Phenotyping of asthma by multidimensional analysis

Focus on peripheral blood characteristics of severe asthma patients

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Fenotypering van astma door multidimensionele analyse

Met de nadruk op kenmerken in perifeer bloed van patiënten met ernstig astma

(met een samenvatting in het Nederlands)

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Ter nagedachtenis aan mijn opa Chris
Aan mijn vader en moeder

CONTENTS

List of abbreviations

ACQ	asthma control questionnaire
AQLQ	asthma quality of life questionnaire
AHR	airway hyperresponsiveness
ASM	airway smooth muscle
AUC	area under the curve
BAL	bronchoalveolar lavage
BB	bronchial biopsy
BMI	body mass index
CI	confidence interval
COPD	chronic obstructive pulmonary disease
CXCR2	interleukin-8R-beta
DA	discriminant analysis
EBC	exhaled breath condensate
ECP	eosinophilic cationic protein
FeNO	fraction of exhaled nitric oxide
FEV1	forced expiratory volume in 1 sec
f-MLF	formyl-methionyl-leucyl phenylalanine
FSC	forward scatter
GCMS	gas chromatography mass spectrometry
GINA	global initiative for asthma
GWAS	genome-wide association study
ICS	inhaled corticosteroids
IgE	immunoglobulin E
IL-5	interleukin-5
ILC	innate lymphoid cell
IQR	interquartile range
LABA	long-acting beta(2)-agonist
MARS	medication adherence reporting scale
MFI	mean fluorescence intensity
MPO	myelo peroxidase
NLPCA	non-linear principal component analysis
NO	nitric oxide
NOS	nitric oxide synthase
NPV	negative predictive value
OCS	oral corticosteroids
OVA	ovalbumin
PC	principal component
PCA	principal component analysis
PEF	peak expiratory flow
PPV	positive predictive value

RCT	randomized controlled trial
ROC	receiver operating characteristics
SD	standard deviation
SSC	side scatter
TSLP	thymic stromal lymphopoeitin
VEGF	vascular endothelial growth factor
VOC	volatile organic compound

INTRODUCTION

Asthma is a heterogeneous systemic inflammatory disease mainly affecting the airways. It is a common disease that affects ~334 million people worldwide [1]. Many patients can be treated well and reach adequate control with inhaled corticosteroids (ICS) and beta-agonists, but 5-10 % suffers from severe disease requiring intensive treatment or remain uncontrolled despite treatment [2]. In The Netherlands severe-asthma prevalence was found to be a bit lower, around 3.6% [3]. This small subgroup accounts for most of the morbidity and mortality and consumes an estimated 60% of total health care costs of overall asthma care [4].

Asthma is defined by symptoms and variable expiratory airflow limitation. In the 2015 guidelines of the Global Initiative for Asthma (GINA) asthma is defined as [1]:

> *(..) a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.*

Importantly, neither the symptoms nor the variable expiratory airflow limitation are uniquely observed in asthma. Respiratory infections (including post-viral wheeze), chronic obstructive pulmonary disease (COPD) and heart failure (historically named 'asthma cardiale') are often characterized by the same clinical symptoms and variable expiratory airflow limitation. Therefore, the short definition is further specified within the guideline by exclusion of other respiratory diseases based on timing, specific symptoms and additional diagnostic tests. Despite adequate elimination of these other airway diseases the remaining patients with asthma can be classified in different subtypes, each supposedly with different underlying pathophysiological mechanisms. These 'asthma phenotypes' are not taken into consideration in the GINA guidelines, which is explicitly stated in one of the six key points:

> *Recognizable clusters of demographic, clinical and/or pathophysiological characteristics are often called 'asthma phenotypes'; however, these do not correlate strongly with specific pathological processes or treatment responses.*

This is a strong statement indicating that the authors of the guideline choose to ignore studies which showed a correlation between biomarker based treatment approaches and disease outcome in asthma (Chapter 1.2). Importantly, the biomarker based clinical studies that showed a desirable clinical effect identified a subgroup of asthma patients with a response to current treatment modalities as will be further explained in this introduction and in Chapter 1.2. Such phenotyping of asthma is based on a description of clinical and/or pathophysiological markers associated with the disease [5]. The term 'asthma endotype' that is alternatively used to describe asthma, refers to the hypothetical situation in which a disease marker is linked to the pathophysiological background of the disease [6]. Endotypes are therefore theoretically superior to phenotypes, but have not yet been truly identified for asthma. Finally, the term 'treatable traits' has been launched to identify aspects of airway disease, which are identifiable and responsive to treatment [7].

Currently, asthma is divided into eosinophilic and non-eosinophilic asthma, either by sputum eosinophilia (≥3%) or by blood eosinophilia (≥0.3x10^9/L). It is clinically relevant to separate patients suffering from eosinophilic inflammation versus non-eosinophilic inflammation for three reasons: (i) patients with airway eosinophilia have a high risk of exacerbations including those requiring ventilation [5]; (ii) corticosteroid treatment adjusted to normalize airway eosinophil counts has been shown to result in reduced exacerbations compared to standard management [8–10]; and (iii) patients with airway and/or blood eosinophilia can benefit from oral prednisolone, anti-IgE and anti-IL-5 treatment [11–14].

Furthermore asthma can be divided into mild/moderate and severe or uncontrolled asthma, with severe/uncontrolled asthma defined as a disease requiring extensive treatment to control it or remaining uncontrolled despite extensive treatment (**Table 1**) [2]. This specification has prognostic implications and is relevant for study purposes, yet it is not a specification by disease mechanism.

Table 1: ERS/ATS criteria for uncontrolled asthma. All criteria mentioned in the table are required for the diagnosis of uncontrolled asthma 2.

Uncontrolled asthma	Objective measures
Poor symptom control	ACQ > 1.5 or ACT < 20
Frequent exacerbations	Two or more bursts of OCS for at least 3 days in the previous year
Serious exacerbation	One or more, defined as hospitalisation, ICU stay or mechanical ventilation in the previous year
Airflow limitation	FEV_1 < 80% predicted and reduced FEV_1/FVC

ATS, American Thoracic Society; ACQ, Asthma Control Questionnaire; ACT, Asthma Control Test; ERS, European Respiratory Society; FEV_1, forced expiratory volume in 1 sec; FVC, Forced Vital Capacity; ICU, intensive care unit; OCS, oral corticosteroids.

Disease markers in asthma

In the past 15 years multiple RCTs have demonstrated certain biomarkers correlate strongly with treatment responses. These clinically validated biomarkers are sputum eosinophilia, peripheral blood eosinophil count and to a lesser extent FeNO (Fraction of exhaled Nitric Oxide), with the latter only tested in primary care setting and secondary care with mild-asthma patients. The identification of biomarkers that correlate with treatment responses has been an important breakthrough in a disease that (i) is poorly defined, (ii) holds several phenotypes and (iii) is treated irrespective of underlying pathophysiology with increasing dosages of corticosteroids, beta-agonists and leukotriene-antagonists. A perfect biomarker for asthma would have three features: (i) high diagnostic value, (ii) be suitable for disease monitoring and (iii) needs to be easily measurable, without patient discomfort.

1. Sputum eosinophilia

In 2002 Green *et al*. demonstrated that a corticosteroid treatment strategy aiming at reducing sputum eosinophil counts lowered the incidence of asthma exacerbations and hospital admissions in patients with moderate to severe asthma [8]. In 2006 this finding was reproduced and enforced by a study by Jayaram *et al*., showing the comparison between a 'clinical strategy' (CS) and a 'sputum strategy'(SS) [10]. In the clinical strategy group, treatment was provided when symptoms were present ≥4 days per week, night-time symptoms were present ≥1 week, there was a need for short acting beta-agonist use (SABA) ≥4 week and FEV1 <80% of personal best was measured. In the sputum strategy group, treatment was performed when sputum eosinophils were ≤2% or >2%, with step-up corticosteroid therapy as long as the sputum eosinophils were >2%. In the sputum strategy group the time to exacerbation was significantly longer and the overall number of eosinophilic exacerbations was lower. A third RCT from 2006 by Chlumsky *et al*. was designed in a similar way and also concluded that a sputum strategy reduced exacerbation frequency and resulted in a longer time to an asthma exacerbation [9]. Unfortunately sputum induction is considered to be technically challenging, laborious and therefore only suitable for use in tertiary centers.

2. Fraction of exhaled Nitric Oxide (FeNO)

In the early nineties gaseous levels of nitric oxide were found to be increased in asthma patients [15,16]. Exhaled nitric oxide (FeNO) is since then considered to be a surrogate marker for eosinophilic inflammation in the airways that can be measured quickly and non-invasively. Two RCTs tested corticosteroid therapy tapered on FeNO levels in adult patients with asthma. In the first RCT by Smith *et al*. the primary outcome was exacerbation frequency. The study showed that this frequency tended to be lower in the FeNO group, but this observation did not reach statistical significance [17]. In the study by Shaw *et al*. with a similar design the exacerbation frequency and corticosteroid dosage were not lower in the FeNO group compared to the clinical decision group [18]. Based on these two RCTs, Petsky *et al*. did not recommend the use of FeNO in daily clinical routine of asthma treatment [19]. However, recent work from McNicholl *et al*. indicated treatment adherence might have been an important confounder in studies with FeNO and asthma [20]. In this small study directly observed inhaled corticosteroid (DOICS) treatment strongly reduced FeNO levels in patients who were found to be non-adherent. In order to conclusively prove the value of FeNO to make treatment decisions in asthma a study with focus on adherence would be required.

3. Blood eosinophil count

Recent clinical trials with humanized anti-IL-5 antibodies have emphasized the value of blood eosinophil count in severe asthma [21,22]. The cytokine IL-5 has an important role in eosinophil production, differentiation, proliferation, survival, chemotaxis and priming.[36] The initial RCTs testing anti-IL-5 treatment as therapy for asthma failed, likely because of (i) the inclusion of mild to moderate asthma patients instead of severe asthma patients, (ii) the timing of the study (3 months instead of 12 months), (iii) the clinical endpoints (lung

function and bronchial hyper-responsiveness instead of exacerbation frequency) and (iv) absence of airway inflammation in the inclusion criteria [23–25]. Recent trials included severe asthmatic patients with high blood eosinophil levels, high frequency of exacerbations in the previous year and low responsiveness to ICS. These studies proved successful in preventing asthma exacerbations and in reducing oral glucocorticoid treatment [13,26]. Although blood eosinophil counts correlate poorly with levels of sputum eosinophilia (r=0.59), the predictive value of a cut-off level of 0.27x109/L eosinophils in blood for sputum eosinophilia (>3%) is 89% (described as ROC AUC) with 78% sensitivity and 91% specificity [27]. Therefore, there is indirect suggestion that the threshold of blood eosinophilia >0.27x10^9 cells/L can be used to titrate steroid treatment, as was shown in the sputum eosinophilia directed corticosteroid treatment studies 8–10.

Just counting eosinophils?

It is very likely that detailed analysis of eosinophils will provide more relevant information than just counts. Several studies looked at eosinophils in more detail and hypothesized that the activation state of eosinophils in peripheral blood might provide additional information about a patient's inflammatory status. Johansson and colleagues suggested that priming and activation of eosinophils in the peripheral blood is deficient during episodes of tissue eosinophilia in severe and uncontrolled asthma [28]. This hypothesis was partly founded on the upregulation of active FcγRII (CD32) on activated blood eosinophils after segmental lung challenge in mild asthmatics (Utrecht laboratory of Respiratory Medicine) [29,30]. The latter seems to contradict the observation of a deficiency of primed or activated cells in the blood of asthmatics. However, in contrast to the situation in mild disease (Utrecht studies) long-term priming of eosinophils in the peripheral blood of severe asthmatics (Madison study) will most likely support migration of these activated cells to the lung and could lead to a deficiency of primed cells in the peripheral blood [31]. Supportive for this hypothesis is the up-regulation of active integrin-receptors and activation- related receptors found on blood granulocytes in mild to moderate asthma and the low expression of these markers in severe inflammatory disease [32]. Therefore, determination of the granulocyte priming and activation status could well improve the assessment of the inflammatory status of asthma patients.

Scope and outline of this thesis

This thesis will test whether asthma phenotypes can be studied by analyzing sputum and peripheral blood cells at three levels:

1. By assessing the activation state of eosinophils and neutrophils
2. By studying cell counts and percentages in blood and sputum
3. By unbiased multidimensional analysis of flow cytometry data including correlations between cell types present in certain numbers, in ratios and in level of receptor expression

In addition, asthma phenotyping can be improved by combining the three levels of cellular data with usual markers in asthma such as FeNO, allergies, presence of obesity, lung function etc. and analyzing the correlations by multivariate analysis techniques.

These new approaches take into account multiple levels of information: (i) on a cellular level, (ii) on the level of clinical characteristics, (iii) on an inter-individual level and (iv) on a group level *e.g.* in patients and healthy controls. They are based on multivariate advantage to study correlations and patterns and aim to bring asthma phenotyping to a higher level by combining detailed cellular analysis with known clinical markers [33].

Different biomarkers to phenotype asthma and to monitor treatment responses are compared and evaluated in the first part of this thesis by reviewing past studies (Chapter 1.1). This overview is followed by an outline of approaches to the clinical assessment of patients with severe asthma potentially suitable for biological treatment and also contains a detailed description of the expected clinical impact of established and new biological treatments in severe asthma, with a focus on eosinophilic inflammation (Chapter 1.2).

In the second part the additional value of the activation state of eosinophils and neutrophils in peripheral blood for asthma phenotyping is studied with the aim to ultimately replace sputum induction as an invasive and time-consuming test (Chapter 2.1). The value of the same activation state was also studied on sputum eosinophils and neutrophils (Chapter 2.2).

The third part of this thesis reports the development of an analysis method for multi-dimensional flow cytometry data. MFC data is usually analysed through 'bivariate' scatter plots that compare fluorescence intensities of two cell-bound markers for each cell within a sample. With only bivariate scatter plots it is very complicated and time-consuming to compare intensities of more than two markers simultaneously [34,35]. This warrants the need for multivariate analysis methods such as those aimed for in Chapter 3.1. A specific aim in this chapter is to study immune responses. Therefore, in Chapter 3.2 the immune response to exercise is studied based on the method developed in 3.1. Finally, Chapter 3.3 reports on another method developed specifically for diagnosis of disease and for graphical expression of cellular profiles derived from flow cytometry datasets of peripheral blood cells of asthma patients.

The fourth part of this thesis focuses on the relevance of a specific cell type in severe eosinophilic asthma which was also found to be important in Chapter 3.3 by studying the graphical expression of cellular profiles.

Finally, a discussion and future outlook on phenotyping by multi-dimensional analysis in asthma is presented in Chapter 5.

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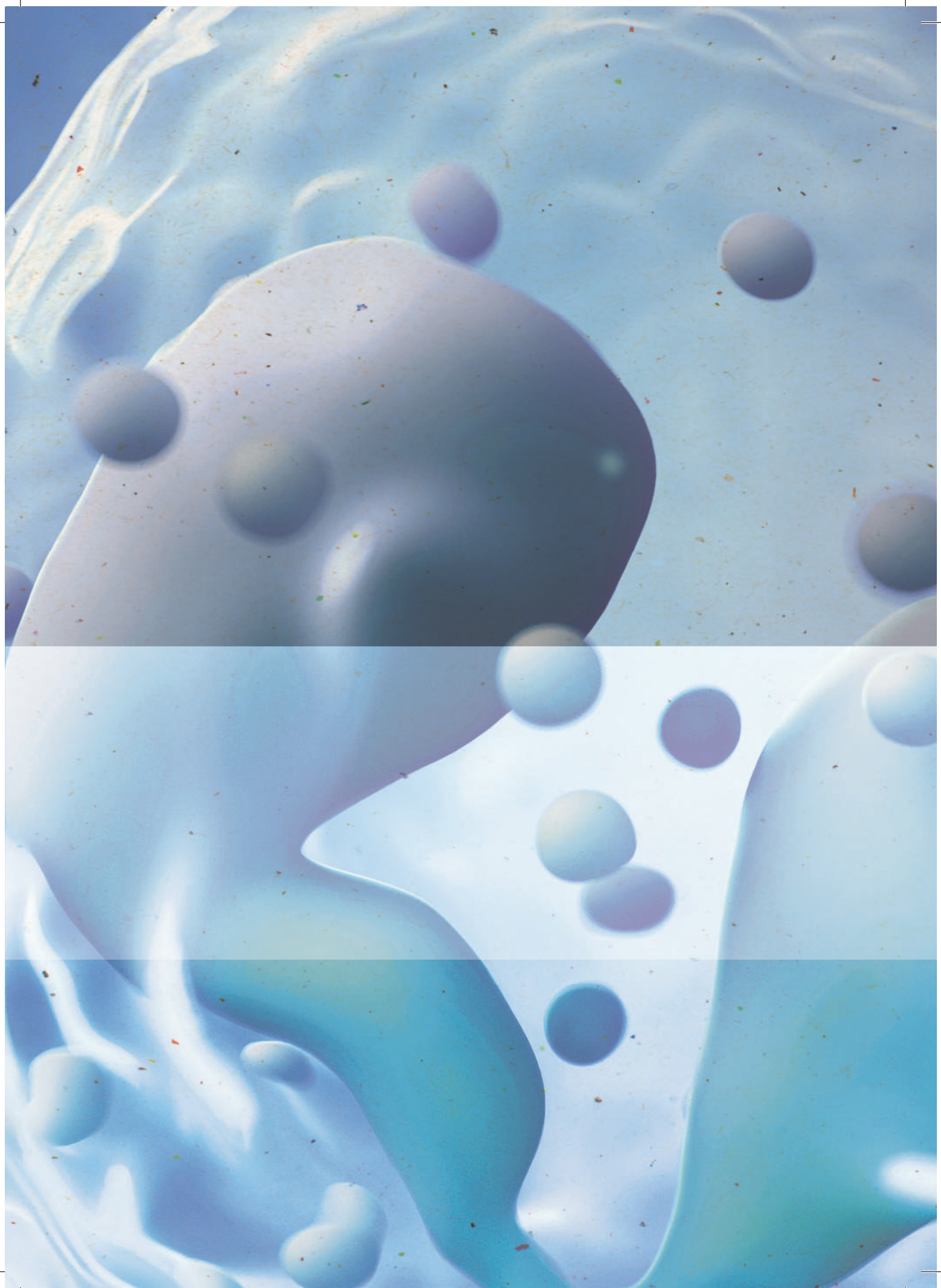

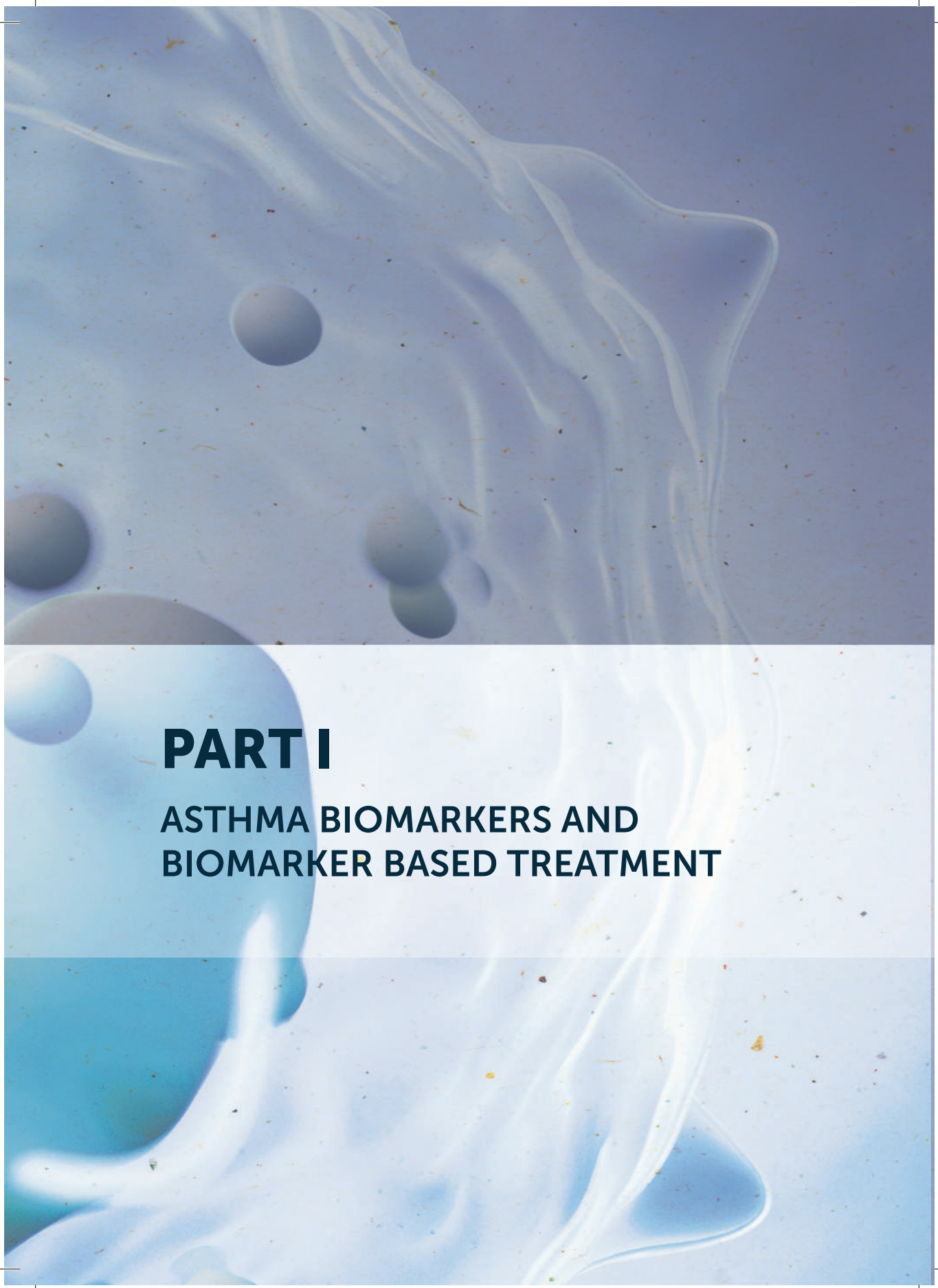

PART I

ASTHMA BIOMARKERS AND BIOMARKER BASED TREATMENT

CHAPTER 1.1

Clinical utility of asthma biomarkers: from bench to bedside

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Abstract

Asthma is a chronic disease characterized by airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction. The disease is very heterogeneous in onset, course and response to treatment and seems to encompass a broad collection of heterogeneous disease subtypes with different underlying pathophysiological mechanisms. There is a strong need for easily interpreted clinical biomarkers to assess the nature and severity of the disease. Currently available biomarkers for clinical practice, for example markers in bronchial lavage, bronchial biopsies, sputum or Fraction of exhaled Nitric Oxide (FeNO), are limited due to invasiveness or lack of specificity. The assessment of markers in peripheral blood might be a good alternative to study airway inflammation more specifically, compared to FeNO, and in a less invasive manner, compared to BAL, biopsies or sputum induction. In addition, promising novel biomarkers are discovered in the fields of breath metabolomics (e.g. volatile organic compounds) and (pharmaco) genomics. Biomarker research in asthma is increasingly shifting from the assessment of the value of single biomarkers to multidimensional approaches in which the clinical value of a combination of various markers is studied. This could eventually lead to the development of a clinically applicable algorithm using various markers and clinical features to phenotype asthma and improve diagnosis and asthma management.

Introduction to the pathophysiology of asthma

Asthma affects over 300 million individuals worldwide [1], making it one of the most prevalent common chronic diseases. Although the respiratory disease is rarely fatal, the economic burden is extensive, due to direct and indirect medical expenses, including prescription drug costs, health care costs and productivity losses [2].

The disease is characterized by airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction. Asthma can be classified as 'atopic' or 'non-atopic' based on the presence (atopic) or absence (non-atopic) of specific IgE antibodies to common environmental allergens. Atopic asthma is the most common form of asthma. In allergen-sensitized patients with atopic asthma, re-exposure to an aeroallergen will lead to an IgE-mediated inflammatory cascade in the airways. Airway resident cells (*i.e.* macrophages and mast cells), newly mobilized immune cells (*i.e.* eosinophils and neutrophils) as well as epithelial cells play an important role in this inflammatory cascade [3]. In allergic inflammation, there seems to be a disturbed balance in Th1-type and Th2-type cytokines - with dominance towards Th2 cytokines [4]. Th2 cells produce cytokines such as Interleukin (IL)-4 and IL-13, which induce a class-switch in B-cells to the production of IgE. Th2 cells also produce IL-5, which recruits eosinophils to the lung and IL-9, which stimulates mast cell proliferation. Upon activation, mast cells start to produce histamine, cysteinyl-leukotrienes (Cys-LT's), and prostaglandin D2 , which on its turn will lead to additional recruitment of eosinophils, Th2 cells and basophils to the tissue [5].

Parallel to the allergic asthma model with airway epithelial cells and the adaptive immune response as important pillars, an additional non-allergic asthma paradigm has been proposed. In the non-allergic asthma model the innate immune system responds to constantly invading respiratory viruses and bacteria. This systemic innate response is driven by sentinel cells such as macrophages, dendritic cells, granulocytes and innate lymphoid cells. A review by Holtzman and colleagues provides a comprehensive overview of both the allergic and non-allergic immune response in asthma [6]. A prolonged presence of activated inflammatory cells in the airways leads to chronic inflammation and induces tissue alterations in composition, content and organization of the airways ('airway remodelling'). Important cytokines released by epithelial cells and associated with remodelling are IL-25, thymic stromal lymphopoietin (TSLP), and IL-33. The remodelling response is characterized by subepithelial basement membrane thickening, epithelial cell disruption, neoangiogenesis, globlet cell metaplasia, enlarged submucosal glands and airway smooth muscle hyperplasia [7]. This airway remodelling is regarded as a continuous process while the number of inflammatory cells infiltrated in the respiratory tract can vary over time. This latter process is evoked by stimuli such as allergens, climate or respiratory tract infections. However, the observation of airway remodelling in young asthma patients suggest that the process may even precede airway inflammation [8].

Asthma biomarkers for diagnosis, phenotyping and treatment efficacy

Asthma diagnosis and management is generally based on reported asthma symptoms, often combined with lung function tests to assess reversible airway obstruction and airway hyperresponsiveness. However, symptoms and lung function measurements may not reflect underlying airway inflammation. Bronchoscopy with biopsies and bronchoalveolar lavage (BAL) are considered the gold standard to assess airway inflammation, but are too invasive for general application in clinical practice [9]. In addition, asthma seems to encompass a broad collection of heterogeneous disease subtypes with different underlying pathophysiological mechanisms [10]. There is a need for asthma biomarkers to identify clinically relevant asthma phenotypes, optimize diagnosis and guide treatment. In this paper we will provide an overview of asthma biomarkers already available for clinical practice and promising biomarkers currently under development (**Figure 1**). In addition, we will address the promises and barriers of the implementation of asthma biomarkers into clinical practice.

