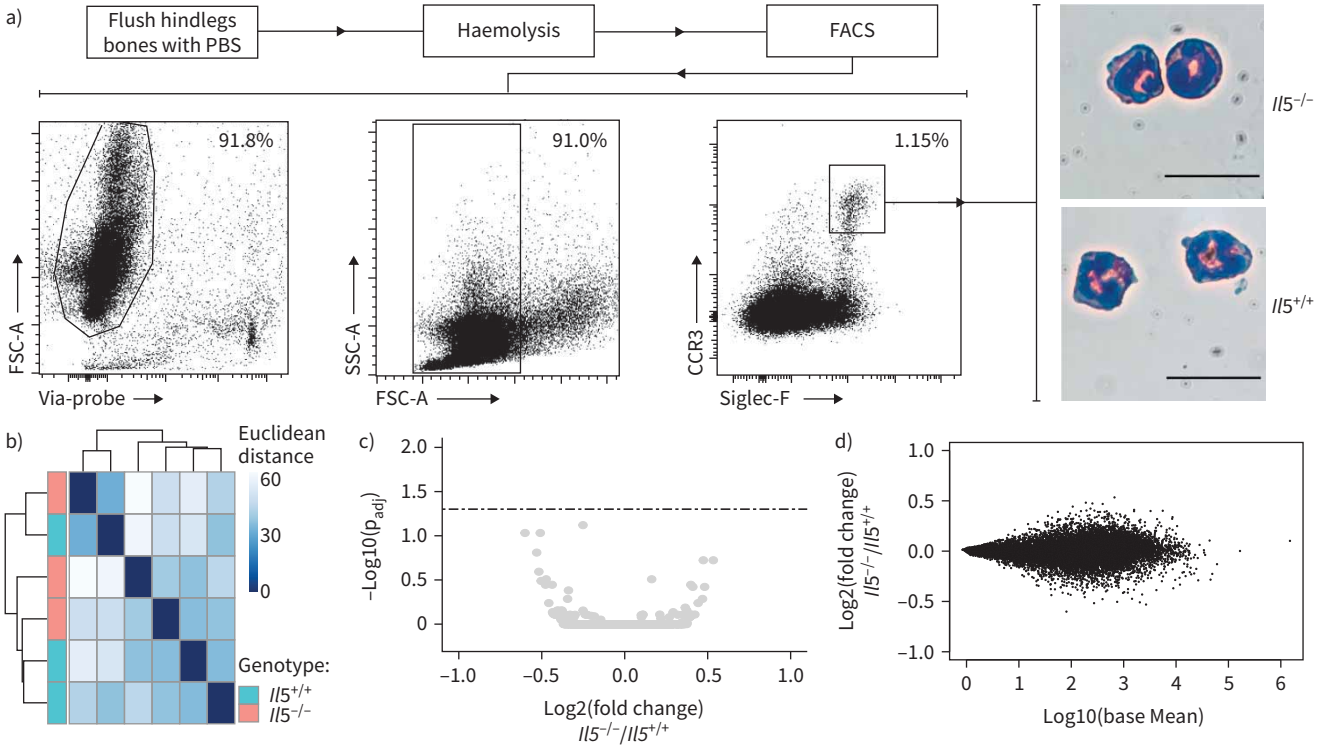

FIGURE 2 Genetic deficiency in interleukin (*Il*)5 has no detectable impact on mouse residual eosinophils. a) Isolation strategy of $Il5^{+/+}$ and $Il5^{-/-}$ mouse bone marrow eosinophils and representative post-sort light microscopy picture. Scale bars=15 µm. b) Sample clustering, c) volcano plot and d) MA plot based on RNA-sequencing of biological triplicates in a).

gene expression programme of steady-state mature BM eosinophils in mice is largely unperturbed by the total absence of IL5 during their development.