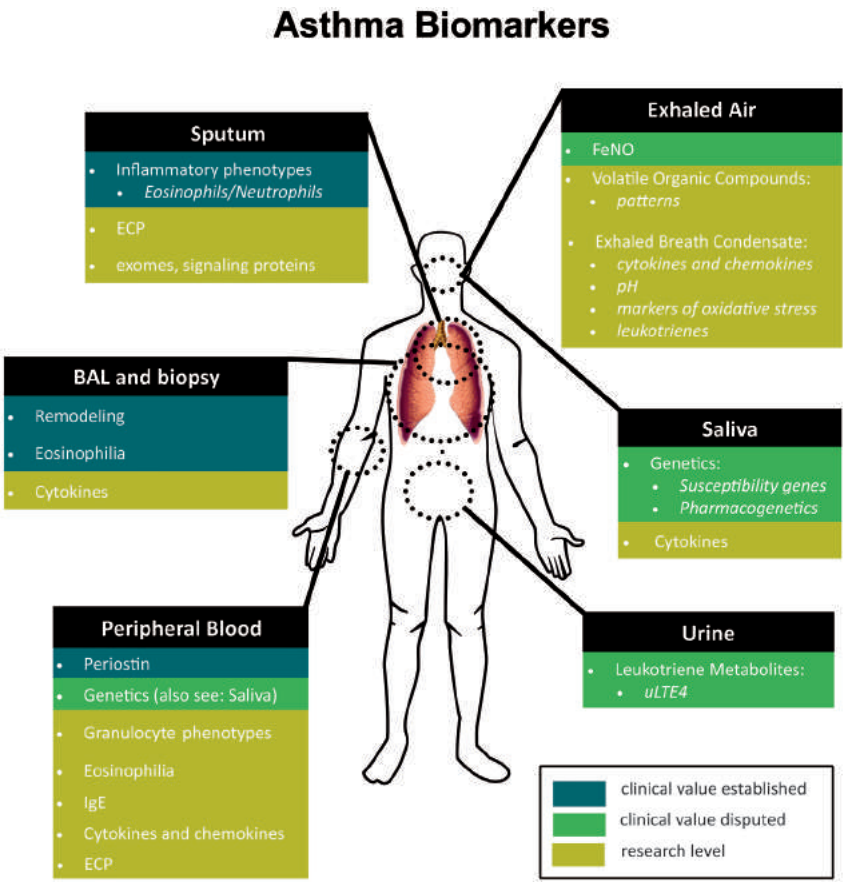

Figure 1: Asthma biomarkers.

Clinically available biomarkers

Sputum Induction, bronchoscopy/biopsy and bronchoalveolar lavage

Tissue-specific diagnostic methods such as bronchoalveolar lavage (BAL), bronchoscopy or bronchial biopsy, used to measure airway inflammation and remodeling, provide reliable and detailed clinical information of asthmatic patients. Airway remodeling has been observed in bronchial biopsies of both adults and children with asthma [11]. BAL fluid of asthmatic patients shows elevated levels of Th2 cytokines compared to healthy individuals [12]. In difficult-to-treat asthma in children, BAL and endobronchial biopsy should be considered to objectify the presence of airway eosinophilia and other typical pathological features of asthma [13]. Thus, invasive and tissue-specific diagnostic methods are valuable in certain patient populations and clinical research settings. However, the invasiveness of these diagnostic procedures limits the use of these methods for daily clinical routine in most asthma patients. Even sputum induction, a diagnostic technique in which the patient inhales nebulised saline solution in increasing concentrations to liquefy sputum, is regarded as too invasive, technically complex and too variable for daily clinical routine. This restricts the procedure to specialized medical centers [14]. There is a strong correlation between cellular components present in airway fluid obtained by BAL and cells present in airway fluid obtained by sputum induction [15,16]. Therefore, compared to BAL, sputum induction is the preferred method to diagnose the inflammatory phenotype of asthma classically based on the presence of different types of granulocytes. Recent studies indicate that the performance of this technique increases when combined with the analysis of other cellular components such as exosomes and signaling proteins [17].

Distinct inflammatory patterns have been established in the sputum of asthmatic adults and asthmatic children based on eosinophil and neutrophil percentages of total non-squamous cells in the sputum. Currently, four inflammatory phenotypes have been identified based on analysis of sputum: the eosinophilic, neutrophilic, mixed and paucigranulocytic types [18]. This is demonstrated in **Figure 2**. It has been suggested that higher levels of sputum eosinophils are associated with a better response to corticosteroids 19-21, but results remain inconsistent [22-24]. Furthermore, the pattern of inflammatory sputum phenotypes seems to be different for adult patients and paediatric patients and the reproducibility of sputum induction measurements over time has been a point of scientific debate since the introduction of this technique [18,25,26].

Other sputum and BAL markers that have been investigated include soluble mediators such as Eosinophilic Cationic Protein (ECP), Hypoxia Inducible Factor-1α (HIF-1α) and VEGF (Vascular Endothelial Growth Factor) [27]. ECP is released during degranulation of eosinophils and can be measured in sputum, BAL fluid and in serum. It is considered to be a non-specific marker for inflammation and, therefore, lacks the specificity for diagnosing asthma. Meijer *et al*. showed that sputum ECP has no predictive value for clinical response to corticosteroids in asthmatic patients [28]. Its added value as a diagnostic tool would be in the measurement of the extent of inflammation and severity of asthma, *e.g.* moderate versus severe asthma [29].

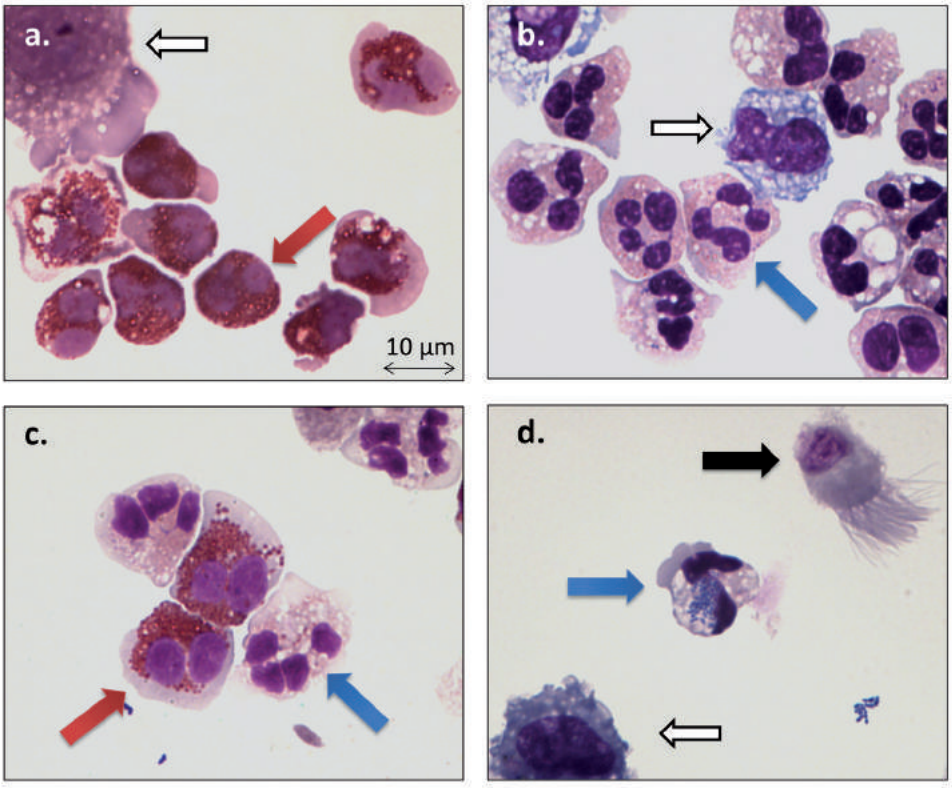

Figure 2: Inflammatory phenotypes of adult asthma patients obtained by sputum induction.

A) Eosinophilic type; marked by the presence of eosinophils >3% (*red arrow*). The hollow arrow indicates an alveolar macrophage, **B)** Neutrophilic type; marked by the presence of neutrophils (*blue arrow*) >61%. *The hollow arrow* indicates an alveolar macrophage, **C)** Mixed type; marked by the presence of both eosinophils (*red arrow*) >3% and neutrophils (*blue arrow*) >61% **D)** Paucigranulocytic type; marked by a lack of eosinophils (<3%) and neutrophils (<61%). The arrow shows a ciliated pseudo-stratified columnar airway epithelial cell (*black arrow*), a neutrophil with phagocytosed bacteria inside (*blue arrow*) and an alveolar macrophage (*hollow arrow*). May-Grünwald/Giemsa staining, photograph at 100x magnification, courtesy of dr. J.A.M. van der Linden (UMC Utrecht, the Netherlands).

HIF-1α and VEGF protein levels have shown to be upregulated in lung specimens from allergen-challenged asthma patients obtained by BAL and endobronchial biopsies [30].

Nitric oxide in exhaled breath

Almost a decade ago the first reports emerged of increased levels of nitric oxide (NO) in exhaled breath (FeNO) in patients with asthma [31,32]. Since then a high number of studies have assessed the clinical value of exhaled nitric oxide in asthma management. Several

FeNO analyzers became commercially available and international guidelines on FeNO measurement were published [33,34].

NO is produced when the amino acid L-arginine is converted by nitric oxide synthases (NOS) into the amino acid L-citrulline. There are three known isoforms of NOS, but especially iNOS (inducible NOS) seems to play a role in the elevated levels of NO in exhaled breath of asthmatics. The activity of the enzyme is upregulated by a wide range of inflammatory cytokines. It remains unclear which cells are responsible for the increased NO production, but airway epithelial cells and eosinophils are thought to be the important candidates [35]. It is thought that inflamed airways will produce increased levels of NO. High FeNO is thought to be a surrogate marker of ongoing eosinophilic airway inflammation and may reflect uncontrolled asthma and predict asthma exacerbations [36].

Despite the initial enthusiasm about FeNO as a new and non-invasive marker of airway inflammation, the clinical usefulness of FeNO to measure asthma control is still debated. Studies that have investigated the association between asthma control and FeNO provide inconsistent results (extensive overview in Supplementary **Table S1**) and studies assessing the relationship between FeNO and other airway inflammation markers, such as sputum eosinophilia or the presence of eosinophils in bronchial specimens, remain inconclusive [37,38]. This may be partly caused by a non-overlap in asthma symptoms and airway inflammation. Furthermore, this relationship is complicated due to various other factors that seem to influence FeNO levels, including age, atopy, medication use, therapy adherence and airway infections [36]. In addition, tailoring asthma treatment based on FeNO measurements did not decrease asthma exacerbations or lead to better asthma control according to a meta-analysis performed by Petsky and collegues [39]. FeNO might, nevertheless, still be a valuable marker in asthma management. Zacharasiewicz *et al.* showed that the combination of increased FeNO and the percentage of sputum eosinophils were significant predictors of exacerbation upon steroid reduction in children with stable asthma [40]. Studies by Szefler *et al.* and Knuffman *et al.* showed that paediatric asthma patients with elevated FeNO were more likely to respond to corticosteroids compared to montelukast [41,42].

Reports on the relationship between FeNO and treatment response remain inconsistent, though there is a suggestion that higher baseline FeNO is associated with a better response to treatment [43]. Although the clinical value of a single FeNO measurement is limited, combining this measure with other markers of airway inflammation may lead to a more accurate assessment of underlying disease state.

Biomarkers under development

Blood

Peripheral blood is easy to obtain and the procedure itself is less invasive in comparison to sputum induction and bronchoscopy. Since inflamed tissue releases chemo-attractants and cytokines, which recruit activated immune cells from the peripheral blood, the dynamic

process of immune cells entering and leaving the blood stream can be used as an indirect readout of the state of disease.

From a cellular point of view, peripheral blood eosinophilia has been described extensively as a potential asthma biomarker [44]. Blood eosinophilia correlates with bronchial hyperresponsiveness and asthma-related inflammation [45]. The specificity of using peripheral blood eosinophilia to diagnose asthma is, however, rather low, as allergies, auto-immune disease and parasitic infections cause blood eosinophilia as well. Therefore, its role as a diagnostic measurement remains limited. The same applies to total and allergen-specific IgE levels in serum [46]. Several studies have evaluated whether the presence of inflammatory soluble mediators such as chemokines and cytokines were applicable as biomarkers for type and extent of asthma phenotypes [47]. Recent studies utilized multiplex analysis allowing the parallel analysis of multiple cytokines within one serum/plasma sample [48,49]. Unfortunately, these studies have neither led to a clinically useful diagnostic tool to identify distinct disease phenotypes, nor to a tool to assess disease severity. A weakness of studies assessing inflammatory chemokine and cytokine profiles lies in the fact that the choice of mediators to be studied determines the (lack of) success of this approach. Anti-inflammatory mediators (such as receptor antagonists) are often neglected. In addition, little consideration has been given to the complex interaction between inflammatory mediators [50].

A different approach is to examine shifts in activation profiles of inflammatory cells in peripheral blood and attempt to link these shifts to clinical phenotypes. These inflammatory cells will integrate all pro- and anti-inflammatory signals and change their phenotypes accordingly. Studies on activation status of peripheral blood cells have provided some insights into the systemic innate immune response in allergic asthma. Many studies have shown that inflammatory cells such as monocytes and granulocytes respond with up-regulation of several activation markers in response to inflammatory signals [51-53]. Many of these markers such as Mac-1 (CD11b), CD63, CD66b, CD69 are typically found in granules that fuse with the plasma membrane upon activation of the cells with inflammatory mediators [54]. Unfortunately most studies [55,56] compared the presence of the markers on blood cells and tissue cells obtained from sputum and BAL and did not take into account that cells homing to the tissue under homeostatic conditions exhibit the same phenotype [57]. The process of homing of the cells towards the tissue compartment is already sufficient to activate the cells both in homeostasis as well as disease. The expression of these markers in the peripheral blood has not lead to a clear link between expression profiles of granulocytes and type of asthma.

Elegant work by Johansson and colleagues has shown that eosinophils change their activation status of membrane bound integrins rather than overall expression in response to inflammatory signals [58]. Application of antibodies specifically recognizing activated states of integrins provided solid data showing that blood eosinophils in poorly controlled asthma are characterized by activated integrins. This situation is consistent with the hypothesis that these cells are primed and prepared to leave the peripheral blood for the tissues. We have obtained similar data by application of antibodies recognizing activated FcγRs [51,59]. These

data demonstrated that eosinophils first become activated in the peripheral blood and subsequently home for the tissue leaving behind unprimed cells [60]. These studies indicated that changes in the phenotype of inflammatory cells can aid in the diagnosis of the type and extent of severity of allergic asthma. But they also show that the differences are very subtle and not yet applicable in the clinical routine.

Closer to clinical implementation might be the biomarker periostin. Periostin is a recently discovered matricellular protein that is secreted by bronchial epithelial cells under the influence of IL-13. The presence of periostin in serum correlates strongly with sputum eosinophilia [61]. A study by Corren *et al*. showed that patients with high levels of serum periostin responded better to Lebrikizumab (anti-IL-13 therapy) compared to patients with low levels of periostin [62].

Air

The measurement of volatile organic compounds (VOCs) in exhaled breath is a novel metabolomic approach to study molecular signatures of respiratory disease. Exhaled breath contains a complex mixture of up to thousands of VOCs. These compounds are produced due to metabolic processes in the airways and the presence and/or concentrations of the different compounds are likely influenced by the presence of airway inflammation. There exist different methods to assess VOCs; one can assess profiles of VOCs ('breathprints') present in exhaled breath using polymer-based gas sensor arrays ('electronic nose') [63] or identify individual molecular components using gas chromatography-mass spectrometry (GC-MS) [64]. Asthma patients can be differentiated from healthy controls based on their breathprints [65], as can asthmatic patients from COPD patients [66]. However, the method was less successful in distinguishing mild asthmatic from severe asthmatics [65]. Breathprints of COPD patients do correlate with the presence of eosinophil and neutrophils in induced sputum, as well as with levels of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) in induced sputum, suggesting that the electronic nose might be capable of assessing distinct types of underlying airway inflammation [67].

Using the other approach, GC-MS, Dallinga *et al*. showed that the measurement of a limited set of VOCs in exhaled air could differentiate asthmatic children from controls with high sensitivity (95%) and high specificity (89%) [64]. A study by Ibrahim *et al*. showed that a set of 15 VOCs could discriminate asthmatic patients from controls, and also could classify patients according to inflammatory sputum phenotype and asthma control (based on the ACQ) [68].

The assessment of VOC in exhaled breath seems to be a very promising approach, especially when knowledge of clinical relevant VOCs is integrated in a user-friendly handheld device such as the electronic nose. However, validation of clinically relevant VOC patterns in a large population of asthmatic patients is necessary, as well as longitudinal assessment of VOC patterns, the assessment of the influence of asthma treatment, and emergence of international guidelines on VOC measurement. A large Europe-wide study to assess the clinical utility of VOCs in asthma in-depth is currently ongoing [69].

Biomarkers in breath can also be measured in exhaled breath condensate (EBC). When exhaled breath is cooled a liquid phase can be obtained, which contains condensed water vapour, as well as non-volatile substances. Various markers in EBC have been found to be elevated in asthmatics when compared to healthy individuals, including adenosine concentration [70], markers of oxidative stress (*i.e.* hydrogen peroxide) [71], cytokines and chemokines [72], nitric oxide-related products [73], isoprostanes and leukotrienes [74]. Furthermore, the pH of EBC has been reported to be decreased in both acute asthmatics and poorly controlled asthmatics [75,76].

In spite of these results, the measurement of markers in EBC is still in its research phase and several important methodological problems complicate the clinical utility of EBC [77]. A standardized methodology for EBC collection is lacking, as are established reference values. Various factors such as the type of condenser equipment used, cooling temperature, condenser tube coating, cleaning procedures, breathing patterns, ambient air pollution and concentrations of relevant cytokines too low for reliable determination influence the measurement and compromise reproducibility.

Urine: leukotriene metabolites

Cysteinyl leukotrienes (LTs) C_4 and D_4 are lipid mediators, which are thought to play a role in asthma pathogenesis. They can be released from various cells, including eosinophils, neutrophils and mast cells. LTC_4 and LTD_4 in the plasma are rapidly converted into the less active LTE_4 metabolite. A fraction of LTE4 is excreted in urine. The urinary LTE_4 ($uLTE_4$) concentration is used as a marker of total body LT production [78]. Studies by Szefler *et al.* and Cai *et al.* showed that asthmatic patients with higher levels of $uLTE_4$ were more likely to respond to leukotriene antagonists (LTRA) when compared to asthmatic patients with lower $uLTE_4$ levels [41,79].

(Pharmaco)Genetics

Twin studies have shown that asthma contains a considerable genetic component [80]. Genome-wide association studies (GWAS) have identified several loci to be associated with asthma risk, including: the *ORMDL3* locus, *ADAM33* and various cytokines and cytokine receptor genes; *IL18R1*, *IL33*, IL2RB, *IL10*, *TGFB1* and *IL6R* [81-84].

A recent review by Dijk *et al.* provides a thorough overview of asthma susceptibility genes that have been found by GWAS [85]. Nevertheless, effect sizes are small (low penetrance) and the identified genetic variants can only explain a small part of the asthma heritability. This could be due to the heterogeneity in asthma phenotypes and the underestimated influence of environmental-gene interactions. For example, recent work by Ierodiakonou and colleagues showed an interaction between variation in *TGFB1* and smoking on asthma severity [86]. Carrying a G-allele of rs6957 in *TGFB1* was associated with higher submucosal eosinophils and basement membrane thickness, but only in current or ex-smoking asthmatics.

A more promising genetic approach for clinical asthma practice might be pharmacogenomics: the association of genomic variations and medication response. Variation in genes coding for proteins involved in the drug metabolism pathway may influence drug concentration and efficacy. Observational studies have found genetic variation to be associated with persistent symptoms as well as with lung function in steroid-treated asthmatics [87-90]. A study by Hawkins *et al*. found a positive correlation with variations in *STIP1*, coding for an adaptor protein in the glucocorticoid receptor complex, and baseline lung function and improvement in lung function upon corticosteroid treatment in 382 adults with asthma [89]. A study by Tantisira *et al*. showed that asthma patients with a variant in the *GLCCI1* have less improvement in lung function upon inhaled corticosteroids (ICS) treatment [90]. *GLCCI1* encodes Glucocorticoid Induced Transcript 1, a protein of unknown function. Furthermore, a SNP in the *FCER2* gene, coding for a low affinity IgE receptor, has been associated with an increased risk of asthma-related hospital visits, uncontrolled asthma and higher daily steroid dosages [87,88], and variation in *TBX21* (encoding transcription factor T-bet) has been related to improved airway responsiveness in childhood asthma upon treatment with ICS [91]. T-bet is thought to be an important regulator of the Th1/Th2 balance [92].

Pharmacogenomic studies on response to LTRA have found most association with *ALOX5* [93,94], a 5-lipoxygenase, and *LTC4S*, a glutathione S-transferase [95,96]. However, a step closer to clinical implementation is assessment of the beta-adrenergic receptor gene (*ADRB2*) in order to determine response to β2-agonists for which randomized clinical trial (RCT) data are available [97-99]. The beta-adrenergic receptor is a G-protein coupled receptor that is expressed in smooth muscle in the airways and activation induces bronchial relaxation. Beta2-agonists are the most frequently prescribed drugs to relieve airway obstruction and act through the beta-adrenergic receptor. Evidence suggests that genetic variations in the gene are associated with an altered treatment response. Recently, a small RCT based on prospective testing of genetic variation in the *ADRB2* gene (alteration in amino acid at position 16; Arg16Gly) showed encouraging results in 62 children with persistent asthma. Asthmatic children homozygous for the variant genotype were randomized to a long acting beta(2) agonist (LABA) plus ICS or to LTRA plus ICS. The group treated with ICS and LTRA scored better on asthma symptoms and quality of life, used less rescue medication and were fewer days absent from school compared to the group children treated with LABA plus ICS [97], suggesting that asthmatic children homozygous for *ADRB2* Arg16Gly substitution (B16 Arg/Arg) benefit more from LTRA compared to LABA as add-on treatment to ICS. Yet, there was no difference in lung function improvement.

On the other hand, RCTs performed in adults found no effect. A *post hoc* pharmacogenetic analysis of two large RCTs in which asthmatic patients were treated with LABA-only or LABA combined with ICS found no differences in exacerbations, use of rescue medication, nights of awaking and lung function when patients were stratified according to differences in *ADRB2* Arg16Gly genotype [100]. In a cross-over RCT asthmatic patients with the B16 Arg/Arg (homozygote for the risk allele) or B16 Gly/Gly (homozygote for the wild type allele) were randomized to LABA plus ICS or placebo plus ICS. There was no difference in lung

function improvement between the groups when ICS was added. Remarkably, airway responsiveness in the patients with B16 Gly/Gly did improve significantly when ICS was added to the treatment, while it did not in the B16 Arg/Arg group [98]. Airway responsiveness was measured as methacholine PC_{20} doubling dose: the dose of methacholine that provokes a 20 percent drop in the volume of exhaled air during the first second of a forced expiratory maneuver (FEV1).

So far pharmacogenetic studies have been limited by small sample sizes, heterogeneous populations and lack of replication. However, the emergence of new sequencing technologies, innovative strategies of analyses and the upcoming of international research consortia may lead to the identification and replication of clinically relevant associations in the near future. In addition, the development of innovative – though expensive – targeted treatment strategies (such as Omaluzimab (anti-IgE), Mepoluzimab (anti-IL5) and Lebrikizumab (anti-IL13)) may provide a novel clinical context for pharmacogenetics in order to identify subgroups of asthma patients that will benefit the most from these treatments.

Ease of biomarker detection and current limitations

Progressive insight into medical biology leads to a layered profile of studying disease mechanisms. Asthma research is shifting from a broad perspective (studying symptom expression, lung function and response to medication) to a narrower focus: cellular profiles, protein analysis and genetic markers, possibly combined with clinical measures. These biological parameters can be measured in different body compartments, and build up to a complexity that has not yet been fully understood. From a biological point of view, there is an almost indefinite number of possible biomarkers that can be measured in the context of asthma. Yet, the clinical applicability (*e.g.* clinical added value, specificity, sensitivity and invasiveness) limits the number of appropriate clinical usable biomarkers. Noninvasive, reliable and easily interpreted biomarkers would ideally be standard in daily clinical routine, but are currently unavailable.

Conclusions and future directions

Single biomarker approaches to phenotype asthma are increasingly regarded to be inaccurate and outdated. To diagnose the presence of eosinophilic inflammation for example, FeNO is a very sensitive biomarker, but not very specific. Intuitively, combining FeNO with markers of eosinophilic inflammation (such as the percentage of eosinophils in peripheral blood or eosinophil receptor expression) or other biomarkers would increase specificity. To test this hypothesis, studies combining multiple known biomarkers, should be performed. Currently, research consortia like U-BIOPRED (Unbiased Biomarkers for Prediction of Respiratory Outcomes, http://www.ubiopred.european-lung-foundation.org/) and SARP (Severe Asthma Research Program, http://www.severeasthma.org) aim to integrate the process of data collection and multidimensional approaches to phenotype asthma.

Single biomarker approaches remain important in the process of biomarker discovery, as newly identified biomarkers can be integrated in a multidimensional approach to strengthen the diagnostic ability of a clinically applicable algorithm to phenotype asthma. Only then personalized asthma treatment will be in reach.

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Table S1: Studies that assessed the association between fraction of exhaled nitric oxide (FeNO) and asthma control

	Study population	Study design	NO device	Asthma control	Outcome	Evidence for association
Vijverberg *et al.*, 2012 [101]	601 children (age: 4-12 yrs) with a reported use of asthma medication	Cross-sectional	NIOX Mino	ACQ -6	Weak positive correlation between FeNO and ACQ score (Rs = 0.13, *P* = 0.002), but poor accuracy to discriminate poor from well-controlled asthma (AUC: 0.56, 95% CI: 0.52–0.61, *P* = 0.008)	+/-
Ozier *et al.*, 2011 [104]	90 asthma patients	Prosp. follow-up of 3 wks for controlled patients and 3-6 months for uncontrolled patients	EndoNO, NIOX Mino	ACQ-6	There was no correlation between NO measurements and ACQ scores. PPV of FeNO < 50% to predict uncontrolled asthma. FeNO had a high NPV to predict loss of control in already controlled patients (>95%)	+/-
Mahut *et al.*, 2010 [102]	200 asthmatic patients (107 children and 93 adults)	Prospective, 12-weeks of follow-up	ENDONO 8000	7-item and 6-item ACQ	FeNO did not correlate with ACQ at inclusion or during follow-up	-
Sardón-Prado *et al.*, 2010 [103]	268 asthmatic children (age: 7-14 yrs)	Cross-sectional	NIOX Mino	Control de Asma en Ninõs (CAN) questionnaire	Weak correlation between FeNO and asthma control (r=-0.3)	+/-
Perez de Llano *et al.*, 2009 [105]	102 asthma patients with suboptimal asthma control	Prospective	NIOX Mino	ACT	FeNO had a PPV of 87.5% and a NPV of 90.6 to predict asthma control.	+
Shirai *et al.*, 2008 [106]	105 asthmatic patients	Cross-sectional	Sievers NOA 280i	ACT	Weak correlation between FeNO and asthma control (r = -0.310, *P* = 0.003)	+/-

Table continues on next page

Table 1: *Continued*

	Study population	Study design	NO device	Asthma control	Outcome	Evidence for association
Khalili *et al.*, 2008 [107]	100 asthma patients (age: 6-86 yrs)	Cross-sectional	NIOX	ACT, ACQ, NAEPP goals of therapy, JTFPP on attaining optimal asthma control, GINA guidelines	No significant association was found between FeNO level and asthma control based on ACQ (P > .99), ACT (*P* = 0.53), NAEPP (*P* = 0.53), JTFPP (*P* = 0.30), or GINA (*P* = 0.86) criteria.	-
Michils *et al.*, 2008 [108]	341 asthma patients	Prospective with post hoc data analysis	LR 2000	ACQ-6	FeNO is a good marker of asthma control over time (especially in patients with low doses of ICS). FeNO decrease <40% or increase <30% precludes asthma control optimisation or deterioration, (NPV: 79 and 82%, respectively). In the low-dose ICS group, a decrease >40% indicated control optimisation (PPV: 83%).	+
Senna *et al.*, 2007 [109]	27 newly-diagnosed asthma patients (age: 16-57 yrs)	Cross-sectional	CLD 88 sp	ACT	Good correlation between ACT score and eNO (r = 0.7, *P* = 0.001)	+
Robroeks *et al.*, 2007 [110]	64 asthmatic children (5-16 yrs)	Cross-sectional	NIOX	Based on GINA	FeNO was associated with poor asthma control, but mainly in a model with markers in exhaled breath condensate (AUC 0.761, *P* < 0.001)	+/-
Rosias *et al.*, 2004 [111]	23 children with mild-to-moderate asthma (age: 6–16 yrs)	Cross-sectional	NIOX	ACQ	No significant correlation between FeNO and ACQ (r = 0.48, *P* = 0.06)	-

Strunk *et al.*, 2003 [112]	144 children with mild to moderate persistent asthma (age: 6-17 yrs)	Cross-over RCT ICS/LTRA	NIOX	Patient diary	No correlation between FeNO and clinical characteristics	-
Franklin *et al.*, 2003 [113]	155 children (mean age: 11.5 ± 2.3 yrs)	Prospective birth cohort	Sievers NOA 280i	Modified American Thoracic Society (ATS) questionnaire	In a multiple regression model recent wheeze was not associated with FeNO	-
Jones *et al.*, 2001 [114]	78 mild/moderate-asthma patients (age: 18-74 yrs)	Prospective, max 6 weeks or to loss of asthma control (LOC)	Unknown	LOC based on: lung function decrease, increase bronchodilator use, nocturnal asthma symptoms, distressing asthma symptoms	Good correlation between LOC and (1)FeNO at final study visit, (2) change of FeNO over time and (3) symptom score (r=0.33, P = 0.004, r=0.45, P < 0.0001, respectively). Single FeNO measurements and changes of FeNO had PPV that ranged from 80 to 90% for predicting and diagnosing LOC.	+
Sippel *et al.*, 2000 [115]	100 asthma patients (age: 7-80 yrs)	Cross-sectional	Sievers NOA280i	Questionnaire based on National Heart, Blood and Lung Institute Epidemiology Standardization project / dyspnea score	FeNO significantly correlated with asthma symptoms within the past 2 weeks (P = 0.02), dyspnea score (P = 0.02) and daily use of rescue medications (P = 0.01)	+

ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test, AUC: Area Under the ROC curve, GINA: Global Initiative for Asthma, JTFPP: Joint Task Force Practice Parameter, NAEPP: National Asthma Education and Prevention Program, LOC: Loss of asthma control, NPV: Negative Predictive Value, PPV: Positive Predictive Value.

CHAPTER 1.2

What goes up must come down: biomarkers and novel biologicals in severe asthma

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Abstract

Asthma is a heterogeneous airway disease characterized by typical symptoms in combination with variable airway obstruction. Most patients with asthma have well controlled symptoms and a low risk of asthma attacks with inhaled corticosteroid treatment. However, a clinically important subgroup (~10%) remains symptomatic and/or at risk of asthma attacks despite maximum inhaled therapy. Patients with severe asthma are responsible for a significant proportion of healthcare costs attributable to asthma and have a large unmet need for better treatments. An important advance in recent years has been the recognition that severe asthma is heterogeneous with respect to clinical problems and the pattern of lower airway inflammation. Identification of eosinophilic inflammation in the airways has become an important priority as novel biologicals that target Th2 cytokines, such as anti-IL5, anti- IL-13 and combined anti-IL-4/13 are showing considerable promise as treatments for this sub-group. It has also become clear that anti-IgE (Omalizumab), the first monoclonal antibody registered for treatment of severe asthma, is only active in patients with active eosinophilic airway inflammation. The future will be identification of potentially responsive patients on the basis of raised biomarkers and, as suggested by the title of this review, targeted treatment with specific cytokine blockade that has a direct effect on the biomarkers. In this review we outline an approach to the clinical assessment of patients potentially suitable for biological treatment and describe in detail the likely clinical impact of established and new biological treatments.

Introduction

Asthma is a highly prevalent chronic inflammatory disease of the bronchial airways affecting 5-10% of all adults and children. An estimated 235 million people are diagnosed with asthma worldwide and the WHO estimates that a significant proportion is undertreated or undiagnosed. In developed countries the clinical manifestations of asthma are controlled with inhaled therapy in most patients [1,2]. However, around 10% of patients have severe asthma that is uncontrolled despite intense treatment regimens. This subgroup suffers from significantly higher morbidity and mortality [2], and accounts for more than half the asthma-related health and economic impact [3,4]. Asthma attacks, or acute or sub-acute episodes of worsening of symptoms and airflow obstruction make a significant contribution as they require immediate clinical attention and when severe, hospitalization [5]. Patients with severe asthma therefore have a high unmet need and should be offered alternative treatments.

There has been intense interest from the pharmaceutical industry in finding better treatments for patients with severe asthma. Progress has been frustratingly slow and some treatments now known to be effective failed initial clinical trials. However, a better understanding of the heterogeneity of severe asthma and its lower airway pathology has resulted in more successful treatment outcomes lately. Most of the more promising treatment approaches specifically target eosinophilic airway inflammation and its identification has therefore become an important assessment of patients.

In this review we outline an approach to the clinical assessment of severe asthma, emphasize methods used to identify eosinophilic airway inflammation and overview some of the most promising new treatments targeting this process.

Clinical assessment

The diagnosis of asthma is based on recognition of typical symptoms (dyspnoea, cough and wheeze) in association with variable airflow obstruction [6]. The latter can be demonstrated by the response to a bronchodilator (>12% improve of FEV1), by monitoring of peak expiratory flow (PEF) or by identification of airway hyperresponsiveness (*i.e.* a provocative concentration of Metacholine required to cause a 20% fall in FEV1 of <8 mg/ml). Although asthma is the most common cause of episodic wheeze, cough and dyspnoea, there are other potential causes including dysfunctional breathing, vocal cord dysfunction, rhinitis, cough syndromes, and structural airway abnormalities like tracheomalacia and bronchomalacia [7]. These conditions are prevalent in patients presenting to a severe asthma clinic as the accompanying symptoms do not respond to asthma treatments [8]. The clinical assessment therefore has to be sufficiently rigorous to exclude these conditions with confidence [9]. This is not straightforward as abnormal PEF variability can occur independently of asthma and all the conditions listed above can co-exist with asthma [9]. Assessment of airway responsiveness and eosinophilic airway inflammation are generally more informative than more traditional tests [9]. Chronic obstructive pulmonary disease (COPD) can be difficult to

distinguish from severe asthma with confidence and can be associated with eosinophilic airway inflammation. Our approach is to describe rather than categorise patients with shared features and focus the clinical assessment on identification of treatable aspects. Asthma can also be apparently severe and treatment resistant in patients who have not mastered the basics of asthma management such as treatment adherence, correct inhaler technique and self-management. The clinical assessment should therefore include a thorough assessment of these aspects.

Phenotypes

After confirming the diagnosis of severe asthma the next step is to identify the inflammatory phenotype of asthma in order to direct treatment and risk reduction strategies. Severe asthma is characterized by a marked dissociation between symptoms and disordered airway function on the one hand and lower airway pathology on the other. Therefore, it is necessary to assess both to get the full picture (**Figure 1**).

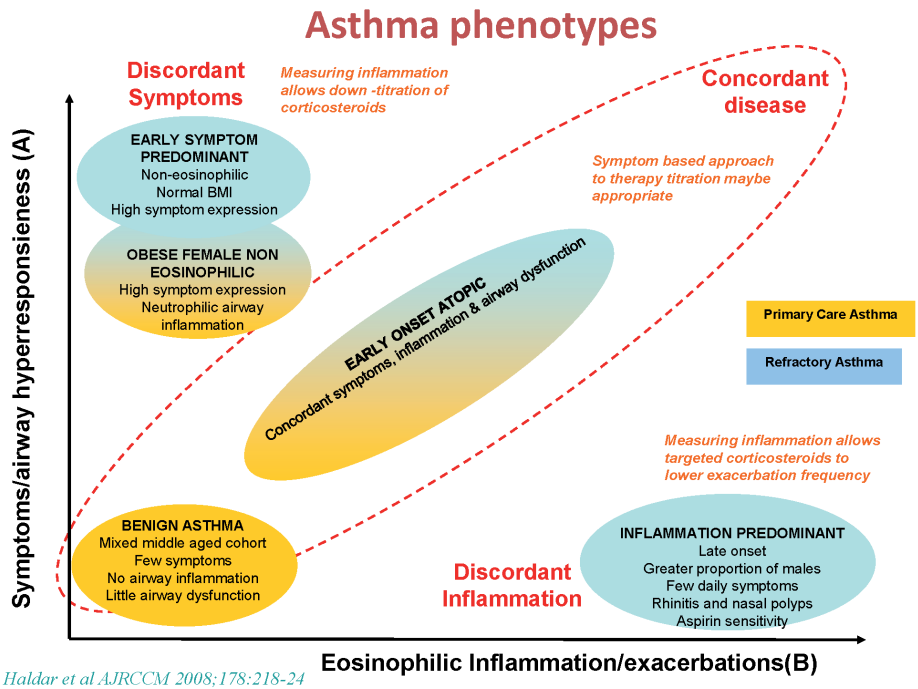

Figure 1: Asthma phenotypes.

Schematic representation by Haldar et al. with eosinophilic inflammation/exacerbation frequency plotted on the x-axis and symptom expression/AHR on the y-axis [10]. The existence of a variety of disease subtypes has been analysed and graphically displayed using unbiased clustering approaches on large cohorts of asthma patients. Notably, there are two subtypes of disease with discordant symptoms and one with discordant inflammation. These subtypes are missed if symptom expression only is taken into account.

Distinguishing eosinophilic inflammation from non-eosinophilic inflammation is important for several reasons: 1) patients with eosinophilic airway inflammation are at high risk of severe attacks, including episodes requiring ventilation [10]; 2) a treatment model based on identification and treatment of eosinophilic airway inflammation has been proven to reduce attacks [11-13]; and 3) prednisolone, Omalizumab and a number of new biological agents are highly active in patients with this pattern of lower airway inflammations. Eosinophilic airway inflammation can be assessed directly by bronchoscopic techniques or by induced sputum or indirectly using the peripheral blood eosinophil count or fraction of exhaled nitric oxide (FeNO). The pros and cons and measurement characteristics of these methods are summarised in **Table 1**.

Table 1: Biomarkers of eosinophilic inflammation used in RCTs with monoclonal antibodies to preselect patients in adult asthma

Biomarker	Association with treatment response	Invasiveness	Comments
FeNO	**Corticosteroids, anti-IL-13, anti-IL-4&13, anti-IgE**	Non-invasive	Easy, quick, not specific, cheap, generally available
Serum IgE	**Not associated**	minimal	Although recommended to measure, there is no clear association between IgE as a biomarker and treatment responses or clinical outcome
Serum Periostin	**Anti-IL-13***	minimal	Effect shown with Anti-IL-13, high costs
Blood eosinophil count	**Anti-IL-5*, anti-IL4/13 (?)**	minimal	Generally available, high clinical impact, predicts anti-IL-5 response. Could be a predictor in anti-IL4/13 treatment.
Sputum eosinophil count	**Corticosteroids, anti-Il-5**	moderate	Specialist centers, tissue specific, time-consuming.

*Proven clinical efficacy in combination with this treatment.

Th2–cytokines as potential treatment targets

Classically, the Th2 pathway is associated with eosinophilic inflammation triggered by allergens or parasites that come into contact with the epithelial barrier. As a first line of defence, dendritic cells pick up the antigens and cause a response cascade after binding to T-helper cells at regional lymphoid sites. In case of a Th2 response, CD4+ lymphocytes become Th2-cells and start producing the cytokines IL-4, IL-5, IL-9 and IL-13 [14].

IL-4 produced by Th2-cells causes a general shift in Th0 cells to differentiate into Th2 cells and an immunoglobulin class switch, resulting in IgE production by B-cells. The produced

IgE by differentiated B-cells (plasma cells) subsequently binds to mast cells and eosinophils that reside in the tissue enabling them to release their toxic granules upon antigen binding. Another Th2 cytokine, IL-5, is a very important systemic regulator of eosinophil dynamics in humans as it is a critical cytokine leading to maturation and activation of eosinophils. Locally it acts as a chemo-attractant and causes migration of eosinophils to sites of damage. IL-5 functions also in combination with IL-9 to recruit mast cells and eosinophils to an affected tissue site. IL-13 has various effector functions: it acts similarly to IL-4 in inducing IgE production by B-cells and it induces physiological changes in mucus production by epithelial cells and causes goblet cell metaplasia. However, compared to IL-4, IL-5 and IL-9 it is has a more extensive effect on airway smooth muscle and airway hyperresponsiveness in animal models [15]. Whether these effects can be translated directly to human asthma remains to be established.

Interestingly, a large subgroup of patients with severe asthma and eosinophilic inflammation is not atopic and has normal serum IgE. This counterintuitive finding complicates the understanding of the pathophysiology of eosinophilic inflammation, and implies that the pathology can be induced independently of exogenous allergens. A newly recognised class of cells might be the missing link between the 'allergic' Th2 response and persistent eosinophilic inflammation in patients without allergies. These cells were discovered in 2010 as nuocytes and eventually named innate lymphoid cells (ILCs) [16]. ILC-2s, a subclass of ILCs, are able to produce large amounts of IL-5 and IL-13 but not IL-4, making them an attractive candidate for orchestrating the immune response in patients with non-atopic eosinophilic airway inflammation. The current paradigm for the role of ILC-2 is that disruption of the epithelial barrier by an external trigger, for example a virus, causes epithelial damage and enhances production of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) by epithelial cells [14]. These cytokines cause ILC2 activation in the tissue, thereby starting the production of Th2 cytokines. Production of prostaglandin D_2 and LTE_4 by recruited and activated eosinophils and mast cells might then enhance responsiveness of ILC-2 cells leading to a perpetuating cycle [17].

Th2 cytokine blockers

Anti–IgE

Omalizumab is a recombinant humanized monoclonal antibody directed against Immunoglobulin E (IgE) and registered for treatment of patients with severe persistent allergic asthma [18]. In a recent Cochrane review, Normansell and colleagues reviewed 25 studies involving 6382 patients [19]. The most important findings were that Omalizumab reduced exacerbations by about 40%, reduced steroid use, improved ACQ scores and improved health related quality of life scores. Side effects were similar in placebo groups and Omalizumab groups, except for some local skin reactions at the injection sites. An important comment by the authors is the fact that most studies included patients with moderate asthma, while the drug is registered for severe asthmatics. Therefore, the effect of

the drug has to be evaluated further in a group that might benefit more from the treatment than patients with moderate asthma.

A more practical difficulty is that most patients with severe asthma are not suitable for Omalizumab treatment as treatment is restricted based on the presence of a perennial allergy, serum IgE and weight. This is unfortunate as none of these characteristics has been shown to be associated with the response to Omalizumab and it is now clear that treatment efficacy is largely a function of the presence of eosinophilic airway inflammation and is accurately predicted by FeNO and serum periostin but not serum IgE [20,21].

Anti–IL–5 or Anti–IL–5R

Anti-IL-5 or Anti-IL-5R treatment influences eosinophil dynamics throughout the human immune system. Both basophils and eosinophils express the IL-5 receptor and can be targeted by blocking IL-5 or the IL-5 receptor. The first RCT with the IL-5 antagonist Mepolizumab in patients with asthma showed a strong biological effect [22]. A single dose of Mepolizumab (10 mg/kg) reduced blood eosinophil count for 16 weeks and sputum eosinophil count for up to 4 weeks. Moreover, Mepolizumab was able to prevent blood eosinophilia during the late phase response after allergen challenge. Although the biological effect was strong, no effect was seen on AHR and the late asthmatic response.

Two larger RCTs with Mepolizumab in patients with moderate asthma taking ICS confirmed the strong biological effect [23,34]. However, both were disappointing in respect of the clinical effect on AHR, lung function, ACQ or rescue medication use. Interestingly this latter group did show a non-significant trend in the reduction of asthma exacerbations after Mepolizumab treatment.

The fact that these studies did not clearly show a clinical effect led many to question whether eosinophilic inflammation is of any importance for the key clinical outcomes in asthma. The alternative possibility that treatment did not have a sufficiently large effect on eosinophilic airway inflammation was unlikely as the effects of treatment on sputum and airway biopsy eosinophil numbers were at least similar to those observed on corticosteroid treatment.

A third possibility was that eosinophilic airway inflammation contributes to exacerbations of asthma to a greater degree than disordered airway function and symptom expression. In support to this, exacerbations and symptom expression are to some extent disconnected within patients and respond to treatment differently [25]. Symptoms and lung function are more responsive to a long-acting beta2-agonist and exacerbations are more responsive to a 4-fold increased dose of inhaled corticosteroids [26].

In addition, studies which have examined the clinical effects of a management strategy which controls eosinophilic airway inflammation as well as symptoms have shown a large reduction in exacerbation numbers but not much change in lung function and symptoms [11,12]. It is now clear that asthma symptoms and traditional physiological tests of asthma are at best weakly associated with the presence of eosinophilic airway inflammation. As many

as 80% of patients with asthma who remain symptomatic despite inhaled corticosteroids have no evidence of eosinophilic airway inflammation and are thus unlikely to respond to a treatment which targets this feature [27].

These findings provided a strong basis for further evaluation of the effects of blocking IL-5 with two essential modifications to the clinical trial design: i) treating patients with evidence of active eosinophilic airway inflammation rather than an arbitrary and unrelated clinical measure such as a significant acute bronchodilator response; and ii) assessing the effect of treatment on asthma exacerbations, an outcome measure which is directly and potentially causally related to eosinophilic airway inflammation.

These studies showed that monthly IV injections of 750 mg Mepolizumab had a beneficial effect on exacerbations and an oral steroid sparing effect [28,29]. In a large multi-center GSK sponsored study (DREAM), different doses of Mepolizumab were evaluated in a population with eosinophilic asthma identified with more permissive criteria including a raised blood eosinophil count and/or FeNO [30]. This study confirmed that treatment reduced asthma attacks by about 50% and showed that equivalent efficacy was apparent with a ten-fold lower dose of Mepolizumab. The study was large enough to begin investigating factors associated with treatment efficacy. Of the many variables assessed, only the blood eosinophil count and the prior frequency of asthma attacks were associated with the effect of treatment [30]. Neither the initial proof of concept study or DREAM showed much effect of Mepolizumab on asthma symptoms, quality of life, FeNO or lung function.

However, two recently published studies focused on severe-asthma patients with a blood eosinophilia and >2 exacerbations in the past year found clear evidence of improvement in these measures as well as a significant OCS sparing effect (**Figure 2**) [31] and a clear decrease in exacerbation rate (**Figure 3**) with monthly subcutaneous injections of 100 mg Mepolizumab [31,32]. The more complete effect of Mepolizumab in these phase III trials may reflect better selection of a responsive population using the blood eosinophil count. No particular safety issues have been identified with long-term use of Mepolizumab and it is therefore a promising therapy for the patients who suffer from severe eosinophilic asthma.

Alternative anti–IL–5 treatments

Reslizumab is another type of IL-5 inhibitors that was used in RCTs for asthma. It was shown to significantly improve lung function, reduce sputum eosinophils and improve ACQ in a subgroup with nasal polyps [33]. Benralizumab, the anti-IL5-receptor alpha drug, causes eosinophil cytotoxicity and has been shown to induce eosinopaenia in a study with mild atopic asthmatics and it radically reduced tissue and sputum eosinophilia in another [34,35]. More recently, a Benralizumab Phase IIb dose-ranging RCT in patients with eosinophilic asthma and non-eosinophilic asthma showed a lowered exacerbation rate in the patients with eosinophilic asthma that received Benralizumab compared to placebo. Eosinophilic asthma was defined according to the ELEN-index, which predicts sputum eosinophilia based on non-sputum parameters. Interestingly, the *post hoc* analysis revealed significant

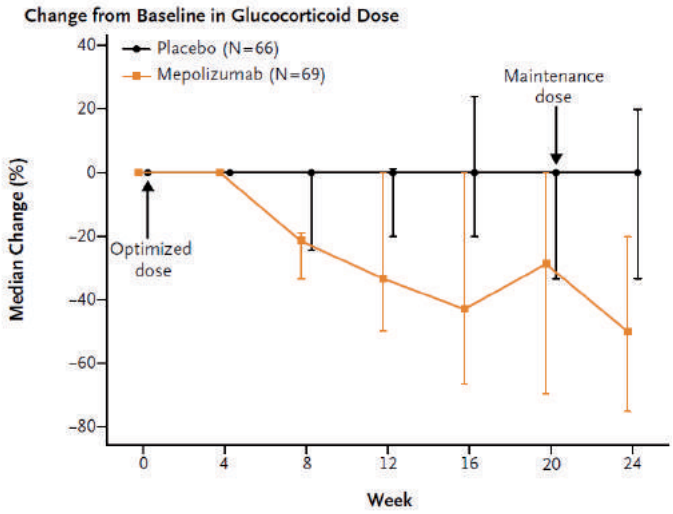

Figure 2: Median percentage reduction from baseline in the daily glucocorticoid dose in the two study groups.

At 24 weeks, the median percentage reduction was 50% in the Mepolizumab group, and there was no reduction in the placebo group (P=0.007). The I bars represent 95% confidence intervals. Courtesy of Bel EH et al. *N Engl J Med* 2014;371:1189-1197.

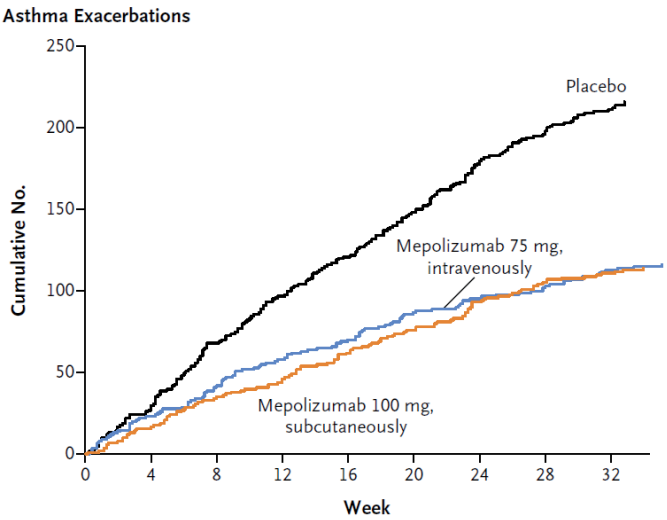

Figure 3: Numbers of asthma exacerbations in patients receiving either intravenous or subcutaneous Mepolizumab or placebo.

The rate of exacerbations was reduced by 47% (95% confidence interval [CI], 29 to 61) among patients receiving intravenous Mepolizumab and by 53% (95% CI, 37 to 65) among those receiving subcutaneous Mepolizumab, as compared with those receiving placebo (P<0.001 for both comparisons). Courtesy of Ortega et al. *N Engl J Med* 2014, doi:10.1056.

improvements in exacerbation rates, ACQ-6 and FEV1 for patients with blood eosinophilia (>300 eosinophils/μL) [36]. Benralizumab did not reduce exacerbations in COPD patients with sputum eosinophilia and an exacerbation in the previous year [37]. However post-hoc analysis of the data showed a similar pattern compared to its severe-asthma equivalent, namely a trend towards lower acute exacerbations of COPD and improvement of the SGRQ-C (St. George Respiratory Questionnaire-C), CRQ-SAS (Chronic Respiratory Questionnaire-Self Assessed Standardised), and FEV1 in the patients with higher blood eosinophil counts. In summary, the various ways of blocking IL-5 and its receptor are promising and might increase treatment effect in the future.

Anti–IL–13

IL-13 is closely linked to IL-4 and exerts similar functions by binding and activating the alpha subunit of the IL-4 receptor. This linkage is the subject of an ongoing debate about the relevance of singularly targeting either IL-4 or IL-13 in asthma. However, in the first trial in humans testing two anti-IL-13 compounds, using allergen challenge in mild atopic asthma, anti-IL-13 attenuated the drop in FEV1 in the late phase [38].

A randomised controlled clinical trial of anti-IL-13 treatment (Lebrikizumab) in a cohort of moderate to severe asthma patients showed a small but significant improvement in FEV1 [39]. The *post hoc* analysis of this study was particularly noteworthy as patients with serum periostin concentrations above the median or a high FeNO had a greater FEV1 improvement and a strong trend to reduced exacerbations with treatment. This emphasizes again the importance of selecting the right endotype of asthma before starting specific treatment. Another IL-13 monoclonal antibody Tralokinumab, also showed an effect on FEV1 but no effect on clinical markers [40].

In a more recent study, the anti-IL-13 compound, GSK679586, showed no clinical effect in a well-defined cohort of 198 severe asthma patients, even though the authors retrospectively stratified the patients by periostin levels and by the presence of blood eosinophilia suggesting that anti-IL-13 might be less effective in more severe asthma [41].

IL–4 receptor–alpha blockers

Blocking the alpha subunit of the IL-4 receptor affects both IL-4 and IL-13 signalling. The first trial with anti-IL-4-alpha blockers was a RCT that studied the effect of the fully human monoclonal antibody AMG 317 in patients with moderate to severe asthma and showed no beneficial effect on the main endpoint, which was reduction in the ACQ scores of the overall population [42]. However, it did show an effect in a subgroup that had high ACQ baseline scores, which therefore could be regarded as a more uncontrolled group. In the most recent trial by Wenzel *et al.*, a subgroup of patients with persistent moderate to severe asthma who had blood eosinophilia >300/ microliter received either placebo or anti-IL-4-alpha treatment (Dupilumab) [43]. During the study, patients were withdrawn from long-acting beta-agonist (LABA) treatment first and secondly from ICS to observe differences in exacerbation rate

(main endpoint). There was a significant difference in exacerbation rate observed after withdrawal, in favour of the Dupilumab group. Secondary endpoints, such as FEV1 improved significantly and ACQ also dropped in the treatment arm with evidence of a treatment effect on these measures before LABA and ICS were withdrawn. Treatment reduced FeNO, IgE levels, TARC (Thymus and activation regulated chemokine) and eotaxin-3 levels, providing evidence of a biological effect of the drug. One concern with the main endpoint is the definition of an exacerbation, also described by the authors. An exacerbation was defined as the need for systemic corticosteroids or doubling of the ICS dose, which differs from the current consensus statement in which >3 days of oral corticosteroids is used as a definition.

Prospects for Th–2 low disease

Prospects for modifying airway inflammation in Th-2 low disease is much more uncertain as the patterns of airway inflammation and its likely cause are poorly understood. Some encouragement is provided by the beneficial effects of long-term low-dose macrolides in patients with non-eosinophilic asthma and by preliminary evidence of efficacy of a CXCR2 antagonist [44]. Neutrophilic airway inflammation might be driven by Th-17 mediated processes [45].

In a first clinical trial anti-IL17, Brodalumab did not improve ACQ scores (primary endpoint) in a group of moderate to severe asthmatics [46]. However, treatment did have beneficial effects in a subgroup with high reversibility to albuterol. Patient selection was not optimal as the presence of neutrophilic airway inflammation was not confirmed and there was no marker of IL-17 involvement included for selection. More work is needed to understand the mechanisms and phenotypes of Th-2 low asthma.

Conclusion

The success of the introduction of novel biological agents in asthma largely depends on the ability to select the appropriate asthma patients. All ‘successful’ clinical studies that involved novel biological agents included a specific subgroup that was likely to be responsive to the treatment. Ideally, patients are selected by an easily measurable biomarker that is directly influenced by the treatment. Thus, the everyday saying used in the title of this review very much applies for biological treatment of severe asthma.