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 *Il5*$^{-/-}$ eosinophils could consequently only become apparent following their activation. To address this possibility, we stimulated BM eosinophils from *Il5*$^{-/-}$ and *Il5*$^{+/+}$ mice *ex vivo* with both IL5 and the alarmin IL33, 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 *Il5*$^{-/-}$ and *Il5*$^{+/+}$ 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% of variance in gene expression (figure 3b). Compared with their unstimulated counterparts, 2660 genes were differentially expressed (adjusted p<0.05, |log2(fold change)| >1) in stimulated eosinophils from both *Il5*$^{-/-}$ and *Il5*$^{+/+}$ 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 NF-κB pathway through its ST2 receptor [25]. Notably, PCA suggested that the response of eosinophils from *Il5*$^{-/-}$ and *Il5*$^{+/+}$ 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 *Il5*$^{-/-}$ *versus Il5*$^{+/+}$ eosinophils (figure 3f).

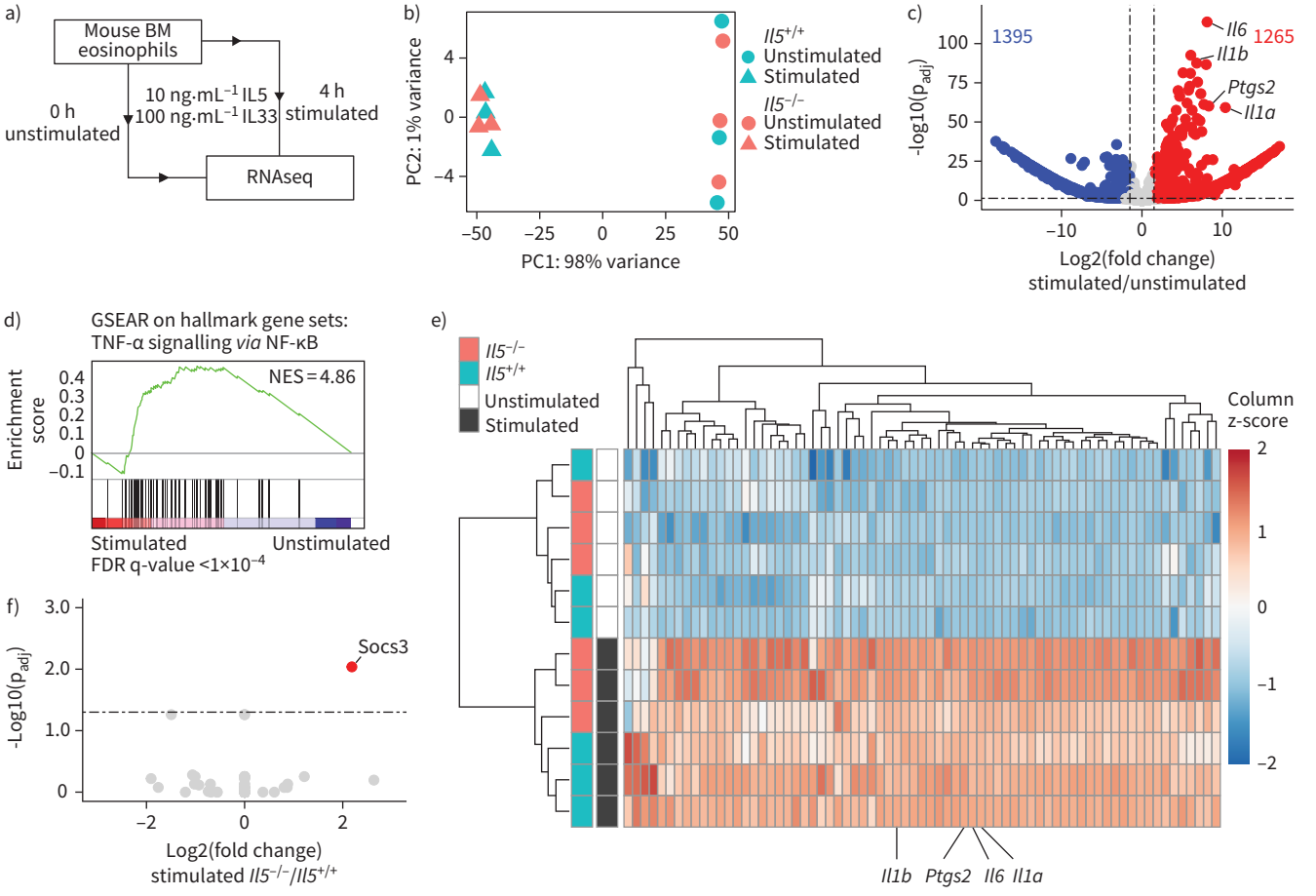

FIGURE 3 Response to stimulation of interleukin (*Il*)*5*$^{+/+}$ and *Il5*$^{-/-}$ bone marrow (BM) eosinophils. a) Experimental outline; b) principal component (PC) analysis; c) changes in gene expression of *Il5*$^{+/+}$ and *Il5*$^{-/-}$ eosinophils in response to stimulation; d) ranked gene set 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 *Il5*$^{-/-}$ *versus Il5*$^{+/+}$ eosinophils in response to stimulation. TNF: tumour necrosis factor; NES: normalised enrichment score; FDR: false discovery rate.

These observations suggested that deprivation of IL5 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 p<0.05, |log2(fold change)| >1) 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 IL5-depleted *versus* IL5-replete conditions. To this end, we collected blood eosinophils from an additional three mepolizumab-treated and three omalizumab-treated severely asthmatic