It is interesting that existing data shows that FeNO and serum periostin are good biomarkers of treatment response to Omalizumab and biological agents targeting IL-13 and IL-4 whereas the blood eosinophil count is most closely associated with a response to anti-IL-5. Moreover, treatment with IL-5 reduces the blood eosinophil count but not FeNO whereas the reverse is true for Omalizumab, anti-IL-13 and anti-IL-4&13 (**Table 2**). Whether these biomarkers can be used to identify sub-groups of patients within the Th-2 high population who are particularly suited to different cytokine blockade, will be an important research question for the future.

Table 2: Effect of Th2 associated monoclonal antibodies on clinical markers and biomarkers in adult asthma patients

Mono-clonal antibody	Biomarker used for patient selection	FEV_1	AHR	ACQ	Exacer-bations	OCS-sparing effect	QLQ	Blood eos.	Sputum eos.	FeNO	Serum IgE	Comments
		Effect on clinical endpoints						Effect on biomarkers				
Anti-IL-5	PB and sputum eos. count, exacerbation rate	0/+	0	0/+	++	++	+	↓↓	↓	0	0	Clinical effects in specific subgroup of severe asthma
Anti-IgE	Blood IgE*, spec. IgE level and positive SPT*, FeNO, periostin	+	0	+	++	unclear	+	Stable or small ↓	↓	↓↓	↓↓	Most RCTs focused on moderate asthma, sparse evidence in severe asthma
Anti-IL-13	Periostin level, FeNO	+	unclear	0/+	unclear	N/A	0	↑	unclear	↓	↓	Partially based on subgroup analysis
Anti-IL-4/ IL-13	Periostin and sputum eos., IgE, FeNO	+	Exp.	unclear	unclear	unclear	N/A	Stable or ↑	unclear	↓	↓	Promising combination, one RCT

+ = clinically improved; 0 = measured and no effect observed; N/A = not attributable/not measured; unclear = measured, not enough data points for conclusion. Exp, expected; FEV1, Forced Expiratory Volume in 1sec.; AHR, airway hyperresponsiveness; ACQ, asthma control questionnaire; OCS, oral corticosteroid; ICS, inhaled corticosteroid; QLQ, quality of life questionnaire; eos., eosinophils; FeNO, fraction of exhaled nitric oxide; IgE, Immunoglobulin E; RCT, randomised controlled trial; IL, Interleukin; *Blood IgE does not correlate with treatment response to anti-IgE.

Another important priority is to understand the pathophysiology of asthma in patients with no evidence of Th-2 mediated inflammation since patients with this phenotype of asthma have few treatment options.

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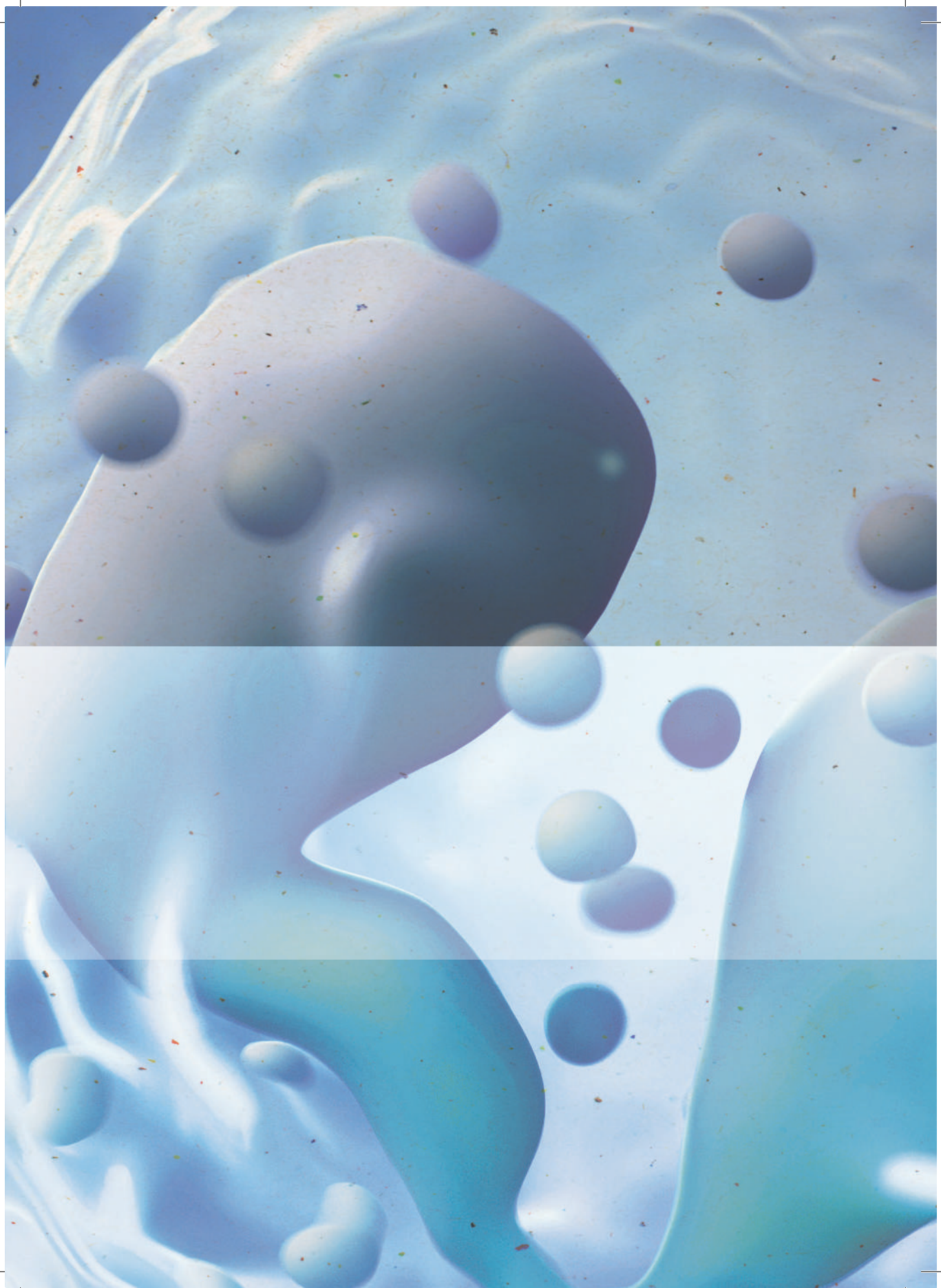

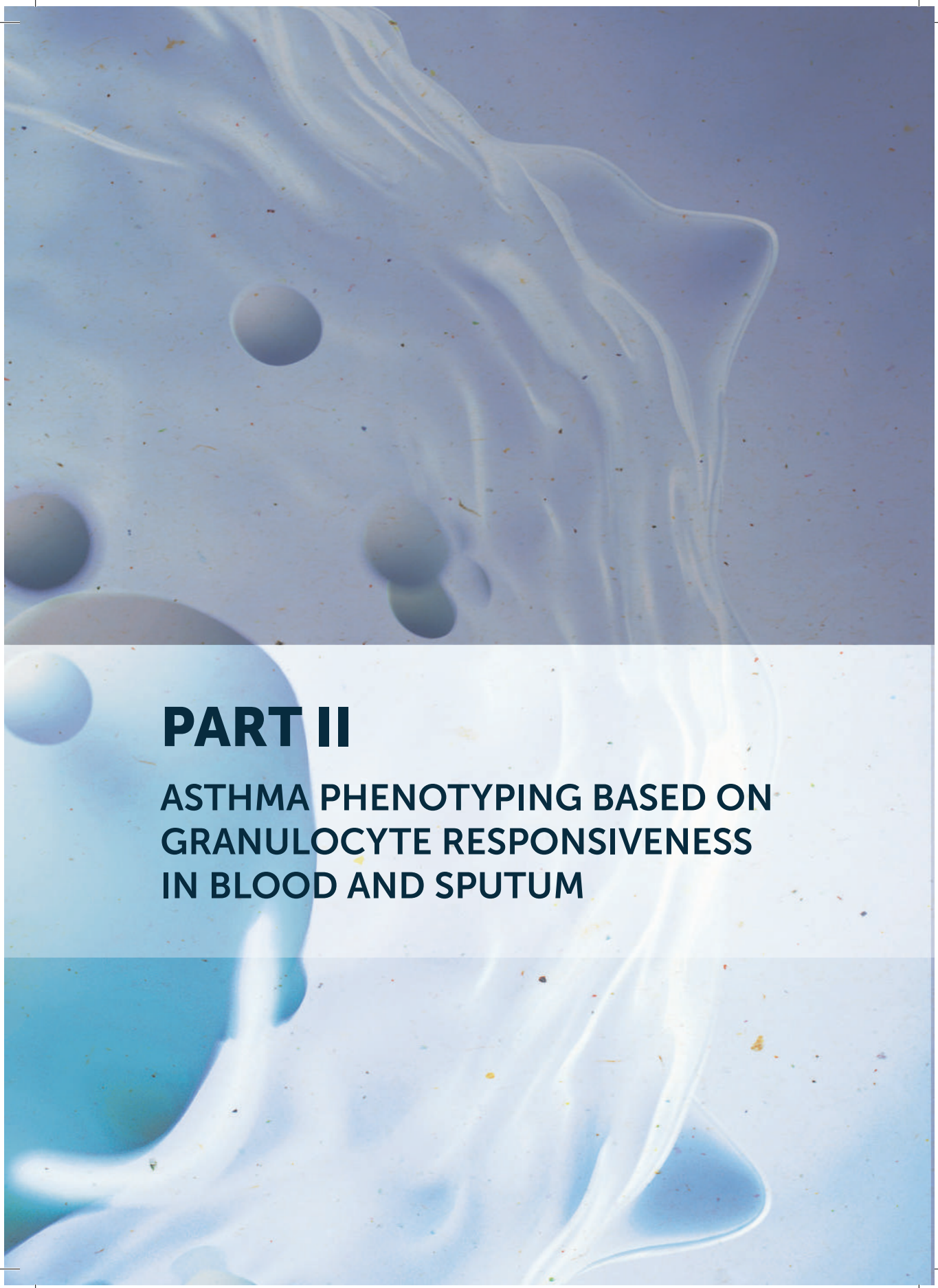

PART II

ASTHMA PHENOTYPING BASED ON GRANULOCYTE RESPONSIVENESS IN BLOOD AND SPUTUM

CHAPTER 2.1

Diagnosing eosinophilic asthma using a multivariate prediction model based on blood granulocyte responsiveness

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Abstract

Background: *The identification of inflammatory asthma phenotypes, using sputum analysis, has proven its value in diagnosis and disease monitoring. However, due to technical limitations of sputum analysis there is a strong need for fast and non-invasive diagnostics. This study includes the activation state of eosinophils and neutrophils in peripheral blood to phenotype and monitor asthma.*

Objectives: *To (1) construct a multivariate model using the activation state of blood granulocytes, (2) compare its diagnostic value with sputum eosinophilia as gold standard and (3) validate the model in an independent patient cohort.*

Methods: *Clinical parameters, activation of blood granulocytes and sputum characteristics were assessed in 115 adult asthma patients (training cohort/Utrecht) and 34 patients (validation cohort/Oxford).*

Results: *The combination of blood eosinophil count, FeNO, ACQ, medication use, nasal polyposis, aspirin sensitivity and neutrophil/eosinophil responsiveness upon stimulation with fMLF, was found to identify sputum eosinophilia with 90.5% sensitivity and 91.5% specificity in the training cohort and with 77% sensitivity and 71% specificity in the validation cohort (relatively high percentage on OCS).*

Conclusions: *The proposed prediction model identifies eosinophilic asthma without the need for sputum induction. The model forms a non-invasive and externally validated test to assess eosinophilic asthma in patients not on OCS.*

Introduction

An estimated 334 million people worldwide suffer from asthma, while its prevalence is still rising [1]. The majority of patients are well-controlled with beta-agonist combined with inhaled corticosteroids. However, 5-10% of the patients suffer from poorly controlled asthma, consume ~60% of total asthma-related health care costs and experience long-term side effects of oral glucocorticoids use. This group needs better asthma treatment and identifying inflammatory phenotypes is essential to choose the right treatment option.

Since the introduction of sputum induction to obtain cellular samples from the airways, it has been one of the most accepted methods to assess airway inflammation and thereby diagnosing the asthma inflammatory phenotype [2]. Its clinical value in asthma management was established in three randomized controlled trials that tailored treatment based on sputum eosinophilia [3-5]. These studies independently showed a reduction of asthma exacerbations after treatment adjustments that were based on sputum eosinophilia. In addition the presence of eosinophils in airway epithelium [6] and sputum has been shown to correlate with exacerbation frequency [3-5]. However, sputum induction is considered to be an invasive, time-consuming diagnostic test that needs to be performed only in specialized centres. Another disadvantage is the procedure fail rate (10-30%). These limitations restrict this type of adequate inflammatory phenotyping to a cohort of severe asthma patients [7,8].

Measuring peripheral blood eosinophil count is a promising alternative for sampling in the airways. In the past, cohort-studies that focussed on relations between blood eosinophilia and asthma found correlations between blood eosinophilia and asthma diagnosis, asthma events, emergency department (ED) visits, sputum eosinophilia and wheeze [9,10]. In the DREAM study, blood eosinophilia correlated with a reduction in exacerbations after anti-IL5 (Mepolizumab) treatment and was a predictive indicator for reduction in sputum eosinophil count [11]. Later on, blood eosinophilia was the basis for patient selection in two large phase III studies that looked into the effect of Mepolizumab on exacerbation frequency and glucocorticoid sparing [12,13].

In contrast to sputum eosinophilia it is yet unclear if glucocorticoid treatment strategies based on blood eosinophilia can reduce exacerbation frequency or improve other outcome measures in asthma. Blood eosinophil count does not correlate perfectly with sputum eosinophilia. This stretches the importance to identify fast and accurate measures to predict airway eosinophilia. The blood compartment is favourable because it is easily accessible, already part of routine clinical workup and with technical advances in measurements such as multi-colour flow cytometry has increased potential for inflammatory phenotyping.

In addition to eosinophil count, the activation state of eosinophils could be a promising biomarker. Johansson and colleagues indicated that priming and activation of eosinophils in the peripheral blood is deficient during episodes of tissue eosinophilia in severe and uncontrolled asthma [14]. This hypothesis was partly founded on the upregulation of active FcγRII on activated blood eosinophils after segmental lung challenge in mild asthmatics [15].

The latter seems to contradict the putative deficiency of primed or activated cells. However, long-term priming of eosinophils in the peripheral blood of severe asthmatics and the subsequent migration to the lung could lead to a deficiency of primed cells within the peripheral blood [16]. An up-regulation of active integrin-receptors and activation-related receptors are found on blood granulocytes in mild to moderate asthma and in contrast to this low expression profiles of these markers are found in severe inflammatory disease [17]. These findings indicate relevance of granulocyte priming and activation for assessment of the inflammatory status of asthma patients.

Not only additional biomarkers could improve asthma phenotyping; combined analyses of known clinical and biological characteristics provided important insights in airway disease mechanisms by using the multivariate advantage [18]. Multivariate advantage refers to classifications based on multiple, combined features that outperform the combined classifications on the separate features. In asthma a key finding was the absence of correlation between eosinophilic inflammation and symptoms [19]. Haldar and colleagues furthermore showed the value of inflammation-driven treatment decisions based on an unbiased approach for patient selection. Two other studies that made use of the multivariate advantage evaluated the power of blood eosinophil count, FeNO and periostin level to predict sputum eosinophilia [20] and to predict the response to anti-IgE treatment [21]. Both conclude that the combination of the three markers might be a good way to assess the inflammatory status of asthma patients, while the value of the single parameters FeNO, blood eosinophils or total IgE to predict sputum eosinophilia has been regarded to be moderate. In a meta-analysis of 24 studies overall sensitivity and specificity in detecting sputum eosinophilia in adults were: 0.66 and 0.76 respectively for FeNO; 0.71 and 0.77 for blood eosinophils; and 0.64 and 0.71 for IgE [22].

We designed a cross-sectional study to investigate whether the classification accuracy of a multivariate prediction model for sputum eosinophilia benefits from including measurements of peripheral blood granulocyte activation status and whether such a non-invasive prediction model has sufficient diagnostic value to replace expertise-dependent sputum analysis. The multivariate prediction model is based on a training cohort (Utrecht, The Netherlands) and prospectively validated on independent data from a validation cohort (Oxford, United Kingdom). Sputum eosinophilia was set as gold standard.

Methods

Subjects

Training cohort

Asthma patients aged 18-75 were recruited at the respiratory outpatient clinics of the University Medical Center Utrecht (UMCU), and the Central Military Hospital Utrecht (CMH), The Netherlands between May 2012 and December 2013. Inclusion and exclusion criteria are provided in the Supplementary Material (**Figure S1**, flow chart). Written informed

consent was obtained, and the local ethics committee of the UMCU and CMH approved the study protocol.

Test/Validation cohort

Adult asthma patients were recruited at the respiratory outpatient clinic of the Churchill Oxford University Hospital between September 2014 and June 2015. The same inclusion and exclusion criteria were used as in the test cohort. The study protocol was ethically approved and written informed consent was obtained from all patients.

Study design

Asthma patients (see **Table 1** for demographics) underwent lung function measurement, sputum induction, blood withdrawal and FeNO (Fractional exhaled Nitric Oxide) measurement. Their medical history was taken and both the Asthma Control Questionnaire (ACQ) [23] and the Medication Adherence Report Scale (MARS) [24] were filled out.

Sample size

For the sample size calculation we refer to Supplementary Material, Methods, sample size.

Measurements

Blood

Blood was obtained in 9mL tubes containing sodium heparin, transported at room temperature and processed and analysed within 2 hours. Eosinophil and neutrophil priming was tested *in vitro*: Four polystyrene tubes with 50µL blood were incubated for 5 minutes at 37°C. Hereafter, two of the tubes were stimulated with 5µL 0.001 mM N-formyl-methionyl-leucylphenylalanine (fMLF) for 5 minutes. Subsequently, whole blood in all tubes was stained with fluorescein isothiocyanate (FITC) labeled monoclonal phage antibodies (Abs) [A17 or A27] [31] and with phycoerythrin (PE)-labeled αM (CD11b) and incubated for 30 minutes on ice. Hereafter, red cells were lysed in ice-cold isotonic NH_4Cl and cells were centrifuged at 1500 rpm for 5 minutes. The cell pellet was washed twice and resuspended in ice-cold PBS/1% human serum albumin. In the test cohort, cells were measured using a Gallios flow cytometer (Beckman Coulter, Brea, California, US). In the validation cohort cells were measured using a Cyan flow cytometer (Beckton Dickinson, Franklin Lakes, New Yersey, US). Prior to the analysis blood was stained with Krome Orange (KO)-labeled CD16 antibody. Eosinophils could be distinguished from neutrophils by low FcγRIII (CD16) expression. Data from individual experiments are reported as fluorescence intensity in arbitrary units (AU) or in n-fold change from baseline.

Lung function and FeNO

FEV1 measurements were performed by using the PiKo-1 (nSpire™) device and FeNO was determined using NIOX MINO® (Aerocrine, Solna, Sweden) with an expiration time of 10 seconds.

Sputum

Sputum induction was performed with hypertonic saline according to the European Respiratory Society (ERS) guideline [25]. Cytospin slides of sputum cells were stained with May-Grünwald Giemsa and cells were differentiated and counted by an experienced technician. A cut-off value of 3% eosinophils in sputum was used to classify patients with eosinophilic or non-eosinophilic inflammation. A cut-off value of 61% neutrophils in sputum was used to classify patients with neutrophilic inflammation. A mixed phenotype was assigned if >3% eosinophils and >61% neutrophils were counted. Eosinophils <3% and neutrophils <61% was regarded as a paucigranulocytic phenotype.

Statistical analysis

Non–linear Principal Component Analysis (NLPCA)

Principal Component Analysis (PCA) is a widely used unsupervised method to reduce dimensionality in data sets. However, PCA is only suitable to analyse data consisting of continuous variables. We used Non-linear PCA (NLPCA) because the majority of variables in our study were either categorical or nominal. Linting et al. described a stepwise approach for NLPCA and applied the technique in clinical cohorts [26,27]. Applying this technique, we were able to take into account the correlated variance from 26 clinical and immunological parameters simultaneously. Briefly, the method applied entails transposition of all parameters to a linear scale, followed by reduction of the number of parameters by a two-step selection process based on correlation of variances and by PCA of the resulting dataset to produce a simplified description of the data that retains as much variance as possible using only a small number of principal components.

After creating a final model with NLPCA using the Utrecht cohort as a 'training set', the Oxford cohort was plotted in this PCA-model as test set in SPSS using >Data >Weight of 0.01 per patient. Thus, the Oxford cohort was used as validation set.

Discriminant Analysis (DA)

Discriminant Analysis was used on the NLPCA scores. A class for eosinophilic asthma and a class for non-eosinophilic asthma was set (≥3% sputum eosinophils), this is a supervised step. External validation of the Oxford data was performed by weighting the NL-PCA scores of these patients by 0.01 in the DA. For an overview of both NLPCA and DA steps see **Figure 1**.

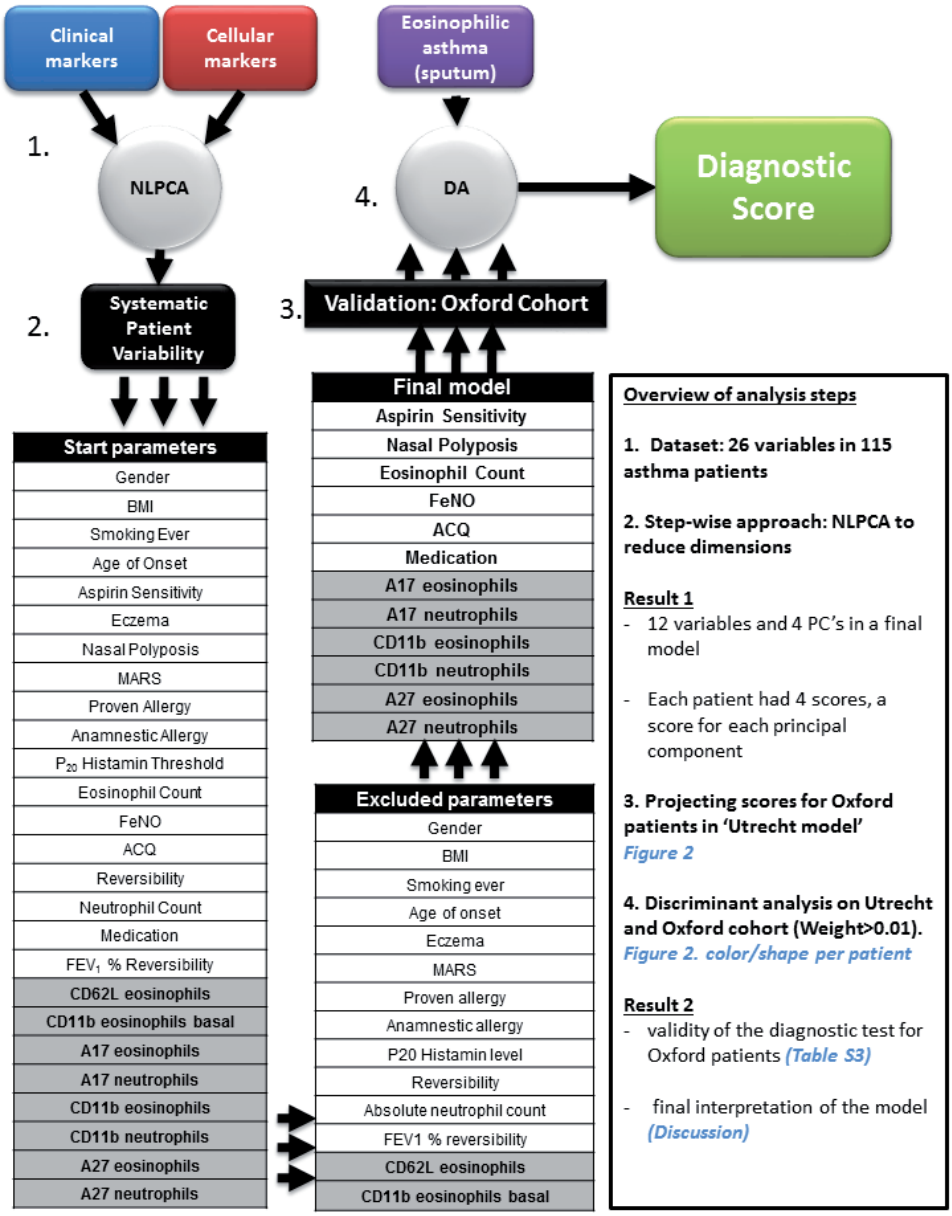

Figure 1: Overview of the data analysis procedure.

Step 1 and 2: Clinical (in the white cells) and peripheral blood markers (in the grey cells) were combined to build a model by using dimension reduction (NLPCA, unsupervised). After step 1 and 2, the Oxford Cohort was added to the NLPCA model to validate the prediction model for airway eosinophilia. Subsequently, DA was performed by setting a class for eosinophilic asthma and a class for non-eosinophilic asthma (≥3% sputum eosinophils), this supervised step was performed to obtain a diagnostic score.

Results

One hundred fifteen asthma patients were recruited in the Netherlands (see Supplementary Material **Figure S2** for the inclusion process). In total 76 patients could be classified by sputum analysis and 39 asthma patients (34%) were not able to cough up or had sputum samples that showed >80% buccal squamous cells. Twenty of the 34 patients recruited in the United Kingdom (59%) could be classified by sputum induction. Demographic details are presented in **Table 1**.

Multivariate diagnostic model

Out of 26 parameters, NLPCA identified 12 important parameters that together described most variance within the cohort of patients in Utrecht (see **Figure 1**). Six of the final parameters were classical 'clinical' parameters and the other six were peripheral blood parameters that describe responsiveness of eosinophils and neutrophils to fMLF. The six parameters with the highest variance accounted for (VAF) were: aspirin sensitivity, CD11b response on eosinophils and neutrophils, nasal polyposis, ACQ and A17 response on neutrophils, in decreasing order. The remaining parameters explained less variance, being A27 response on eosinophils, medication, A17 response on eosinophils, FeNO, eosinophil count and A27 response of neutrophils. The stability of the NLPCA model was tested by performing a bootstrapping procedure on the test cohort (Utrecht). Ten cohorts were created that separately underwent NLPCA. The loadings from these NLPCA analyses were compared to the original loadings and the RV-coefficient of this comparison was 0.84. This high correlation coefficient (value between 0 and 1) indicates the NLPCA loadings are highly stable. Technical details of the performed NLPCA are supplied in paragraph I of the results section of the Supplementary Material 'NLPCA' and in **Figure 1**.

Interpretation of the model

The result of NLPCA is a set of 'scores' and 'loadings'. As there are four principal components in this model (see Supplementary Material for the origin of this number), each individual patient is represented by four scores. **Figure 2** (middle) shows both the loadings of the 12 most relevant parameters and the scores of the patients on the first two principal components of the model. These two principal components together define the 2D-projection of the data in which the most variability can be presented. The position of a patient indicates its score on PC1 (horizontal axis) and its score on PC2 (vertical axis). The 12 loadings per PC each represent the contribution of a single parameter, such as for example eosinophil count, to the variability among the patients described by the PC: The higher the correlated variance of a parameter, the higher the loading and the longer the vector in **Figure 2**. Parameters pointing in the same direction are likely to be correlated.

The distribution of patients within the score/loading-plot (**Figure 2**) is largely determined by markers of eosinophilic inflammation as indicated by the direction of the markers: Patients with sputum eosinophilia plot in areas indicated by the direction of the loading vectors of the

Table 1: Baseline characteristics of subjects in Utrecht cohort

Utrecht cohort n = 115			Oxford cohort n = 34	
Age (mean)	43		56	
Gender (M/F)	55/60		19/15	
BMI, kg/m^2	27		31	
Smoking ever (%)	27		41	
Pack years	1.28		4	
Aspirin Sensitivity (%)	5		24	
Eczema	20			
Nasal Polyposis (%)	19		29	
ACQ	1.4		1.6	
Proven allergy (anamnestic and spec. IgE) (%)	59		71	
History of allergy	77			
FeNO (ppb)	23*	16-36	18*	19-45
% predicted FEV1 (L)	86	82- 89	68	60-75
Total eosinophil count in PB * 10^9/L	0.22*	0.13-0.41	0.25*	0.12-0.39
Sputum cell profile		%		%
Eosinophilic (>3% eosinophils)	21	18	11	32
Neutrophilic (>61% neutrophils)	14	12	8	24
Mixed (>3% eos. and >61% neutr.)	8	7	2	6
Paucigranulocytic	33	29	0	0
Epithelial (>80% epithelial cells)	39	34	13	38
Treatment		%		%
No medication (currently)	3	3	1	3
SABA	1	1	2	6
Low-dose ICS	5	4	1	3
Low-dose ICS + LABA or medium dose ICS	69	60	1	3
High dose ICS + LABA (and/or LTRA)	24	21	19	56
High dose ICS + LABA + OCS	13	11	10	30
MARS (non adherence in percentage)	27			

BMI = body mass index, MARS = medication adherence report scale, ACQ =Asthma Control Questionnaire, FeNO = Fraction of exhaled nitric oxide. FEV1 = Forced Expiratory Volume in 1sec, PB = peripheral blood, SABA = short-acting beta-agonist, LABA = long-acting beta-agonist, ICS= inhaled corticosteroids, LTRA = leukotrien-receptor-antagonist. *Median and IQR.

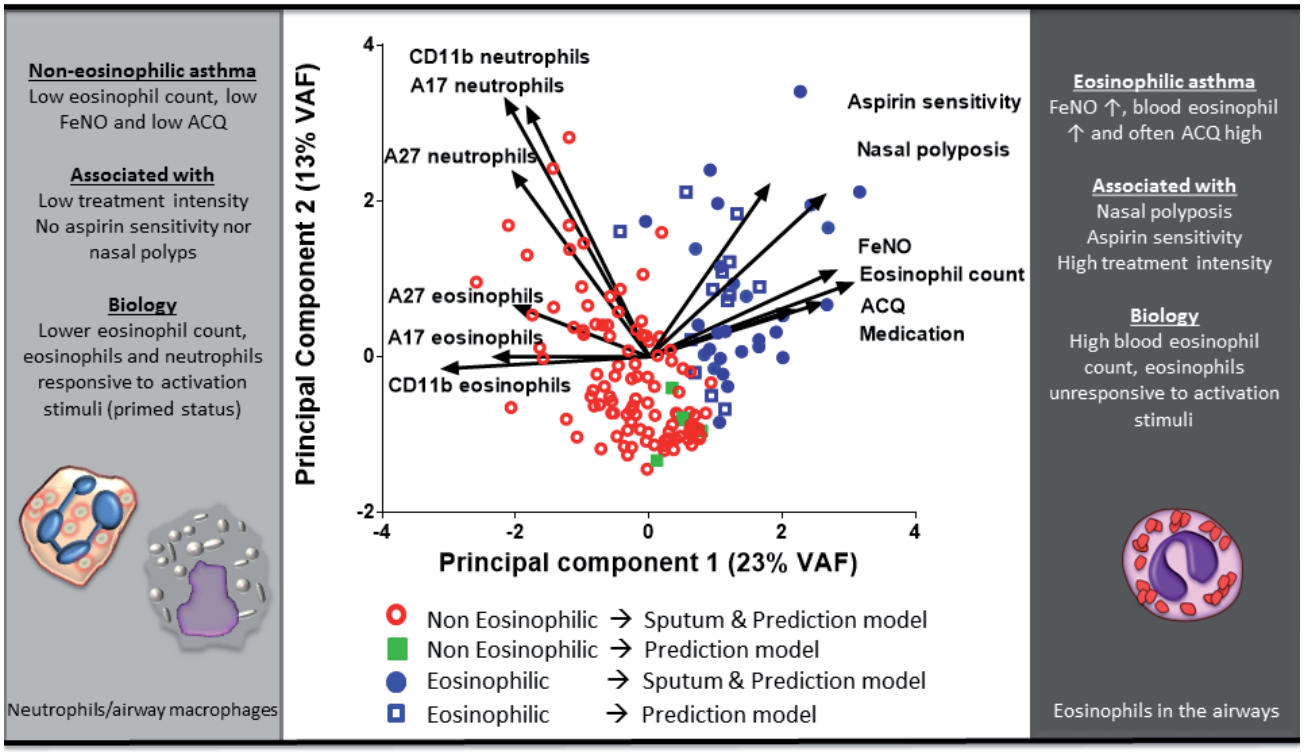

Figure 2: Combined scoring and loading plot after non-linear principal component analysis.

Each data point represents a patient. Each vector represents a variable, in total 12 in this model. The more variability a variable has, the longer the vector. Each patient's score depends on the value of these 12 variables. Therefore, eosinophilic patients (in blue) are high in FeNO, ACQ, eosinophil count and low in eosinophil responsiveness, while their non-eosinophilic counterparts (in red and green) exhibit low values in these clinical markers, but higher values of granulocyte responsiveness and appear on the opposite side (left and bottom) of the graph. CD11b, A17 and A27 on eosinophils and neutrophils represent fold-induction of receptor expression (fluorescence intensity after fMLP stimulation divided by baseline fluorescence intensity).

parameters FeNO, ACQ, eosinophil count, medication, nasal polyposis and aspirin sensitivity. On the contrary, these patients have low values of blood eosinophil responsiveness (A17, A27 and CD11b) and therefore plot in the opposite direction of these vectors. In short, if there is a high percentage of eosinophils present in sputum, a patient has blood eosinophilia with cells that are refractory to stimulation. At the same time, patients with a neutrophilic and a paucigranulocytic sputum phenotype have lower values of for example FeNO and ACQ and higher values of responsiveness of eosinophils and neutrophils and therefore plot on the other side of the graph. Notably, sputum characteristics were not part of the selection of parameters for the multivariate model (**Figure 1**) and were used as a gold standard. The technical details of the performed DA are supplied in 'Statistical Analyses, paragraph Discriminant Analysis' section of the Supplementary Material.

Validation

An internal cross-validation was used to test the classification accuracy of the NLPCA/DA hybrid model with sputum eosinophilia as the dependent variable. Based on a Leave-One-Out cross validation of the Utrecht cohort, sputum eosinophilia could be predicted with a sensitivity of 90.5 and a specificity of 91.5 (**Table S1**, of the Supplementary Material), In the next step, by using the 'Utrecht cohort' as a test set and subsequently adding the 'Oxford cohort' as a validation set it was possible to classify sputum eosinophilia with 77% sensitivity and 71% specificity (**Table 2**, tested by cross-validation).

The discriminant analysis results in four classes (**Table 3**) by means of positivity or negativity for sputum eosinophilia (gold standard) and positivity or negativity predicted by the model.

Four ROC curves (**Figures 3A-D**) were created by using the discriminant function and sputum eosinophilia as state variable. Notably, the fourth ROC curve (**Figure 3D**) was created by leaving out patients who were taking OCS.

Table 2: 2x2 contingency table with diagnostic score of the prediction model with respect to the Oxford Cohort

The number of patients correctly classified with eosinophilic disease is 10 out of 13 (76.9%). The number of patients with non-eosinophilic disease is correctly identified in 15 out of 21 (71.4%). On average, 73.5% of original grouped cases is correctly classified (leave-one-out cross validation accuracy). Eos: eosinophilic asthma, Non-eos.: non-eosinophilic asthma.

Prediction model for sputum eosinophilia		Predicted group membership		Characteristics
		Eos.	Non-eos.	
Sputum Analysis	Eos.	10	3	Postive Predictive Value (PPV): 62.5%
	Non-eos.	6	15	Negative Predictive Value (NPV): 83.3%
Characteristics		76.9% Sensitivity	71.4% Specificity	Accuracy: 73.5%

Finally, the dataset was rerun without the granulocyte responsiveness data. The sensitivity dropped from 90.5% to 47.6% and specificity increased slightly from 91.5% to 95.7% (**Table 4** and **Figures 4A-B**).

Table 3: Clinical characteristics of the four groups identified by the prediction model

All values are represented in mean and 95%CI lower/upper limit, or in number (n) except for FeNO which is expressed in median and interquartile range.

	Eosinophilic by Sputum &Pred. Model	Non-eosinophilic by Sputum & Pred. Model	Eosinophilic Pred. model	Non-eosinophilic Pred. model
n	29	102	13	5
FeNO, median (IQR)	48 (215)	20 (81)	25 (120)	16 (21)
ACQ (CI)	2.71 (3.86)	1.29 (4.43)	1.0 (3.57)	1.29 (1.86)
Eosinophil count $*10^9$/L(CI)	0.49 (1.16)	0.16 (0.8)	0.27 (0.75)	0.18 (0.62)
Aspirin sensitivity % (n)	24 (7)	1 (1)	46 (6)	0
Nasal polyposis % (n)	66 (19)	6 (6)	54 (7)	0
Medication, % on OCS (n)	28 (8)	8 (8)	46 (6)	20 (1)

Table 4: 2x2 contingency table with diagnostic score of the prediction model without blood markers, using sputum analysis as reference test

The number of patients correctly classified with eosinophilic disease is 10 out of 21 (47.6%). The number of patients with non-eosinophilic disease is correctly identified in 90 out of 94 (95.7%). On average, 87% of original grouped cases is correctly classified (leave-one-out cross validation accuracy). Eos: eosinophilic asthma, Non-eos.: non-eosinophilic asthma.

Prediction model for sputum eosinophilia		Predicted group membership		Characteristics
		Eos.	Non-eos.	
Sputum Analysis	Eos.	10	11	Postive Predictive Value (PPV): 71.4%
	Non-eos.	4	90	Negative Predictive Value (NPV): 89.1%
Characteristics		47.6% Sensitivity	95.7% Specificity	Accuracy: 87.0%

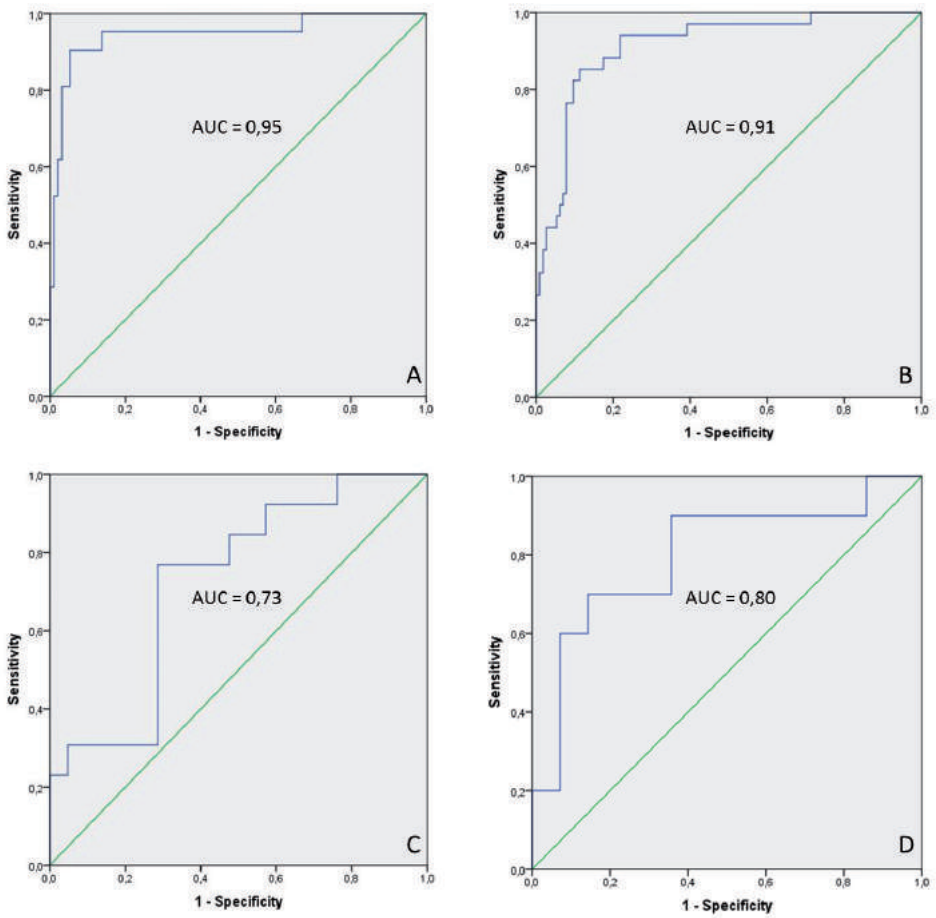

Figure 3: ROC curves.

A. Based on the Utrecht dataset. This ROC curve has an AUC close to 1 as it is the base of the NLPCA model. AUC = 0.946, the reported p-value is: <0.001. **B. ROC curve based on the combined Utrecht and Oxford datasets.** This combined set has a high AUC of 0.914, the reported p-value is: <0.001. **C. ROC curve based on the Oxford validation cohort only.** The AUC is lower compared to the test set and indicates a difference between test set and validation set. AUC = 0.725, the reported p-value is: 0.029. **D. ROC curve based on the Oxford validation cohort only without BTS treatment group 6.** For this ROC curve patients in BTS treatment group 6 (oral steroid treatment) were excluded. The AUC increased from 0.73 to 0.80. AUC = 0.800, the reported p-value is: 0.014.

Discussion

The findings of this cross-sectional study in a 'training' cohort of 115 asthma patients and a 'validation' cohort of 34 patients visiting university medical centres in respectively Utrecht and Oxford, underline the value of cellular markers in peripheral blood to classify asthma phenotypes. fMLF-induced upregulation of activation-associated receptors on eosinophils

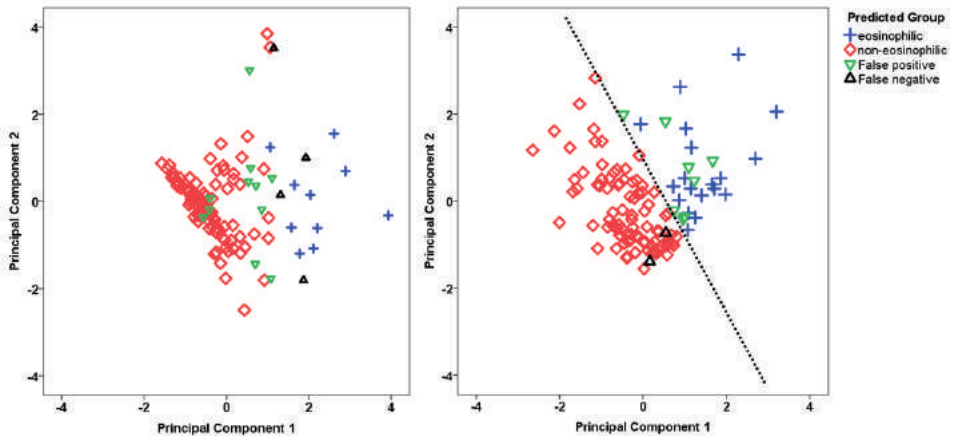

Figure 4: Prediction models.

A. without blood markers. According to this model differences between groups of patients are not clear; true non-eosinophilic asthma patients (red diamonds), true eosinophilic asthma (plus sign), false positive patients (green diamonds) and false negative (black triangles). The model has a poor diagnostic value. **B. Prediction model based on blood markers.** The model discriminates accurately between eosinophilic and non-eosinophilic asthma. The dotted line indicates the discrimination between eosinophilic (plus sign) and non-eosinophilic (red diamonds) disease according to the prediction model based on clinical parameters and blood granulocyte measures. The two false negatives (black triangles) are not identified by the model, however the eight false positive cases (green diamonds) that have a high symptom and high eosinophilic inflammation-profile, illustrate the improved classification capability of the prediction model. These false positives would have been missed by sputum analysis only.

and neutrophils, together with a limited set of clinical parameters, can serve as an accurate read-out for eosinophilic asthma. Results of the unbiased analysis of both cellular and clinical parameters confirm the important role for already established measurements in asthma, such as eosinophil count, ACQ and FeNO. However in this study, adding measurements of blood granulocyte responsiveness significantly increased the predictive accuracy, improving the sensitivity from 47.6% to 90.5%.

Interestingly, the 'eosinophilic patients by prediction model' (i.e. patients without sputum eosinophilia) have distinct characteristics; these 13 eosinophilic patients have higher blood eosinophil counts, higher values of FeNO and a higher incidence of aspirin sensitivity and nasal polyposis compared to the non-eosinophilic patients. More patients in this group are using oral glucocorticoids compared to the other groups (~46%, **Table 3**). Oral corticosteroids are known to induce apoptosis in eosinophils and can explain the 'false' low number of sputum eosinophils [28]. To strengthen this, the Oxford cohort has a ~3-fold higher percentage of patients on OCS compared to Utrecht. Therefore, these patients are particularly less likely to have sputum eosinophilia, leading to the 'false' conclusion they do not suffer from

eosinophilic asthma. Based on known steroid effects and high OCS use in the group of 13 patients that were 'false positive', OCS use is the most likely explanation for the relatively low sensitivity and specificity of the prediction model in the Oxford cohort and suggests the prediction model as developed here is more suitable for asthma classification in patients not on OCS. This was validated by excluding patients on OCS from the Oxford cohort, which led to an increase in predictive power: 79.2% compared to 73.5% without this group. The sensitivity lowered from 76.9% to 72.7% and the specificity increased extensively from 71.4% to 84.6%. The ROC-curves with the full Oxford cohort (**Figure 3C**) and the cohort with patients on oral steroids (**Figure 3D**) also shows a great improvement in AUC-value.

Instead of using a single parameter approach, an unbiased multidimensional approach was used to evaluate the experimental data. This is generally regarded as a promising analysis strategy for the understanding of heterogeneous diseases such as asthma [29,30]. Large asthma cohorts, such as SARP and the Leicester cohorts, already brought more insight in disease phenotypes using clustering techniques [19,31,32]. One strong determinant of the quality of multidimensional models is number of included parameters. Therefore, it is important to include and test new parameters such as granulocyte responsiveness. In this study we were able to improve the sensitivity of our prediction model from 47.6% to 90.5% by adding granulocyte responsiveness to the model. By using Non-Linear Principal Component Analysis correlations between many of the measured clinical parameters were taken into account. These correlations may be clinically valuable but on the other hand also complicate multiple linear regression models. NLPCA provides a consistent, widely used and quantitative way to merge parameters measured on different levels.

Our prediction model is based on 12 clinical and cellular parameters and does not depend on several common asthma parameters such as atopy, gender and BMI. These latter parameters showed little discriminative value in our cohort. This finding is in agreement with findings in the larger Leicester and SARP cohorts [19,31-34]. The Leicester cohorts showed that atopy, gender and BMI were not significant determinants for the secondary care factor model. Similarly, the SARP cohorts also had low variability within the datasets for atopy, gender and BMI. These collective findings are also in line with insights from the DREAM cohort [11]. Atopy in the DREAM cohort was not a predictor for the response to Mepolizumab, whereas peripheral blood eosinophil count and exacerbation frequency in the past year, both hallmarks of eosinophilic inflammation, had predictive value for the response. In summary, the model focuses attention on relevant parameters and is in line with data from earlier unsupervised multivariate models.

Flow cytometry analysis is the required technique to perform cell counts and in in this study also to measure granulocyte responsiveness. State-of-the-art bench top flow cytometers are able to perform a stimulation step on whole blood, such as adding fMLF. A blood tube has to be loaded into the cytometer and the pipetting step is performed automatically by the cytometer, after which it measures fluorescence intensity. This important advancement makes it possible to use complex flow cytometry for clinical diagnostic tests, such as testing granulocyte responsiveness in asthma patients.

In conclusion, the proposed prediction model identifies eosinophilic asthma with peripheral blood analysis, FeNO measurement and assessment of routine clinical data. Responsiveness of peripheral blood granulocytes was essential to come to a sensitive diagnostic test and adds to the ongoing scientific debate about the biological relevance of granulocyte responsiveness in asthma. The prediction model was prospectively tested in an independent patient population visiting a specialised asthma centre in Oxford (UK) and identified an important group of patients with potentially eosinophilic inflammation that rendered non-eosinophilic in sputum most likely due to OCS use. Finally, this study underlines the potential of unbiased approaches to support clinical decision making in complex diseases such as asthma.

Acknowledgements

Our gratitude goes to research nurse Simone Sluis who contributed greatly during the study and to the lung function department of the UMCU and the Central Military Hospital Utrecht for their technical support. Research nurses Clare Connolly and Catherine Borg of the Nuffield department of Medicine, contributed greatly to the inclusion of patients. We thank the respiratory trial unit of Oxford University for their technical expertise.

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Supplementary Material

Methods

Sample size

Preliminary results of an earlier study by our research group formed the basis for the sample size calculation [1]. In this study fluorescein isothiocyanate (FITC) labeled monoclonal phage antibody A17 was used, which binds to the active form of the FcγRII receptor. Eosinophils of asthma patients expressed higher levels of active FcγRII than healthy controls. Therefore, the capacity of eosinophils to bind A17 was chosen as a diagnostic test. The mean of the non-eosinophilic group for A17 binding was 3.41 AU (log transformed arbitrary units), the mean of the eosinophilic group was 3.95 AU. The standard deviation (SD) was 0.85. The power (β) was set at 0.8 and the α at 0.05. Taking into account a 1:3 eosinophilic/non-eosinophilic ratio in the asthma cohort, the comparison of the two independent means resulted in a required sample size of 104 patients in total; an expected 26 patients with eosinophilic asthma patients and 78 controls had to be included. In order to correct for possible drop-outs or missing data, 115 asthma patients were planned to be included in order to have sufficient power (Power and Sample size Program, version 3.0 jan 2009).

Inclusion and exclusion criteria

Specific criteria were set to include patients with adult asthma, defined as having the following clinical features: episodic shortness of breath, particularly at night, often accompanied by cough and wheezing heard by auscultation. Moreover, patients needed to have reversibility in FEV1 to 400 µg inhaled salbutamol (>12% predicted and >200 ml) or airway hyperresponsiveness to histamine (PC20 <8 mg/ml).

Exclusion criteria were smoking at present or in the last 12 months and/or a past history of more than 10 pack years. If the patients received antibiotic treatment for a respiratory tract infection <4 weeks prior to the study, he or she was also excluded. Finally, proven allergic bronchopulmonary aspergillosis was regarded as an exclusion criterion [2].

Utrecht Cohort (training set)

Ninety-two of 115 patients in the Utrecht were diagnosed with asthma according to the GINA guidelines which requires proven reversibility or a positive histamine or metacholine test in combination with classic asthma symptoms. The other 23 patients had asthma according to an experienced chest physician, which meant they suffered from symptoms, while reversibility was not proven by lung function tests or a histamine or metacholine challenge test within the University Medical Center itself. Often reversibility had been tested before in another medical center, before referral to this tertiary center and was never repeated.

Oxford Cohort (validation/test set)

All 34 recruited patients had asthma according to the GINA guidelines.

Sputum induction

Sputum was induced via inhalation of a hypertonic saline aerosol, generated by an ultrasonic nebulizer (Untraneb 2000; DeVilbiss, Somerset, PA, USA) by a standardized internationally approved protocol [3]. The nebulizer was calibrated to an output of 1.5 ml/min and production of particles with a diameter of 4.5 µm. Solutions of sodium chloride of 0.9, 3, and 5% were nebulized at room temperature (20-22°C) for 5 min within each step, and administered through a mouthpiece connected to a 100 cm-long tube with an internal diameter of 22 mm.

For mild-to-moderate asthmatics the exact description of the protocol was used. In high-risk asthmatics (*e.g.* severe asthma, highly reactive airways, exacerbation and using increasing doses of beta2-agonist) a lower dose of saline was used, always starting with 0.9% and in these patients the increasing saline dosages were sometimes stopped before reaching the inhalation step with 5% saline solution if a >20% fall in FEV1 was measured.

Sputum analysis

Sputum samples were kept at 4°C and processed within 2 hours of expectoration. Sputum plugs were selected for analysis to reduce squamous contamination. The sample was weighed to ensure accurate volumes of liquids were used. A two-step lysis process was followed: The sample was first broken down by repeated pipette aspiration in the presence of PBS, centrifuged and half the supernatant volume removed and stored at -80°C. A 0.2% dithiothreitol (DTT) solution was then added and the sample was left to incubate at 4°C for 15 minutes to ensure complete homogenization. The samples were then filtered through 48-mm nylon gauze (Thompson, Ontario, Canada). A total cell count was performed on a cell counter (Abbott Labs Cell Dyn 1800®), viability and level of squamous cell contamination were measured using a Burker-Turk haemocytometer and the Trypan blue exclusion method. Cells were classified as viable leukocytes, dead leukocytes and squamous epithelial cells. A cytospin slide was stained with either May-Grunwald Giemsa (Utrecht) or Rapi-Diff II (Oxford) for further cell differentiation. The filtered sample was then centrifuged for 5 min at 1500RPM at 4°C. The DTT supernatant was aspirated, transferred to Eppendorf tubes and stored at -80°C. Within the two centers, the same technician evaluated the cytospin slides. Both these technicians were highly experienced in processing clinical samples and also tested samples from external academic centers to cross-validate their own analyses.

Venipuncture

A 9 mL venous blood sample was collected in a sodium heparine tube and full blood was analysed on a cell counter (Abbott Labs Cell Dyn 1800®).

Statistical analyses

Non–linear principal component analysis (NLPCA) on Utrecht cohort

NLPCA was selected, because it can be applied to a combined set of categorical, nominal and continuous variables. Applying this technique, we were able to take into account the correlated variance from all 26 clinical and immunological parameters simultaneously.

The repeated stepwise approach described by Linting *et al*. was followed [3,4]. In short, before running PCA, all values in the dataset were transformed into positive values. Then all 26 parameters were labelled as a specific type of data: numerical, ordinal or nominal (**Table S1**). Missing values were regarded as 'passive values' within the analysis. This has the advantage that none of the patients had to be excluded and that no value had to be fabricated to replace a missing data point if one of the values within the 26 parameters was absent. NLPCA implementation was performed with IBM SPSS® Statistics 22 (SPSS, IL, USA) via the pre-programmed options 'dimension reduction methods', 'optimal scaling' and finally 'Categorical Principal Component Analysis'. We ran the NLPCA algorithm repeatedly to reduce the number of parameters and stopped it at an optimal cut-off point, at which the least number of parameters explained the most variance within the dataset. Normalization of the quantitative variables is integrated into the CATPCA procedure of Linting *et al.* as implemented in SPSS. For the programme code in SPSS: http://www.ibm.com/support/knowledgecenter/SSLVMB_22.0.0/com.ibm.spss.statistics.reference/spss/categories/syn_catpca.htm (website accessed at 16th of October, 2016).

Initially, 6 Principal Components (PCs) were chosen after examining the scree plots. After the first PCA on 26 parameters, the parameter 'smoking history' had the lowest VAF(Variance Accounted For) and was excluded from the set, after the second run the expression of L-selectin (CD62L) on eosinophils was left out and by repeating this step we ended with 15 parameters with a VAF higher than 0.250 in 6 PCs. After examining the scree plots again, we set the amount of PCs suitable for this dataset to 4. After this step we had to exclude eczema, gender and age of onset (VAF < 0.25) to end up with 12 parameters (**Figure 1** 'final model') and 4 PCs with 24% in PC1, 14% in PC2, 12% in PC3 and 9% in PC4, that totalled 59% of VAF within the model.