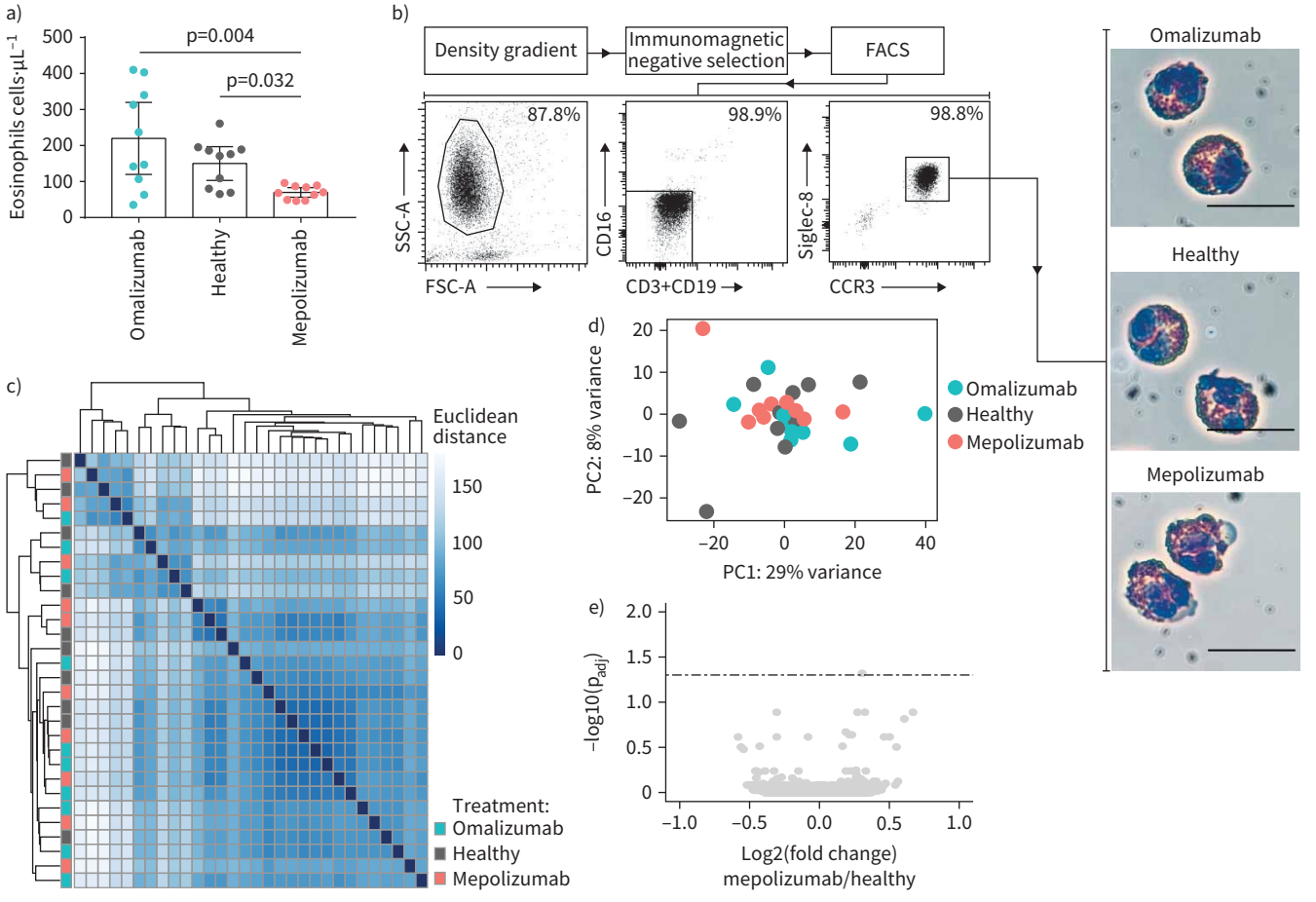

FIGURE 4 Transcriptomic impact of mepolizumab on human blood eosinophils. a) Eosinophil blood counts in healthy controls, and in severely allergic asthmatic patients receiving mepolizumab or omalizumab treatment (one-way ANOVA followed by Tukey honestly significant difference tests); b) blood eosinophil sorting strategy and representative post-sort light microscopy pictures of eosinophils from the three groups of donors in a); c) sample clustering by RNA-sequencing; d) principal component (PC) analysis; e) differentially expressed genes in eosinophils from mepolizumab-treated *versus* healthy control donors. Scale bars=15 μm. FACS: fluorescence-activated cell sorting.

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 IL33 before processing. We stimulated human eosinophils with IL33 alone to stay closer to the *in vivo* environment encountered by eosinophils in mepolizumab-treated patients, in whom IL5 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 IL33 had a very marked impact on human eosinophil gene expression. The first principal component in a PCA captured 56% of variance in gene expression and separated IL33-cultured samples from their unstimulated counterparts in each patient, whereas PC2 did not separate patient samples based on treatment and captured only 23% of the variance. This suggested that IL33 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 IL33 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 IL33 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 IL33 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 IL33 differed depending on the patients' treatment. Of potential interest still, one gene, *SOCS3*, was more robustly induced by IL33 in IL5-depleted eosinophils in both mice and human.