Additional clarification for excluded parameters by NLPCA

With regard to BMI, Desai and colleagues also reported that obesity in asthmatic patients is not associated with sputum eosinophilia [5]. However, overweight and obese patients do express higher levels of sputum IL-5 and higher submucosal numbers of eosinophils. These two parameters were not measured in our study, which may provide an explanation for the lack of predictive value of BMI within our cohort. Markers of bronchial hyperresponsiveness, histamine threshold and reversibility, did not have a significant discriminatory value either. This is not surprising since the patients in our study either had lowered histamine threshold and/or reversibility of >200 mL, which means there already was selection by applying the inclusion criteria. Smoking and the amount of pack years were also included in the exclusion criteria, only current non-smokers with <10 pack years were included and therefore it is a logical consequence that these parameters were excluded from the final model.

Discriminant analysis: from dimension reduction to a clinical prediction model for sputum eosinophilia

The NLPCA scores represent the maximized variability among the patients, only part of which will relate to distinction between disease phenotypes. In principle, the discriminant analysis could be performed on the original variables, *i.e.* the 'raw data'. However, these variables comprise categorical, nominal and continuous values that are likely to be mutually related and like 'ordinary' PCA, DA cannot extract such correlations as it can only handle continuous and dichotomous variables. The covariance matrix 'separate-groups' was chosen within the SPSS implementation and prior probabilities were corrected for group size.

Analysis of Utrecht 'test' cohort

DA of the 4 PCs shows a significant difference between the group of patients with sputum eosinophilia >3% (n =21) compared to non-eosinophilic patients (n=94). The results of the DA on clinical parameters only and on clinical + peripheral blood parameters combined are depicted in **Figures S3A** and **S3B**.

The test characteristics of the final model (clinical + peripheral blood) are depicted in **Table S2**. Overall the test predicts 91.3% of all patients correctly. The test sensitivity is 90.5% and specificity is 91.5%. For comparison, the test characteristics of solely the clinical parameters are depicted in **Table S3**, which show a lower overall accuracy of 87.0%, a sensitivity of 47.8% and a specificity of 95.7%.

The eigenvalue of the DA performed at the non-blood parameters was 0.567 and of the final model, including the blood parameters, it was 1.014. A larger eigenvalue indicates a stronger discriminative effect of the discriminant function. This change underlines the additional value of the blood parameters.

To test the stability of the DA-based classification in addition to the test of the stability of the NLPCA model, we tested each bootstrap sample against the Oxford cohort. Patients in the Oxford cohort were correctly classified in 0.71 of total (95%CI 0.69-0.74). The small 95% CI indicates a stable model.

Description and validation with the Oxford cohort

To investigate whether the NLPCA model based on the Utrecht cohort could predict if patients within the Oxford cohort suffered from airway eosinophilia, the Oxford cohort was projected on the PCA model that was built with Utrecht data. Discriminant analysis was performed on the combined dataset, with both Utrecht and Oxford data, to validate the prediction model. We did this by setting the weight for Oxford patients to a negligible minimum of 0.01 (using >Data >Weight>0.01) and by running NLPCA and DA as described earlier. The sensitivity and specificity that followed from this step are an indication of the predictive value of the *training set (Utrecht)* for the recognition of airway eosinophilia in the *test set (Oxford)*, **Table 3**. Cross-validation of the table shows an overall classification accuracy of 74%.

Missing values

The amount of missing values in the 12 variables included for NLPCA are given in **Table S4**.

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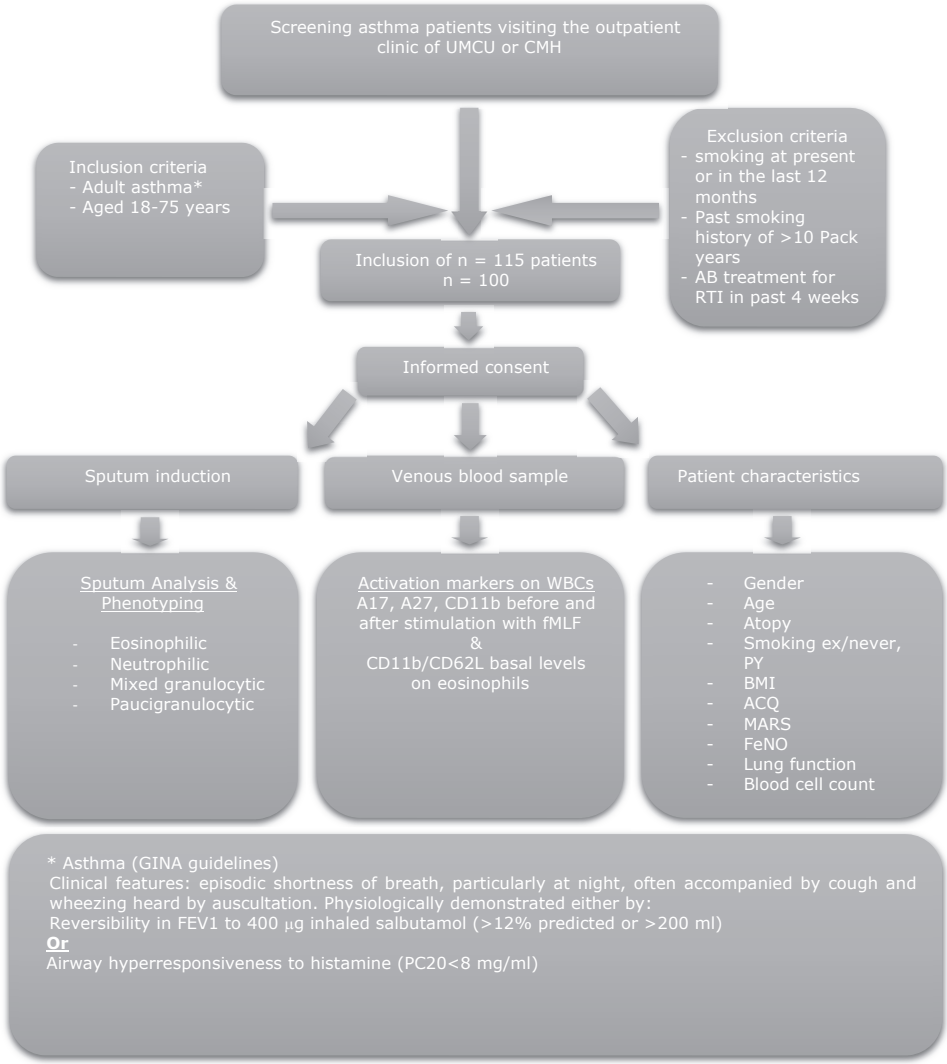

Figure S1: Flow chart with study design.

UMCU = University Medical Center Utrecht, CMH = Central Military Hospital, RTI = Respiratory Tract Infection, fMLF = N-Formylmethionine leucyl-phenylalanine, BMI = body mass index, MARS = medication adherence report scale, ACQ =Asthma Control Questionnaire, FeNO = Fraction of exhaled nitric oxide, FEV1 = Forced Expiratory Volume in 1 sec, ABPA = Allergic Broncho-Pulmonary Asgergillosis, AB = Anti-Biotic treatment.

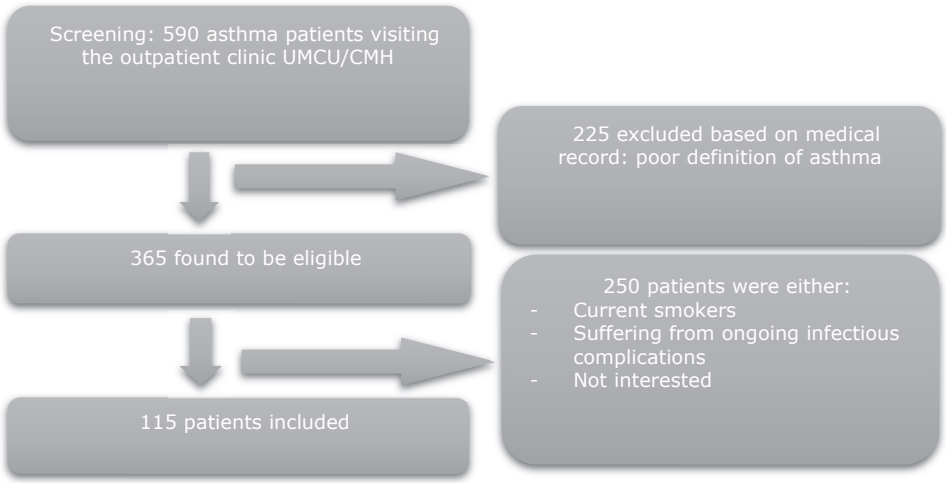

Figure S2: Inclusion process.

UMCU, University Medical Center Utrecht; CMH, Central Military Hospital.

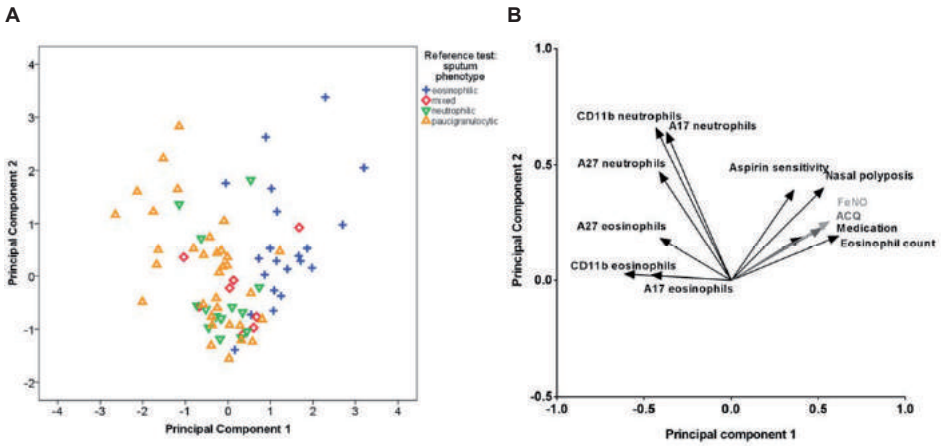

Figure S3A: Scoreplot of asthmatic subjects in the NLPCA model.

Patients with eosinophilic asthma according to sputum analysis (plus sign) and non-eosinophilic asthma phenotypes (other signs). Sputum phenotypes represent the result of sputum analysis as an overlay for the NLPCA-model to compare the results between the model with reduced dimensions and sputum analysis. The patients that were not classified by sputum analysis are not included in this figure.

Figure S3B: Loadingplot of the NLPCA model.

Higher marker variability results in a longer vector and the direction of the vector determines the place of a patient score in **Figure S3A** (one sign = one patient). Therefore, eosinophilic patients (plus sign, **Figure S3A**) are high in FeNO, ACQ, eosinophil count and low in eosinophil responsiveness, while their non-eosinophilic counterparts (triangles and squares) exhibit low values in these clinical markers, but higher values of granulocyte responsiveness and are mostly plotted at the opposite side of the graph. FeNO and ACQ have lighter gray vectors in order to facilitate distinction from the partly overlapping vector for Medication.

Table S1: Analysis levels per parameter

Parameter	Analysis level
Gender	Nominal
BMI	Numerical
Smoking ever	Nominal
Age of onset	Numerical
Aspirin sensitivity	Nominal
Eczema	Nominal
Nasal polyposis	Nominal
MARS	Nominal
Proven allergy	Nominal
History of allergy	Nominal
P20 Histamin level	Numerical
Eosinophil count	Numerical
FeNO	Numerical
ACQ	Numerical
Reversibility	Numerical
Absolute NeutrophilCount	Numerical
Medication	Ordinal
FEV1 %reversibility	Numerical
CD62L Eosinophils	Numerical
CD11b Eosinophil	Numerical
A17 eosinophils	Numerical
A17 neutrophils	Numerical
CD11b eosinophils	Numerical
CD11b neutrophils	Numerical
A27 eosinophils	Numerical
A27 neutrophils	Numerical

Table S2: contingency table with the diagnostic score of the prediction model, using sputum analysis as reference test

The number of patients correctly classified with eosinophilic disease is 19 out of 21 (90.5%). The number of patients with non-eosinophilic disease is correctly identified in 86 out of 94 (91.5%). On average, 91.3% of original grouped cases is correctly classified (leave-one-out cross validation accuracy). Eos: eosinophilic asthma, Non-eos: non-eosinophilic asthma.

Prediction model for sputum eosinophilia		Predicted group membership		Characteristics
		Eos.	Non-eos.	
Sputum Analysis	Eos.	19	2	Postive Predictive Value (PPV): 70.4%
	Non-eos.	8	86	Negative Predictive Value (NPV): 97.7%
Characteristics		90.5% Sensitivity	91.5% Specificity	Accuracy: 91.3%

Table S3: 2x2 contingency table with diagnostic score of the prediction model <u>without blood markers</u>, using sputum analysis as reference test

The number of patients correctly classified with eosinophilic disease is 10 out of 21 (47.6%). The number of patients with non-eosinophilic disease correctly identified is 90 out of 94 (95.7%). On average, 87% of original grouped cases is correctly classified(leave-one-out cross validation accuracy). Eos: eosinophilic asthma, Non-eos.: non-eosinophilic asthma.

Prediction model for sputum eosinophilia		Predicted group membership		Characteristics
		Eos.	Non-eos.	
Sputum Analysis	Eos.	10	11	Postive Predictive Value (PPV): 71.4%
	Non-eos.	4	90	Negative Predictive Value (NPV): 89.1%
Characteristics		47.6% Sensitivity	95.7% Specificity	Accuracy: 87.0%

Table S4: Missing values in the dataset of a total of 149 study participants

Parameter	Total of 149	Missing values
Eosinophil count	148	1
Aspirin sensitivity	149	0
Nasal polyposis	149	0
FeNO	145	4
ACQ	148	1
Medication	149	0
A17 eosinophils	141	8
A17 neutrophils	140	9
CD11b eosinophils	143	6
CD11b neutrophils	146	3
A27 eosinophils	143	6
A27 neutrophils	138	11

CHAPTER 2.2

Similar activation state of neutrophils in sputum of asthma patients irrespective of sputum eosinophilia

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Abstract

Background: *Inflammatory phenotypes of asthma are associated with differences in disease characteristics. It is unknown whether these inflammatory phenotypes are reflected by the activation status of neutrophils in blood and sputum.*

Methods: *We obtained peripheral blood and induced sputum from 21 asthma patients and stratified our samples based on sputum eosinophilia resulting in two groups (>3% eosinophils: n=13, <3%: n=8). Eosinophils and neutrophils from blood and sputum were analysed for expression of activation- and degranulation markers by flow cytometry. Data were analysed by both classical, non-parametric statistics and a multivariate approach, using principal component analysis (PCA).*

Results: *Patients with sputum eosinophilia were characterized by increased ACQ scores and blood eosinophil counts. Both sputum neutrophils and eosinophils displayed an activated and degranulated phenotype compared to cells obtained from blood. Specifically, degranulation of all granule types was detected in sputum cells, combined with an increased expression of the activation markers (activated) Mac-1 (CD11b), Programmed Death-Ligand 1 (CD274) and a decreased expression of CD62L. CD69 expression was only increased on sputum eosinophils. Surface marker expression of neutrophils was similar in the presence or absence of eosinophilia, both by univariate and by multivariate analysis.*

Conclusion: *Sputum neutrophils were highly activated and degranulated irrespective of sputum eosinophilia. Therefore, we conclude that differences in granulocyte activation in sputum and/or blood are not associated with clinical differences in the two groups of asthma patients. The finding of PD-L1 expression on sputum granulocytes suggests an immuno-modulatory role of these cells in the tissue.*

Background

An estimated 300 million individuals worldwide are affected by asthma [1]. Asthma is characterized by airway inflammation, bronchial hyperresponsiveness and reversible airway obstruction. Several different inflammatory phenotypes have been identified in asthma, which are accompanied by different clinical characteristics [2]. Sputum eosinophilia is associated with bronchial hyperresponsiveness, high FeNO and (specific) IgE levels. In addition, the presence of either neutrophils or eosinophils in sputum is associated with a decreased FEV1 [3,4]. These inflammatory phenotypes are identified by microscopically evaluating the percentages of neutrophils and eosinophils in sputum or by transcriptomic profiling of whole sputum samples [2,5,6]. In addition to microscopic evaluation, cells obtained by sputum induction can be analysed by flow cytometry [7-11]. Sputum granulocytes analysed by this technique have been shown to display an activated phenotype, with upregulation of markers for activation and degranulation on either neutrophils [11], eosinophils [12] or both [13]. However, currently there are no reports on in-depth analysis of activation markers comparing blood and sputum granulocytes using multi-dimensional analysis. In addition, no studies have been published that adequately compare sputum granulocyte expression profiles between patients with different asthma phenotypes.

One single study did compare the expression levels of two classical activation markers between patients with moderate and severe asthma, but did not find a correlation between expression and disease severity [13]. Transcriptomic profiling of whole blood and sputum samples showed upregulation of neutrophil defensins and proteases in the blood of neutrophilic asthma patients, and significant differences between sputum samples of patients with different asthma phenotypes [6,14]. It is unknown, however, whether these differences reflect differences in expression of the granulocytes themselves or differences in cell numbers.

Traditionally, granulocyte activation markers include adhesion receptors such as the integrin Mac-1 (CD11b/CD18) and L-selectin (CD62L). Other activation markers described to be upregulated on circulating or sputum granulocytes from asthma patients are the CD11b activation epitope CBRM1/5 [15] and the Intercellular Adhesion Molecule-1 (ICAM-1, CD54). The latter adhesion receptor was proposed as a therapeutic target for antigen-induced acute airway inflammation [16]. The activation marker CD69, which is well known as a T-cell activation marker [17], was only shown to be upregulated on eosinophils from broncheoalveolar lavage (BAL) when compared to blood cells [15].

Neutrophils and eosinophils possess different types of granules containing antimicrobial and pro-inflammatory proteins. For neutrophils, release of these proteins to the outside of the cell (degranulation) occurs sequentially in response to increasing strength of activation signals, with secretory vesicles degranulating by the most mild stimulus, followed by tertiary, specific and azurophilic granules [18-20]. The marker for neutrophil tertiary- and eosinophil secretory granules (CD11b) was shown to be upregulated on cells from both BAL and sputum, compared to blood granulocytes [15]. Markers for specific and azurophilic/crystalloid granules were shown to be upregulated on BAL and on sputum granulocytes in several diseases, but

so far not on sputum cells from asthma patients [15,21,22]. Degranulation of neutrophil secretory vesicles was shown to occur already in the blood of asthma patients [23,24].

Another activation marker of interest, Programmed Death-Ligand 1 (PD-L1, CD274), has been implicated in a murine model of airway hyperresponsiveness [25]. The immunomodulatory protein CD274 is not as well characterized in granulocytes as in lymphocytes, but was shown to be responsible for suppression of T-cell responses by interferon-γ stimulated neutrophils [26] and is highly expressed in granulomas in the lungs of sarcoidosis patients, although its expression on granulocytes was not tested by flow cytometry [27].

Up to now, all immunophenotyping of sputum cells has been performed by analysis of single-dimensional marker expression, which ignores the interaction between multiple markers. To overcome this issue, marker expressions and interactions can be studied using principal component analysis (PCA). PCA is a multivariate analysis method that detects systematic variability within multiple parameters and explores correlations between these parameters [28]. It transforms datasets with a large number of measured parameters into a smaller number of parameters, called principal components. As the resulting smaller number of components is more easily interpreted, it is a preferred technique for analysis of large datasets and forms the basis of cluster approaches used to identify asthma phenotypes [29,30].

In this study we investigate differences in granulocyte activation and degranulation by flow cytometric evaluation of neutrophils and eosinophils isolated from blood and sputum of asthma patients with and without sputum eosinophilia, both by univariate and multivariate analysis.

Methods

Study population and ethics

This study was approved by the local medical ethics committee. Patients were recruited at the pulmonary outpatient clinic of the University Medical Centre Utrecht (**Table 1** patient baseline characteristics) and gave written informed consent in accordance to the Declaration of Helsinki (seventh revision, Fortaleza, 2013).

Patients were included according to the following criteria: age 18-75 years, having adult asthma defined by the GINA guidelines on clinical features (episodic shortness of breath, particularly at night and often accompanied by cough and wheezing) and reversibility of FEV1 upon inhalation of 400μg salbutamol (≥12% predicted or ≥200ml) and/or airway hyperresponsiveness to histamine (PC20 <8mg/ml) [1]. Patient numbers were calculated to be able to distinguish medium effect sizes with a power of 0.8 and an α of 0.05 using G*Power 3.1.3 [31].

Exclusion criteria for the study were smoking in the last twelve months, a smoking history ≥10 pack years, treatment with antibiotics <4 weeks ago or confirmed allergic bronchopulmonary aspergillosis.

Blood and sputum samples were stratified on presence or absence of sputum eosinophilia based on a cut-off value of three percent sputum eosinophils, as determined by differential count (described below). All samples were collected between 9AM and 1PM to minimize effects of diurnal variation such as found in FeNO and haematological parameters [32,33].

Lung function and clinical parameters

After inclusion of patients, the Asthma Control Questionnaire (ACQ) and Medication Adherence Report Scale (MARS) were filled out. FEV1 measurements were performed by peak flow measurement using the PiKo-1 (nSpire Health, Longmont, Co, USA) and FeNO was determined using NIOX MINO® (Aerocrine, Solna, Sweden) with an expiration time of 10 seconds. Absolute blood eosinophil counts were calculated from percentages obtained by FACS-analysis (see below) and WBC counts obtained froma Cell-Dyn haematology analyser (Abbot Diagnostics, North Chicago, Il, USA).

Blood processing

Erythrocytes were lysed from sodium heparin blood using lysis buffer consisting of 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM NA_2EDTA dissolved in ddH_2O. Resulting total leukocyte preparations were washed and resuspended in staining buffer consisting of PBS supplemented with trisodium citrate (0.32% w/v) (both prepared by the UMCU pharmacy) and human pasteurized plasma solution (10% w/v, Sanquin, Amsterdam, the Netherlands).

Sputum induction and processing

Sputum was induced by inhalation of 0.9-5% saline aerosols and processed as published previously using Sputolysin prepared from a 10x stock (Merck Millipore, Darmstadt, Germany) and supplemented with NaCl to reach an osmolarity of 280-290mOsm [34,35]. Cytospin slides of sputum cells were stained with May-Grünwald-Giemsa for differential cell count. Samples containing >80% squamous epithelial cells were excluded from analysis. Percentages of cells in sputum were calculated after exclusion of squamous epithelial cells [36]. Remaining cells were resuspended in staining buffer for FACS staining procedure.

Flow cytometry

Samples were stained with antibodies for 30 minutes on ice at a maximum concentration of $5x10^6$ cells/ml and washed twice before analysis on a Gallios flow cytometer (Beckman Coulter, Pasadena, CA, USA).

Granulocytes in blood were identified based on FSC/SSC (**Figure 1A**) and CD16 was used to differentiate into neutrophils ($CD16^{bright}$) and eosinophils ($CD16^{dim}$). For sputum samples a different sorting strategy was chosen due to the presence of epithelial cells and alveolar macrophages with a high SSC. Epithelial cells do not express CD11b and alveolar macrophages are highly autofluorescent at emission wavelengths of around 450nm when excited by a 405nm laser. Therefore, after exclusion of debris on FSC/SSC, granulocytes

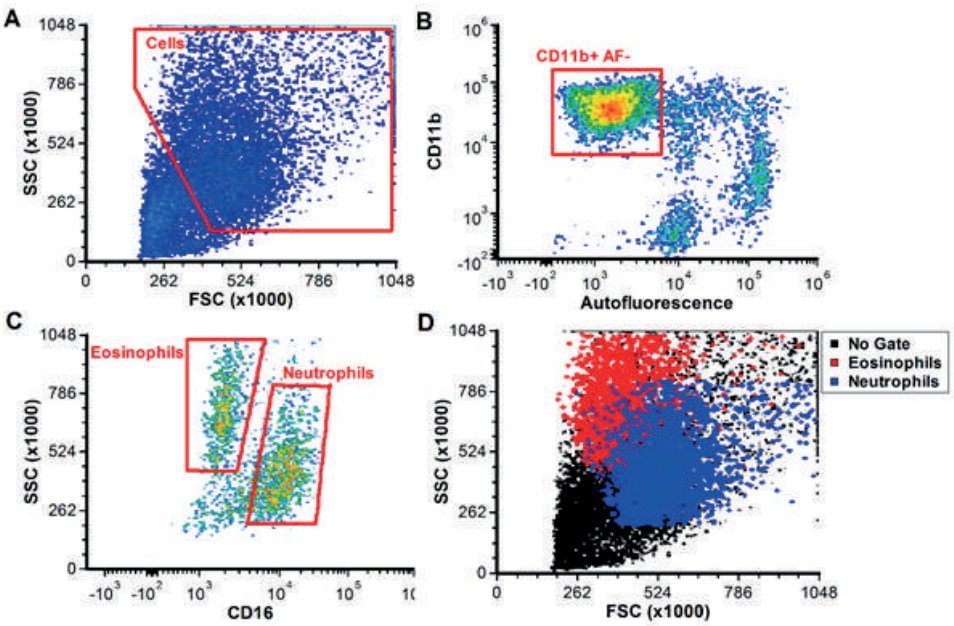

Figure 1: Gating strategy to identify sputum granulocytes.

A. A FSC/SSC gate is used to exclude most debris and lymphocytes. **B.** Subsequently, cells positive for CD11b and negative for ~450nm autofluorescence were gated to exclude residual lymphocytes and alveolar macrophages (which are highly autofluorescent). **C.** The resulting granulocytes were subdivided in neutrophils and eosinophils based on SSC and expression of CD16. D. The resulting neutrophil and eosinophil population show FSC/SSC patterns similar to those in blood, with a higher SSC for eosinophils and higher FSC for neutrophils.

were identified as CD11b^{bright} and 405/450nm-autofluorescence^{dim}. Subsequently, they were differentiated into neutrophils and eosinophils based on CD16 expression and SSC. Analysis was performed on at least 250 neutrophils/eosinophils. Degranulation of neutrophils was measured using CR1 (CD35), integrin αM (CD11b), CEACAM-8 (CD66b) and LAMP-3 (CD63) antibodies for secretory, tertiary, specific, and azurophilic, respectively [19]. For eosinophils, LAMP-3 was used as a marker for crystalloid (specific/secondary) granule degranulation [37] and CD11b as a marker for secretory (sombrero) vesicles [20]. Since CD63 is also expressed on activated platelets, its expression was only determined on cells negative for platelet marker CD41 [38].

Antibodies used were CD11b-APC-Alexa750 (clone Bear1), CD16-Krome Orange (3G8), CD274-PeCy7 (PDL1.3.1) and CD62L-ECD (DREG56) from Beckman Coulter (Pasadena, CA, USA), CD35-FITC (E11) from Biolegend (San Diego, CA, USA. CD63-PE (H5C6), CD69-PeCy7 (FN50), IgG1 isotype control-PE (X40), IgG2a isotype control-FITC (X39) and the Annexin-V PE apoptosis kit I from BD (San Jose, CA, USA), active CD11b-Alexa700 (CBRM1/5) from

eBiosciences (San Diego, CA, USA), CD54-PE (MEM-111) from EXBIO Praha (Vestec, Czech Republic) and CD41-FITC (VIPL3) from Life Technologies (Carlsbad, CA, USA).

Data analysis and representation

FCS express 4.0 (De Novo Software, Los Angeles, CA, USA) was used for evaluation of flow cytometric data and determination of median fluorescence intensities. Data were plotted as boxes representing median +/-IQR with error bars plotted according to Tukey's method in Prism 6.04 (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed in SPSS Statistics 22 (IBM, Armonk, NY, USA). Samples were compared using multiple Wilcoxon-Mann-Whitney tests or Related-samples Wilcoxon signed-rank tests where applicable. Correction for multiple testing was performed using the Bonferroni Method. Correlations between parameters were determined using Spearman's Correlation Coefficient.

Principal Component Analysis (PCA), was performed as described extensively elsewhere [28]. In short, PCA was performed on the MFIs of all measured parameters (except isotype controls) using SPSS Statistics 22. MFIs were classified as numerical data, missing values were mode imputed and discretization was used. Scree plots (not shown) were used to determine the required amount of components. Data points with object scores of >3.5 and <-3.5 were considered as outliers and excluded from the analysis. Graphs of object scores and loading plots were made in Prism 6.04.

Results

Patient characteristics

Sputum and blood samples were obtained from 21 patients (**Table 1** patient baseline characteristics). Thirteen patients (68%) had more than 3% eosinophils in their sputum samples. This sputum eosinophilia was accompanied by higher eosinophil counts in peripheral blood (p=0.007), less well controlled asthma (p=0.035) and trends toward higher FeNO, more medication use and lower absolute FEV1 (p=0.09, 0.09 and 0.053, respectively).

Viability of sputum granulocytes

After processing of a sputum sample, the viability of the cells in the sample was determined by Annexin-V and 7-AAD staining [39]. Sputum neutrophils and eosinophils were typically 90-95% alive (**Figure 2**) and 5-10% necrotic or late apoptotic. Less than 1% of sputum cells was Annexin-V single positive and, thus, early apoptotic.

Expression of activation markers in blood and sputum

Both neutrophils and eosinophils showed an activated phenotype in sputum compared to blood (**Figure 3** and Supplementary **Figure S1**). CD62L was shed from sputum neutrophils and eosinophils. An increased CD69 expression was found only on sputum eosinophils,

Table 1: Patient baseline characteristics at time of sputum induction grouped for sputum eosinophilia

	>3% sputum eo	<3% sputum eo	p-value *
Number	13	8	NA
Gender M/F	9/4	4/4	0.88
Age (years)	45 (19-66)	47 (25-54)	0.45
BMI (kg/m^2)	27 (22-35)	27 (22-32)	0.885
FeNO (ppb)	32 (16-215)	23 (12-42)	0.09
ACQ score	2.3 (0.0-4.4)	1.3 (0.14-2.0)	0.035
Blood Eosinophil Count (10^9/mL)	0.68 (0.1-1.2)	0.18 (0.0-0.5)	0.007
Medication †	4 (3-5)	3.5 (0-4)	0.09
FEV1 (L)	2.4 (1.5-3.9)	3.5 (2.3-4.0)	0.053
FEV1 (% predicted)	76 (61-105)	89 (61-113)	0.16
Reversibility FEV1 ‡	0.47 (0.0-16.6)	0.43 (0.0-4.9)	0.46
Sputum eosinophils (%) §	29 (3-82)	0 (0-1)	NA
Sputum neutrophils (%) §	39 (10-77)	49 (19-83)	0.41

Values are medians +/- range unless indicated otherwise.
* Based on Wilcoxon-Mann-Whitney test or Fisher's exact test where appropriate.
† 5-point ordinal scale based on guidelines of the British Thoracic Society, with 0) no medication, 1) inhaled SABA when required 2) low dose ICS + SABA 3) low/medium dose ICS + LABA, or medium dose ICS + LABA 4) High dose ICS +/- LABA and leukotrien receptor antagonist test and 5) High dose ICS + OCS +/- LABA.
‡ Percentage reversibility in FEV1 (corrected for age, gender, length and bodyweight) after inhalation of 2x 100µg salbutamol.
§ As determined by microscopic evaluation.
Abbreviations: BMI: body mass index, FeNO: forced exhaled nitric oxide, ACQ: Asthma Control Questionnaire, FEV1: Forced Expiratory Volume in 1 sec, NA: Not applicable.

whereas CD11b, CBRM1/5 and CD274 were upregulated on both cell types. Expression of CD54 was not detected, irrespective of cell or sampling location. Increased expression of markers for tertiary (CD11b), specific (CD66b) and azurophilic/crystalloid (CD63) granules indicated a highly degranulated state for sputum granulocytes.

In contrast to the marked differences in expression patterns between blood and sputum granulocytes, no significant differences were detected when comparing expression levels of markers on blood and sputum cells isolated from individuals with high and low eosinophil counts in sputum (**Figure 4**). In addition, no significant correlations were found between ACQ score, FEV1 (percentage predicted and/or absolute value) and expression of any of the granulocyte markers (data not shown). Due to the absence or small numbers of eosinophils

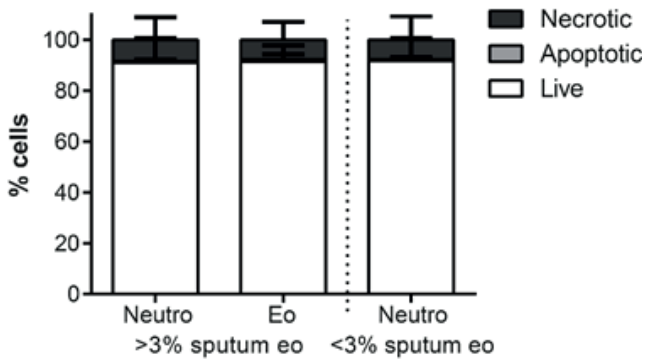

Figure 2: Viability of sputum granulocytes.

Sputum cells are typically >90% viable, with <1% early apoptotic Annexin-V single positive cells and the remainder being necrotic or late apoptotic. Bars depict means with SD patients with (n=13) and without (n=8) sputum eosinophilia.

in patients with <3% sputum eosinophils surface marker expressions of these cells could not be sufficiently compared between patient groups.

Principal component analysis (PCA)

PCA was performed on the neutrophil data and eosinophil data for the patients with adequate numbers of sputum eosinophils (**Figure 5**). Scree plots (not shown) indicated that all PCAs comparing blood and sputum samples required 2 components and the PCA comparing the two asthma phenotypes required 4 components.

PC1 accurately separates samples from blood and sputum for both neutrophils (**Figure 5A**) and eosinophils (**Figure 5B**), demonstrating that receptor expression profiles differ between sampling locations. The first principal component accounted for a total of 55% and 52% of the variation in receptor expression for neutrophils and eosinophils, respectively. PC2 described a further 16% and 23% of the variation.

In contrast, none of the four principal components distinguished between patients with or without eosinophils in their sputum after PCA on the combined data of blood eosinophils, blood neutrophils and sputum neutrophils (**Figure 5C**). Separate PCA of surface marker data for blood eosinophils, blood neutrophils and sputum neutrophils did not distinguish between patient groups either (Supplementary **Figure S2**).

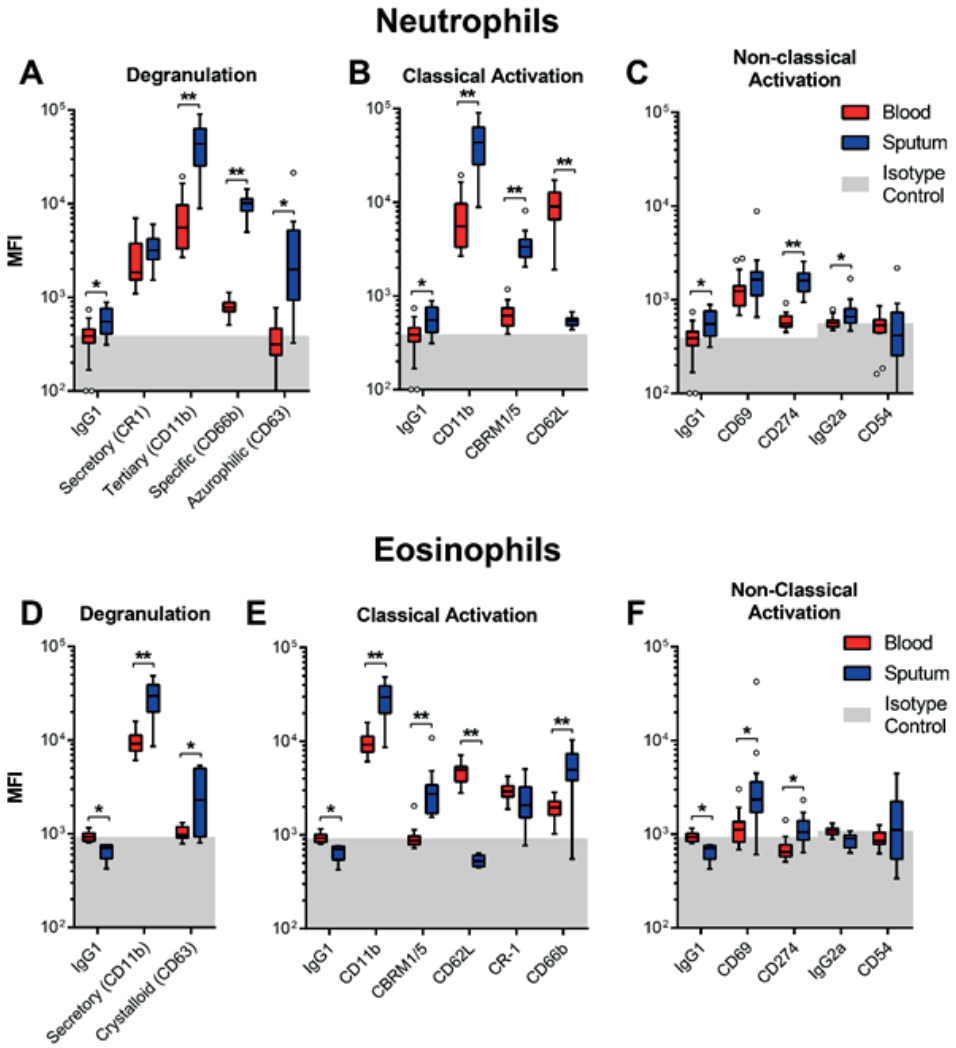

Figure 3: Expression profiles of blood granulocytes compared to sputum.

Expression of activation markers on blood and sputum neutrophils (A-C, N=21) and eosinophils (E-F, N=13). Since CD11b is both a classical activation marker and a marker for degranulation of tertiary granules, it is displayed twice. Boxes represent medians +/- IQR with whiskers of 1.5 IQR as according to Tukey's method. Light grey fills represent data points below the isotype control median measured on blood granulocytes.

* indicates $p<0.05$, ** $p<0.001$ significant differences between blood and sputum as determined by multiple Wilcoxon signed-rank test corrected for multiplicity by Bonferroni correction.

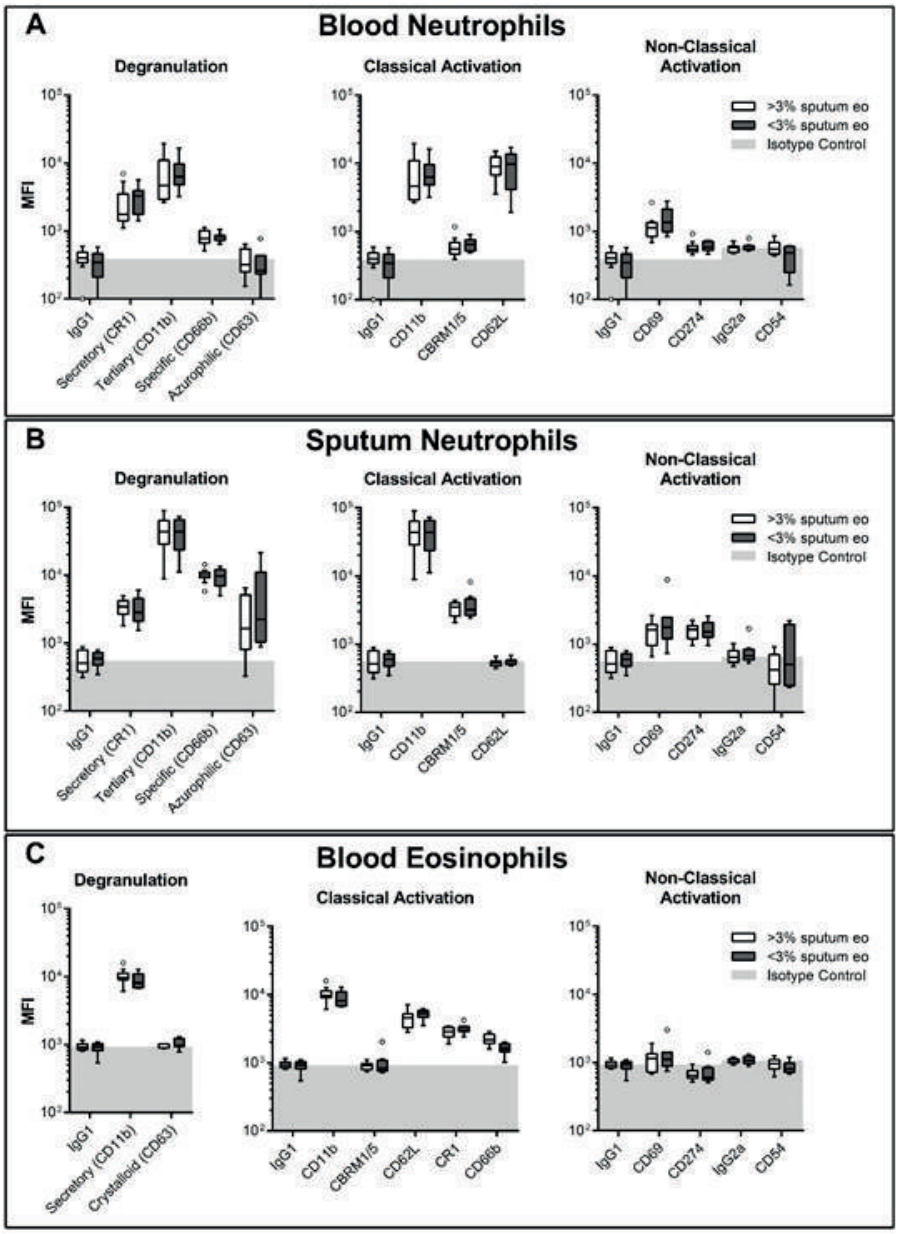

Figure 4: Expression profiles of sputum granulocytes in patients with presence of absence of sputum eosinophilia.

Expression profiles of **(A) blood neutrophils**, **(B) blood eosinophils** and **(C) sputum neutrophils** are similar in patients with presence or absence of sputum eosinophilia. Boxes represent medians +/- IQR with whiskers of 1.5 IQR, as according to Tukey's method. Light grey fills indicate values below the median isotype control. No statistically significant differences were found as determined by multiple Wilcoxon-Mann-Whitney tests with Bonferroni correction ($p<0.05$ after multiplicity correction).

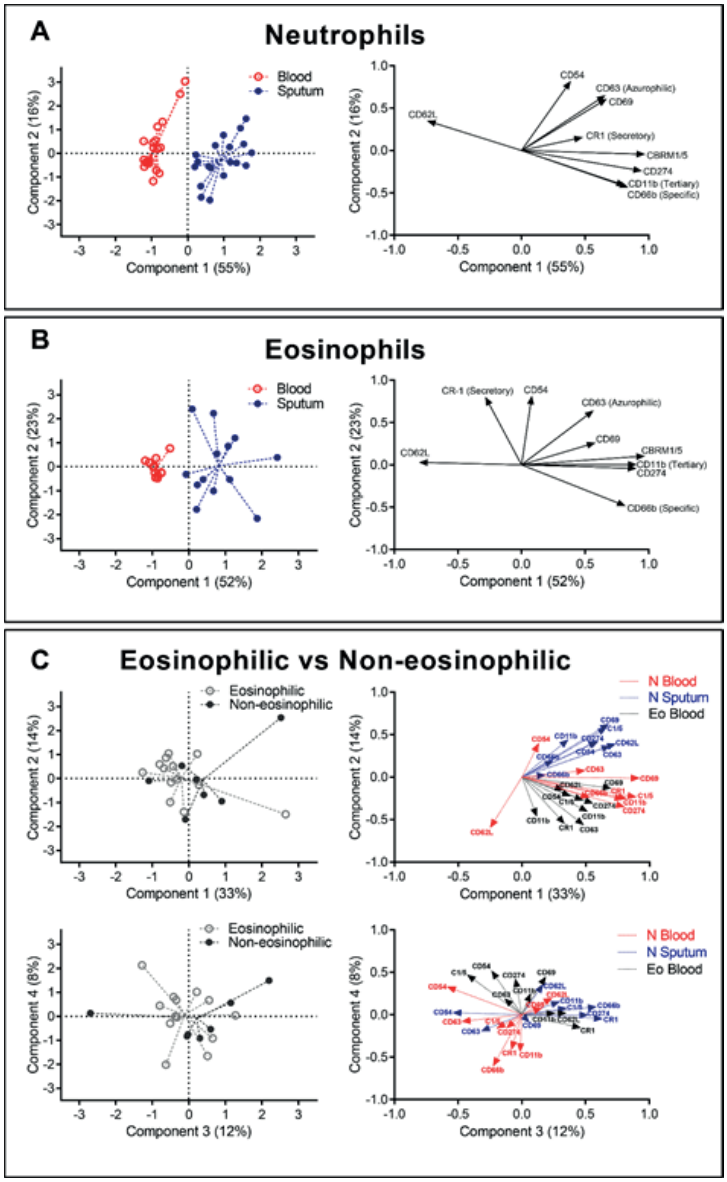

Figure 5: Multi-dimensional analysis of flow cytometric data.

Object scores of each patient (left) and loading plots of each marker (right) after PCA of receptor expression levels on blood and sputum neutrophils **(A)** and eosinophils **(B)**. Dashed lines originate from the mean object scores of a group. In **A and B** PC1 shows a clear separation between blood and sputum cell types with some residual variation explained by PC2. Loading plots indicate the contribution of each marker to a PC, with a large distance from 0 indicating a large influence. Markers which are not (differently) expressed remain close to zero on the x-axis, and therefore play little role in PC 1. Markers upregulated in sputum have positive values for PC1 (*e.g.* CD11b), whereas downregulated markers (CD62L) have negative values. PCA did not discriminate between patient groups (C) in any of the four components. Group sizes were 21 **(A)**, 13 **(B)** and 21 **(C)**.

Discussion

In our study, asthma patients (n=21) were characterized by presence or absence of sputum eosinophilia (>3% eosinophils: n=13, <3%: n=8). The two patient groups showed clear differences in clinical parameters. Patients with sputum eosinophilia had (1) more uncontrolled asthma, (2) blood eosinophilia, and (3) trends towards higher FeNO, increased medication use and lower FEV1.

However, there were no differences in the expression of activation and degranulation markers between the two patient groups. No correlations were found between surface expression markers and markers of disease severity such as ACQ or FEV1. *Post hoc* analysis of the correlations for each marker revealed a median effect size r of 0.146 (range 0.032-0.345), indicating that any effects missed due to the small sample size would have been small. Therefore, we conclude that activation or degranulation of granulocytes in both blood and sputum are not associated with clinical differences in eosinophilic and non-eosinophilic asthma, defined by sputum analysis. The absence of differences in marker expressions on sputum neutrophils is in line with the finding of an activated phenotype of neutrophils in the BAL of both healthy volunteers and COPD patients [40] and supports the hypothesis that the process of recruitment to the airways leads to granulocyte activation. Our results do confirm that sputum granulocytes have increased expression of the classical activation marker CD11b and decreased CD62L expression [8,10-13]. Both neutrophils and eosinophils display a highly degranulated phenotype, with upregulation of markers for tertiary, specific and azurophilic granules on neutrophils, and crystalloid and secretory granules on eosinophils. Interestingly, the expression of secretory granule marker CR1 (CD35) was already high on blood cells and did not increase further on sputum cells. This can be explained by the fact that secretory vesicles are the first to fuse with the membrane and that in the peripheral blood of asthma patients this process has already taken place [23,24]. Alternatively, CR1 may have been shed from the cell surface, as described in other diseases [41].

CD54 has been described as an eosinophil activation marker [42,43], which is expressed on sputum eosinophils [12]. However, in line with another study [8], we did not detect CD54 expression on sputum granulocytes, whilst using the same antibody clone (MEM-111). The reason for this discrepancy remains to be elucidated. We show CD69 to be an activation marker expressed on sputum eosinophils, just as found on BAL eosinophils [15].

Another important finding is the high upregulation of immune-regulatory protein CD274 on sputum neutrophils, and to a lesser extent on eosinophils (see **Figure 3**). Immune suppressive blood neutrophils have been shown to upregulate mRNA for this marker during acute inflammation and employ it for suppression of T-cell proliferation [26]. In the lungs, these cells cannot be as easily identified, as cells have shed CD62L after leaving the bloodstream, but the expression of CD274 does support the view that sputum neutrophils might have a suppressive phenotype. Interestingly, high expression has also been found in sarcoid lung granulomas, even though the expression of CD274 on neutrophils was not studied specifically. In addition, blockade of PD-1 (the receptor for CD274) pathway restores

T-cell functioning *in vitro*[27]. In conclusion, the expression of CD274 in the lungs of asthmatic patients favours the hypothesis that sputum cells can modulate inflammation in asthma rather than merely causing tissue damage and perpetuation of the inflammatory response.

In conclusion, granulocytes in sputum display a highly activated and degranulated phenotype compared to granulocytes in peripheral blood. However, sputum granulocytes receptor profiles do not differ in presence or absence of sputum eosinophilia in patients with asthma. Furthermore, we found the immune-inhibitory protein CD274 to be specifically expressed on sputum cells, supporting the hypothesis that sputum granulocytes can have an immune-modulatory instead of a detrimental role in asthma.

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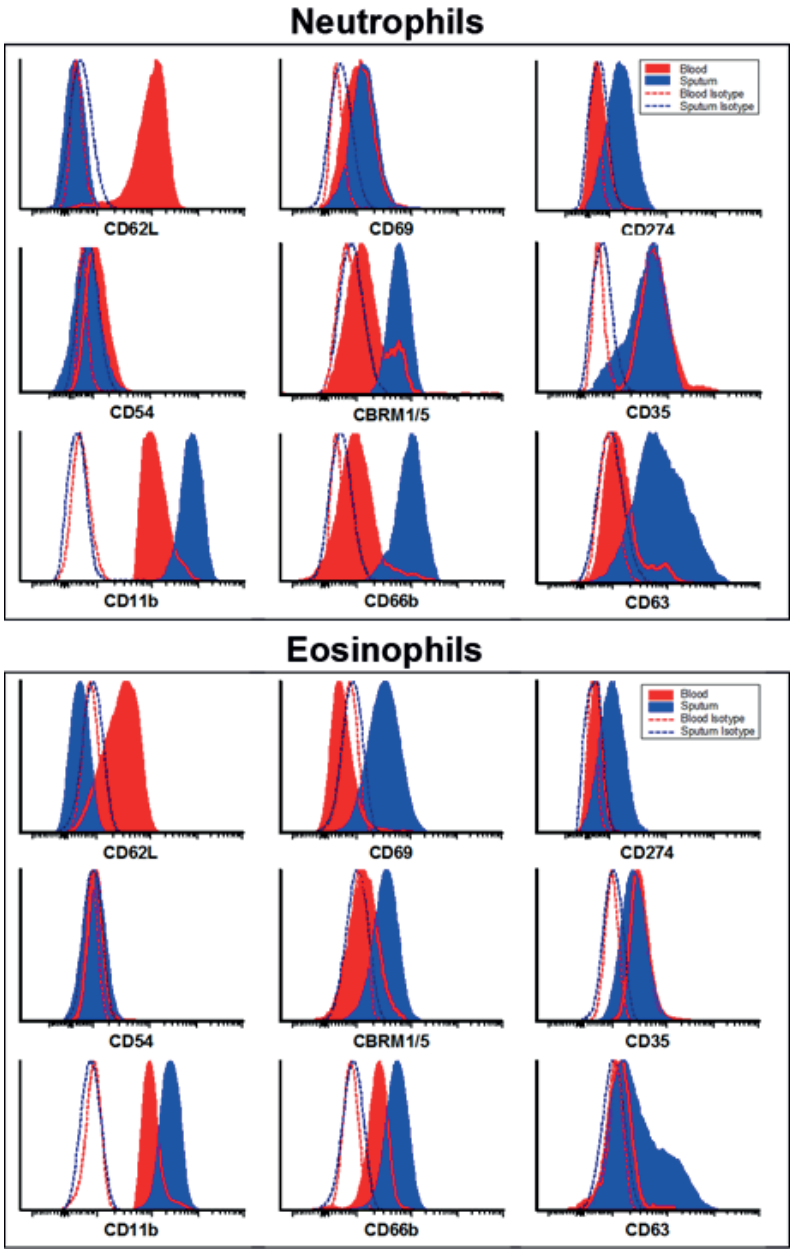
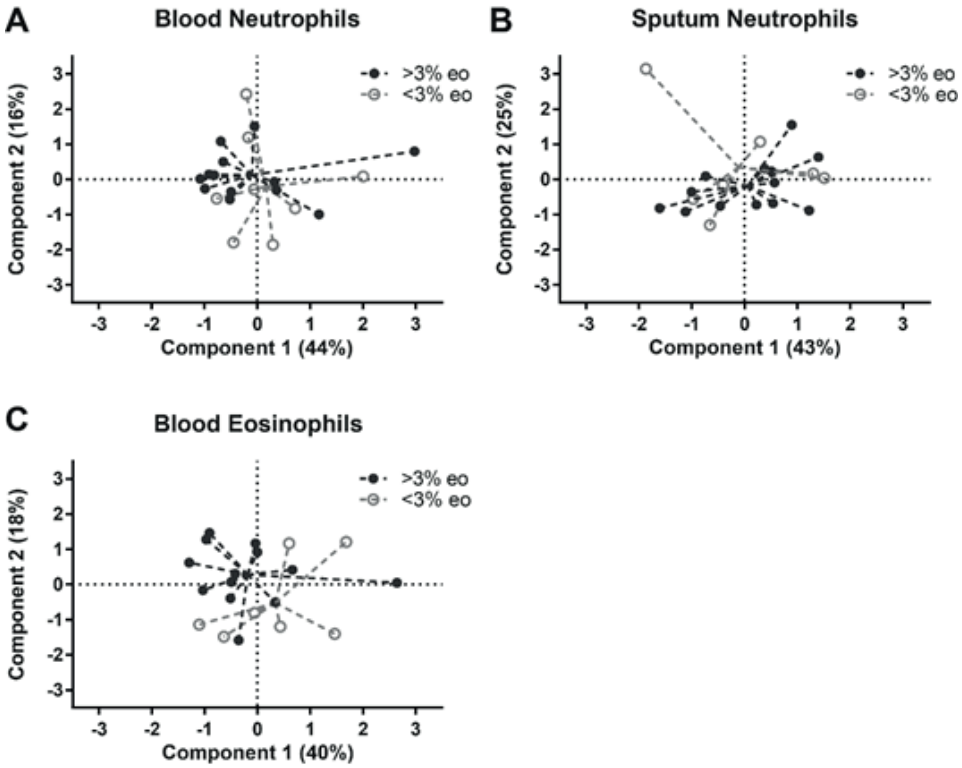
Supplementary Figures



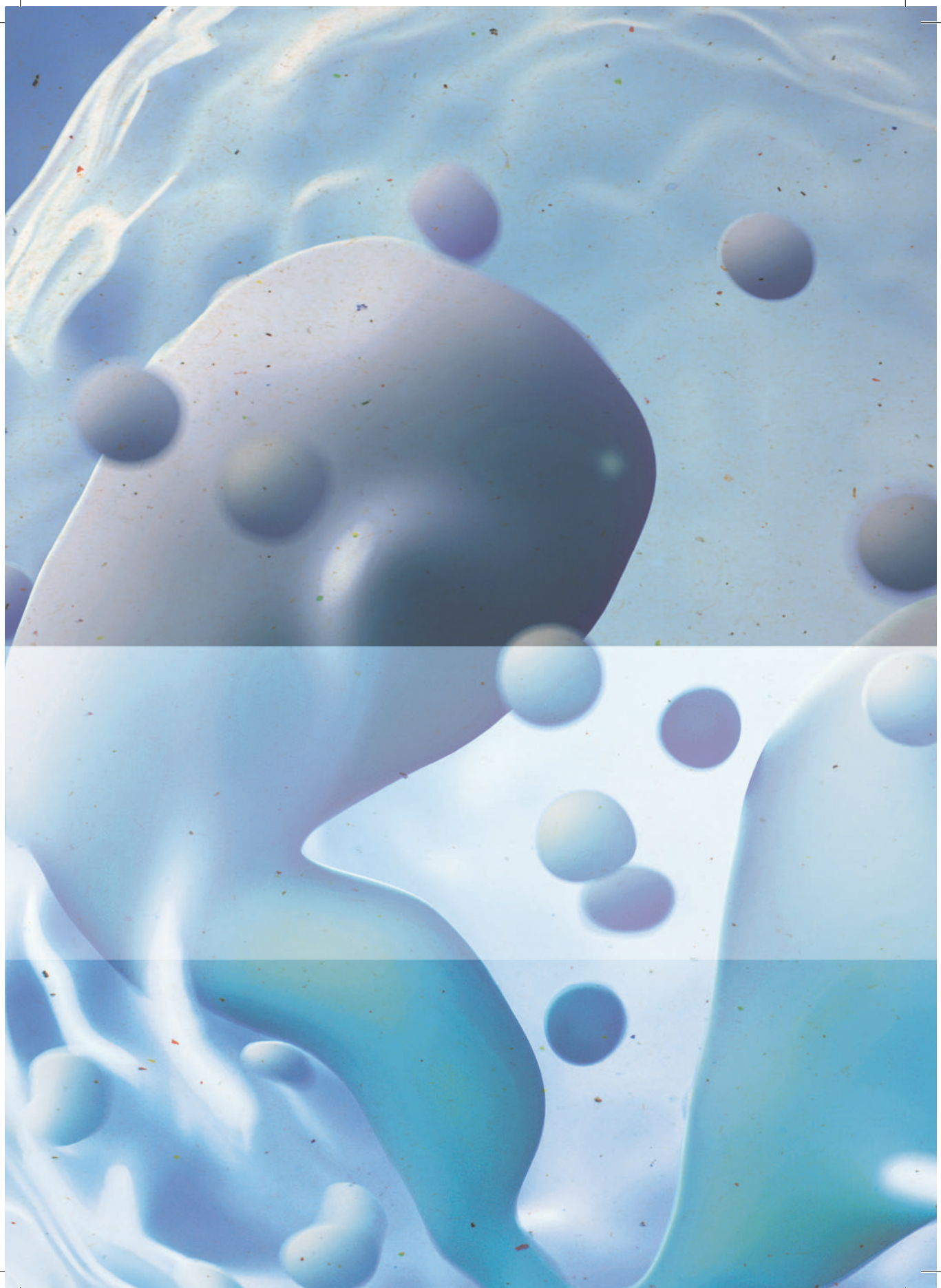
Supplementary Figure S1: Representative histograms of all measured markers in blood (red) and sputum (blue).