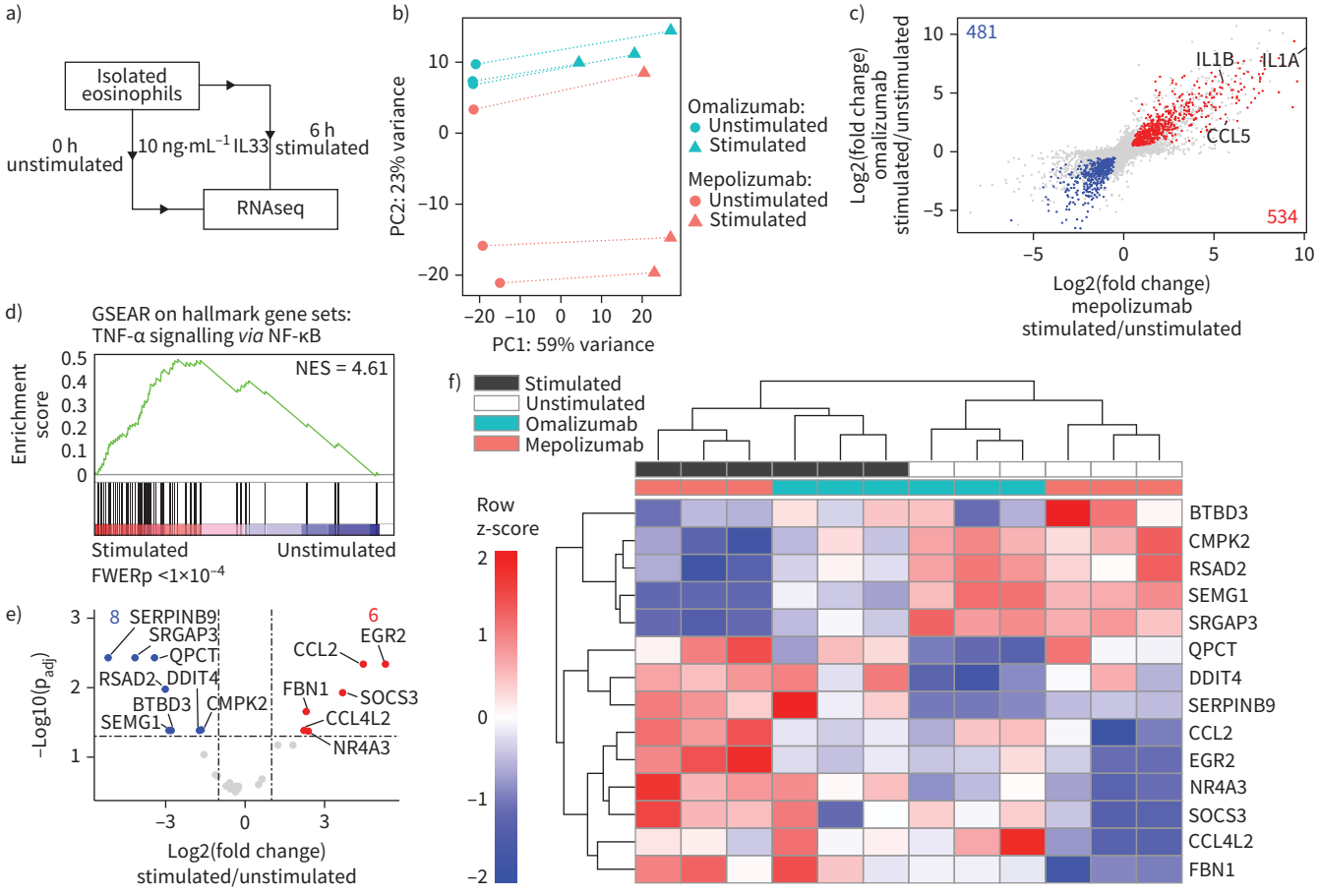

FIGURE 5 Transcriptomic response to stimulation of blood eosinophils from mepolizumab- or omalizumab-treated patients. a) Experimental outline; b) principal component (PC) analysis; c) correlation of changes in gene expression in response to interleukin (IL)33 in eosinophils from both patient groups; 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 IL5, through genetic deficiency in mice or through the administration of anti-IL5 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-IL5 biological therapies leave residual circulating eosinophils largely unaltered, albeit in reduced numbers.

This conclusion is based on congruent observations of the role of IL5 in two distant organisms. First, we studied syngeneic mouse strains differing only for their genetic proficiency or deficiency at producing IL5. Second, we compared severe allergic asthmatic patients receiving anti-IL5 or anti-IgE biological treatments. In both cases, depletion of IL5 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 IL5-depleted *versus* IL5-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 IL5 restriction, namely Socs3, was also more robustly induced in stimulated eosinophils from mepolizumab-treated patients. Altogether, these results suggest that IL5 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 IL12, 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 IL5-depleted conditions has biological consequences would be worth pursuing.

The absence of a major impact of the absence of IL5 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 IL5 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 IL5 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 IL5 on residual eosinophil function. We do not exclude that IL5 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 IL5 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 IL5, showed striking similarities with our analyses in human eosinophils.

Altogether, our results indicate that the restriction of IL5 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 IL5-neutralising antibodies spares a pool of circulating residual eosinophils largely resembling those of healthy individuals.

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