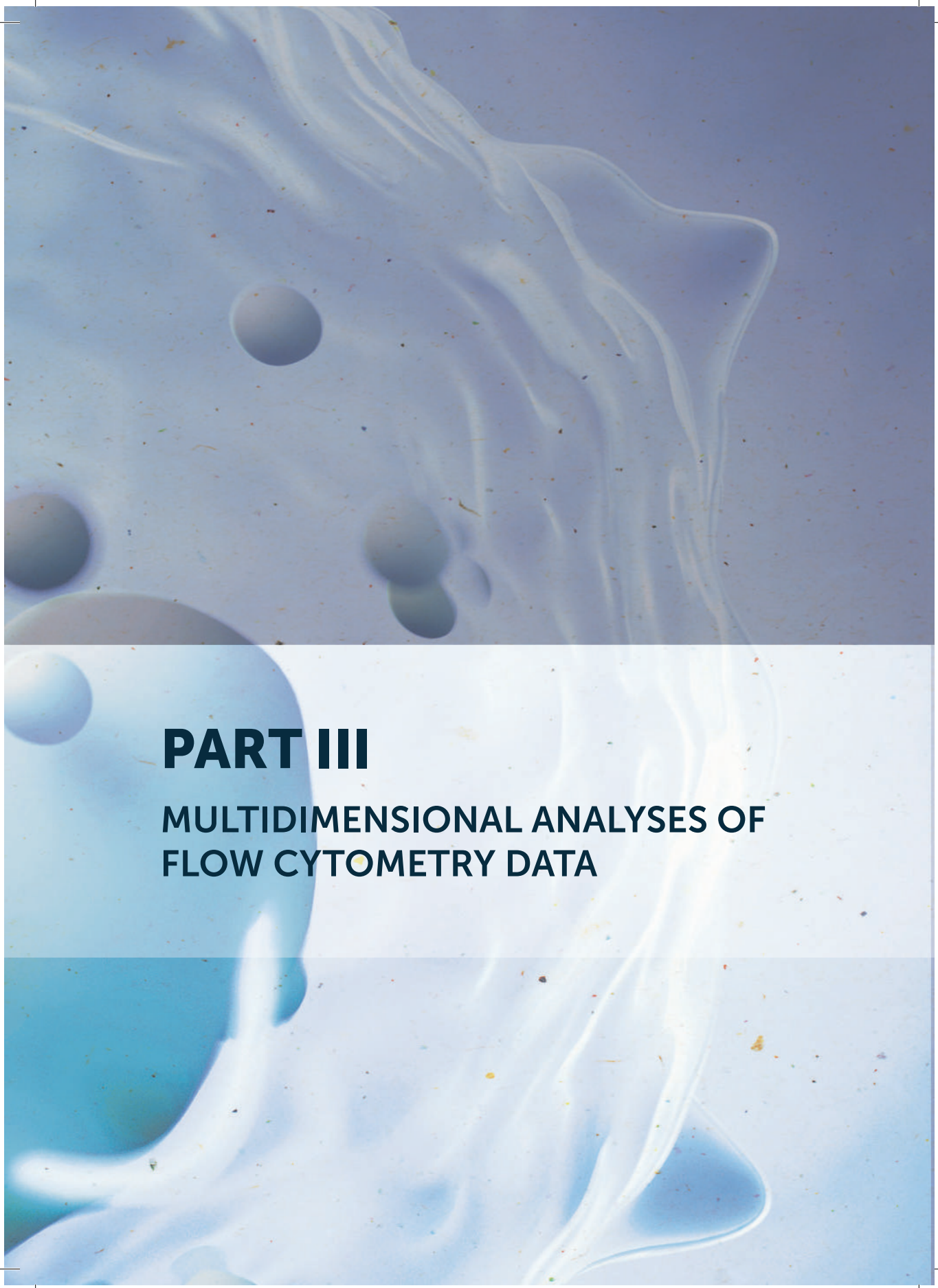
Solid lines (and filled areas) represent the relevant marker/antibody, dashed lines indicate isotype controls. Histograms are binned and normalized for peak values. The horizontal axes display the labels' intensities on a logarithmic scale, and the vertical axes the normalized number of cells.



Supplementary Figure S2: Object scores on PCA of marker expression for cell type and source.

PCA was performed on **(A)** blood neutrophils, **(B)** sputum neutrophils and **(C)** sputum eosinophils separately. None of these analyses was able to discriminate between patient groups. Dashed lines originate from the median object score of the two patient groups. N=13 for >3% sputum eosinophils and N=8 for <3% sputum eosinophils.





PART III

MULTIDIMENSIONAL ANALYSES OF FLOW CYTOMETRY DATA

CHAPTER 3.1

FLOOD: FLow cytometric Orthogonal Orientation for Diagnosis

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Abstract

Multi-color Flow Cytometry (MFC) is widely used for single-cell analysis and employs a vastly increasing number of markers. It can be used for disease diagnosis, research of disease mechanisms and the identification and isolation of individual cells based on their surface marker profile. However, data analysis methods exploiting all these advantages are lacking. Our novel FLow cytometric Orthogonal Orientation for Diagnosis (FLOOD) method reveals disease specific marker patterns. The method constructs a benchmark from surface marker abundances that is used to highlight deviations of challenged from unchallenged individuals. We demonstrate its power in an in vivo study of the response of healthy humans to lipopolysaccharide (LPS) challenge. FLOOD reveals a reproducible pattern of challenge specific markers on blood neutrophils. The method both provides new mechanistic insights and confirms established knowledge on LPS-response, which demonstrates the high potential of FLOOD for both clinical and research application.

Introduction

Multi-color Flow Cytometry (MFC) has become indispensable for characterization of individual cells in immunological or disease-mechanistic studies [1]. Its use increasingly extends to disease diagnosis [2,3], where it has been crucial for decades in phenotyping monoclonal diseases such as myeloid leukemia and gradually became promising for phenotyping heterogeneous diseases. The 'single-cell heterogeneity' [4,5] concept was proposed to classify tumors based on the differences between individual cells. This concept is considered a novel frontier in unbiased diagnostic approaches to better characterize human diseases. The potential to analyse an increasing diversity of fluorescently labeled antibodies that bind to specific binding sites on single cells [6,7], so-called 'deep-profiling' [8], will bring MFC up to par with other broad-spectrum 'omics' technologies [9-11]. MFC thereby calls for multivariate, unbiased analysis of correlations of cell-marker abundances.

MFC data reflect the abundance of multiple selected markers attached to binding sites on each cell; the fluorescence intensity and diversity of the markers used to detect the selected binding sites reflect the cellular functional characteristics. The data from one sample thereby comprises a large number of such single-cell marker readouts: a high-dimensional, multi-cellular 'marker profile'. The multivariate data analysis of these profiles is of strongly emerging interest and importance [12-14], specifically in personalized diagnosis and monitoring as well as in research of disease mechanisms. Multivariate methods may facilitate visualization of high-dimensional data, and at the same time reveal relationships between the measured characteristics that otherwise remain hidden.

Conventionally, MFC data is interpreted through 'bivariate' scatter plots that compare fluorescence intensities of two cell-bound markers for each cell within a sample. Cells can then be selected —'gated'— for subsequent analysis [15]. This strategy can however not be conveniently used to compare intensities of more than two markers simultaneously, which makes the use of multivariate methods essential.

Several dedicated multivariate data analysis methods have been used in MFC, such as Hierarchical Cluster Analysis in hematological oncology [16,17] and the immune response to tetanus [18]. Methods have even been specifically designed for MFC data, such as Spanning-Tree Progression Analysis of Density-normalized Events (SPADE) [2] and cellular hieraRCHY OPTIMization (RchyOptimyx) [19]. These methods rely, however, on prior knowledge about underlying immunological mechanisms. Flow Analysis with Automated Multivariate Estimation (FLAME) [20] constructs discrete cell populations from mixtures of cells, but does not reveal comprehensive relations between markers relevant to the disease mechanism. Frequency Difference Gating constructs differential MFC profiles between different groups of samples, however also without quantifying the underlying correlations between surface markers [21].

Hence, there is a strong need for a MFC data analysis method that simultaneously describes the role of each marker in the multi-cellular profile and uses the correlations between

the markers to facilitate data interpretation specifically for diagnosis. A method called Fisher Information Nonparametric Embedding (FINE) was used for the diagnosis of acute lymphoblastic leukemia from a population that exhibited expansion of physiologic B-cell precursors [22]. FINE transcends the single-cell level for a dedicated description of the diagnosis of patients, although this requires tedious *a posteriori* examination of the single-cell data to discover the disease-associated surface markers.

The viSNE [23] method uses the same principle of dimension reduction to represent the multivariate surface marker profiles, but provides merely a bivariate representation of the similarities between single cells. Using viSNE, healthy bone marrow could clearly be distinguished from leukemic bone marrow. However, because viSNE is inherently nonlinear, the multivariate relationships between different surface markers remain unexploited and abstractly hidden within the model. This leaves such information out of reach for biomedical interpretation.

In the field of chemometric analysis of omics data [24,25], linear dimension reduction techniques [25] have proven highly fruitful [26-28]. Principal Component Analysis (PCA) [29] is the most-widely used dimension reduction method. PCA models describe the single-cell heterogeneity observed in a sample (or in multiple samples), based on the most prominent multivariate correlations between intensities of different surface markers and have already found their way in several highly innovative MFC studies [13,30-33]. However, as PCA aims to describe all variation across the full complement of cells, it may leave highly relevant patterns that are specific to an immune response unexploited [34]. This may be the case for patterns that are concealed, for instance by biological variation.

Several methods have been developed that use external experimental or biological information to focus PCA more on relevant information in high-dimensional biological data. They take into account the experimental design of the study and other prior knowledge of the samples, such as time after treatment or disease state of the individual [35,36]. Such focused PCA-derived methods may reveal biologically relevant patterns within the data that otherwise remain hidden [35,37].

Here we present FLow cytometric Orthogonal Orientation for Diagnosis (FLOOD), a PCA-based method specifically developed to investigate and monitor immune responses within multi-cellular MFC surface marker profiles of multiple individuals. The main hypothesis that underlies the FLOOD method is that crucial diagnostic information resides in how an individual moves away from a 'normal situation' upon confrontation with a challenge. Therefore, the crucial first step in FLOOD is to model cell-to-cell variability in a 'normal' situation without any challenge. Subsequent steps then investigate, from different perspectives, how the cell-to-cell variability within challenged individuals *differs* from the normal situation. We show how focusing upon and investigating *differences* provides unprecedented diagnostic information and disease understanding. The use of the specific PCA features that allow unraveling the underlying correlations is instrumental in the interpretation. FLOOD thereby provides valuable multivariate patterns of challenge-specific

variability among the interrogated surface markers on individual cells. These patterns facilitate gating for diagnosis or further research.

To demonstrate FLOOD, we compare the MFC surface marker profiles of eight individuals that were challenged with Lipopolysaccharides (LPS) with those of eight healthy, unchallenged individuals.

Materials and methods

Flow Cytometry measurements were performed during an endotoxin trial (NCT01374711; www.clinicaltrials.gov). Details regarding the Flow Cytometry experiments that provided the data to illustrate the method are described in the Online Supplement I; source data can be accessed through flowrepository.org ID: FR-FCM-ZZJ3.

FLow cytometric Orthogonal Orientation for Diagnosis (FLOOD)

FLOOD uses multiple steps to systematically distinguish unchallenged from challenged individuals, using the responses observed in single cells. **Figure 1** contains a simplified schematic representation of the method that reveals the most relevant aspects of the model. It demonstrates FLOOD analysis of a hypothetical data set in which the intensities of three surface markers were measured on a multitude of cells. These three surface marker axes are designated by three vectors *I-III* in **Figure 1A**.

Section I: The Control space

The first, crucial step of FLOOD is to model the surface marker profiles of unchallenged individuals by PCA (**Figure 1A**). This model provides 'Control scores' for each cell from an unchallenged individual. The distribution of these scores is represented as a grey cloud in the figure and describes the single-cell heterogeneity in surface marker composition of this individual. This is expressed in a Control space, represented by the two-dimensional black parallelogram. This space captures as much of the cell-to-cell variability on these three surface markers as possible. When the expression of more than three different surface markers has been measured on the cells, the Control space may have more than two dimensions.

The surface marker vectors *I-III* (**Figure 1A**) are projected onto the Control space. The direction and length of the resulting projections—called 'loadings' in PCA— reflect the contribution of each marker to the cell-to-cell variability in the Control space. The length of the corresponding projected vector reflects this. The cells that are positioned in the direction of the vector projection of a specific surface marker, exhibit an above-average expression of this marker. The correspondence between the directions of different projected vectors indicates whether the abundance of the corresponding markers is positively (same direction) or negatively (opposite direction) correlated.

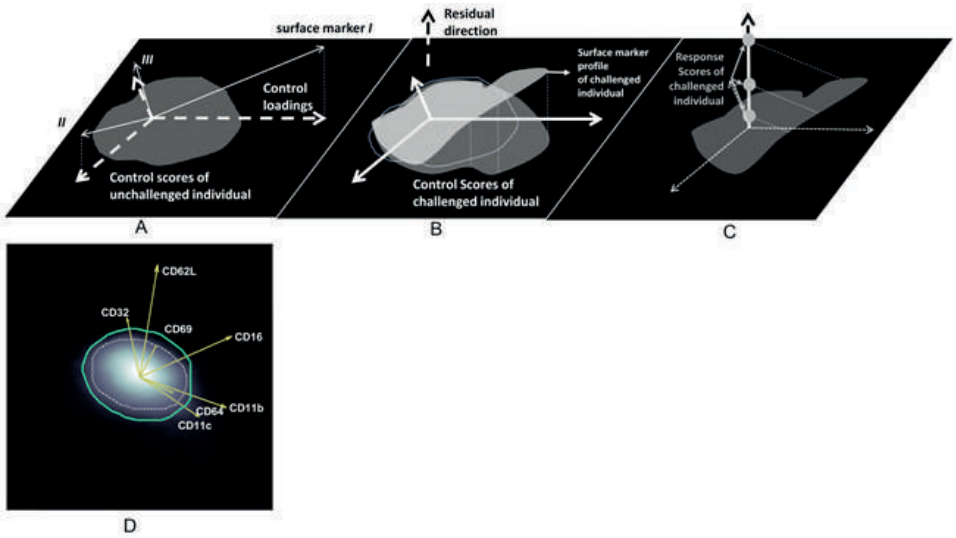

Figure 1: Schematic representation of FLOOD with three hypothetical surface markers.

A. 'Control' PCA model with the surface marker profile of an unchallenged individual. The grey cloud contains the Control scores that span as much variability among the cells as possible in a low number of dimensions (here two). The vectors (thin solid arrows) indicate the intensity distribution on each of the three surface markers I, II and III. The dashed vectors are the loadings of these markers obtained by projection of the marker vectors on the two-dimensional Control space.

B. The white area represents the surface marker profile of a challenged individual. Projection of this area on the Control space defined by the Control loading vectors (thick solid white arrows) results in the grey Control score area. For comparison, the contour of the Control scores of the unchallenged individual from panel A is displayed as a continuous grey circular line. When determined for all unchallenged individuals, this is the Control benchmark. The projection of the surface marker distribution of the challenged individual also renders a 'residual' space that is orthogonal to the Control space. This space is one-dimensional in this example and is indicated by the dashed thick arrow. The residual space would have more than one dimension for MCF analyses with more than three surface markers.

C. The residual surface marker information of each cell not described by the projection on the Control space, *i.e.* in the vertical direction in this example, is then described by a 'Response' PCA model. The scores that result from this projection of the residuals on the Response space of reduced dimensionality are indicated by the dots on the solid vertical arrow (one-dimensional response space). They explain as much remaining variability among cells of challenged individuals as possible. Although not shown here, the original surface marker vectors from panel A may also be projected on this space to reveal the most prominent correlations between them in the Response space as Response loadings. The grey area represents the surface marker profile of the challenged individual; the thin white dotted arrows indicate the Control loading vectors.

D. 'Biplot' of the Control space, *i.e.* analogous to panel A, for the LPS data obtained on a typical unchallenged control individual. The brightness represents the abundance of cells scoring at that position. The white dotted outline encircles 80% of its scores. The thick solid cyan outline corresponds to the collective 'Control benchmark' that encircles 80% of cells in all unchallenged individuals. The arrows represent the loading vectors of each surface marker.

The Control space can be determined on the surface marker profiles of more than one unchallenged individual, by capturing normal cell-to-cell variability among individuals. This collected variability is expressed as a 'Control benchmark' that describes the variability in surface marker expression among cells in unchallenged individuals.

Section II: The Response space: Investigation of cell–to–cell variability introduced by the challenge

The second step of FLOOD then orthogonally projects the surface marker profiles of challenged individuals onto the Control space (**Figure 1B**, for one individual), providing Control scores that describe single-cell heterogeneity observed in challenged individuals. This step facilitates direct and quantitative comparison with the variability in the Control benchmark [38].

The projection on the Control space describes the cell-to-cell variability within challenged individuals only partly, because the loadings of the Control space do not fully describe the surface marker correlations observed in challenged individuals. This 'residual' cell-to-cell variability, present only in challenged individuals, is orthogonal to the Control space.

The third step of FLOOD (**Figure 1C**) explores the response-specific cell-to-cell variability by describing multivariate correlations among surface markers in the orthogonal direction by a second PCA model. This provides a second, 'Response' space that is again spanned by loading vectors that reveal surface marker correlations. However, these correlations are *only* observed in the cell-to-cell variability within challenged individuals. The 'Response' scores thus describe the contribution of each cell to this challenge-specific variability. Since the schematic example is limited to only three surface markers and the Control space is two-dimensional, this Response space can only have one dimension. For more than three measured surface markers, also the Response space may be higher-dimensional and the Response model may have residuals, like the Control space.

In the last step we construct a 'normal' benchmark in the Response space. This is necessary because also in the control population there will be a (small) amount of cells that exhibit a profile that is similar to the typical response profile. To construct a normal benchmark the cell-to-cell variability of unchallenged individuals is projected onto the Response space (This variability was not captured in the Control space and therefore not depicted in **Figure 1A**). The resulting benchmark of 'unchallenged' single-cell heterogeneity in the Response model is used as a second standard to directly and quantitatively compare the Response scores of challenged individuals against.

FLOOD can model a virtually unlimited number of cell characteristics simultaneously, simplifying their representation and extracting challenge-specific correlations. The Supplementary material contains the mathematical representation of the FLOOD algorithm. The schematic representation already shows one of the very strong aspects of PCA, which has not yet been applied to MFC data analysis to such extent. Unlike viSNE and the other advanced methods mentioned before, PCA allows simultaneous representation of the single-cell scores and the surface marker loading vectors in a 'biplot' [39]. **Figure 1D** gives

such a plot for the Control space of an unchallenged individual. Cells in the score plot that are positioned in the direction of a loading vector display relatively high abundances of the corresponding surface marker. Cells positioned in the bottom right of the cloud in **Figure 1D** have a correlated high abundance in the expression of the integrin alpha chain of MAC-1 (CD11b) and integrin alpha X chain, component 3 of complement receptor 4 (CD11c). Analogously, cells to the bottom left have a low abundance of FcγRIII (CD16) and L-selectin (CD62L) as these loadings both direct to the opposite, top right. The length of each loading vector indicates how large the variability associated to each surface marker is within the lower-dimensional Control or Response space depicted in the biplots.

The dotted grey 'benchmark' line in **Figure 1D** encircles the heterogeneity among the 80% of cells that deviate least from the average surface marker composition of that specific individual. This line is constructed by dividing the model space in 100 x 100 equally spaced bins and counting how many cells each bin contains. After the resulting 2D histogram is smoothed [39], the bins with the lowest cell counts are discarded until the remaining bins contain 80% of all cells of that individual; the dotted grey line encircles these bins. The empirical threshold of 80% prevents excluding potentially interesting regions with relatively few cells. It also avoids including bins with strongly deviating surface marker patterns that contain only few cells.

The thick circular line analogously encircles 80% of the cells of *all* unchallenged individuals, to serve as the aforementioned Control benchmark to compare against surface marker profiles of challenged individuals. We determined the 80% threshold to reveal the largest difference in shape and area between the 'unchallenged' benchmark and the lines that encircle the Control scores of the challenged individuals (see **Figure 1** of the Supplementary Material section II). The supplementary material includes a sensitivity analysis of the most important model results for this threshold value. The independent reproducibility of these shapes was statistically validated by cross-validation [24], in which data of each individual was used once as a test set. The data of the individual in the test set was removed from the data set before PCA and projected *post-hoc* to establish whether similar observations could be done without the corresponding data influencing the constructed model. The cross-validation confirmed the robustness of both the Control and the Response model.

Results

The comparison of conventional bivariate histograms of data from an LPS-challenged and an unchallenged individual (**Figure 2**) indicates that LPS challenge results in the appearance of $CD62L^{dim}CD16^{bright}$ and $CD62L^{bright}$-$CD16^{dim}$ cells. However, earlier studies already describe differential expression of several other surface markers involved in this response, using conventional uni- and bivariate interpretation of the same data [40].

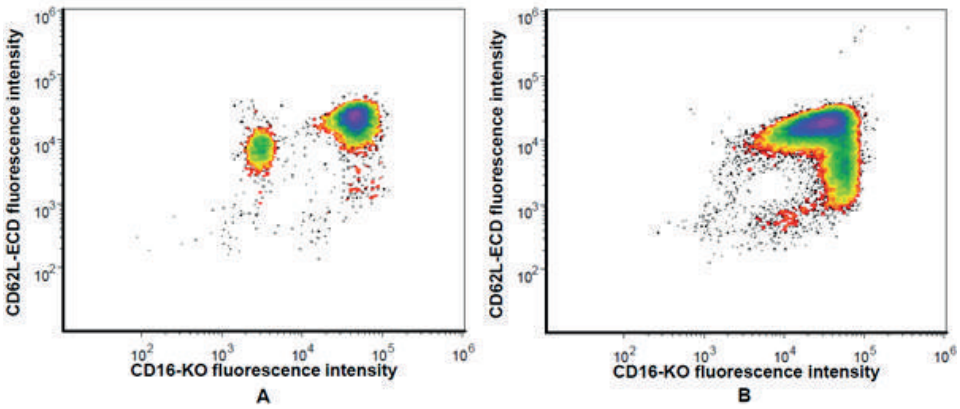

Figure 2: Bivariate histograms of granulocytes plotting CD62L vs CD16 expression.

A: without LPS challenge. B after LPS challenge. Abundance of cells is indicated using 'false colours' (in increasing order: red-yellow-green- blue-purple).

FLOOD model

The first step of FLOOD (**Figure 1A**) provides the biplot of the Control space spanned by the single-cell heterogeneity of unchallenged individuals (**Figure 1D**). The subsequent projection of Surface Marker Profiles of challenged individuals is shown in **Figure 3**. Many of the cells from this typical challenged individual project outside the confinements of the ellipsoid Control benchmark and hence have deviating surface marker profiles compared to unchallenged individuals. The 'bean-like' shape of the projection of single-cell surface profiles is characteristic of the mis-fit of the Control model to the data from challenged individuals. The 80% Confidence Interval is well-reproducible between challenged individuals, as the continuous grey lines indicate (**Figure 3**).

Many of the cells of the challenged individual lie outside the benchmark in the direction of the loadings of CD11b and CD11c, indicating a larger variability in the abundance of these markers. In a similar way, the opposite direction of the left-hand lobe of the bean indicates down-regulation of CD16.

Mis-fits specific to the LPS response are not reflected in the position of the Control scores of cells from challenged individuals alone, as the associated loadings only contain correlations among surface markers present in unchallenged individuals. Correlations that emerge or disappear upon response, lie outside the Control space and are therefore contained in the Control residuals. Cells of challenged individuals may have large residuals even when positioned inside the Control benchmark.

The contours of the projections of the cell populations from challenged individuals on the Response space (**Figure 4**) show that also in this model the scores exhibit a shape that is highly reproducible among challenged individuals. The Response loadings show the

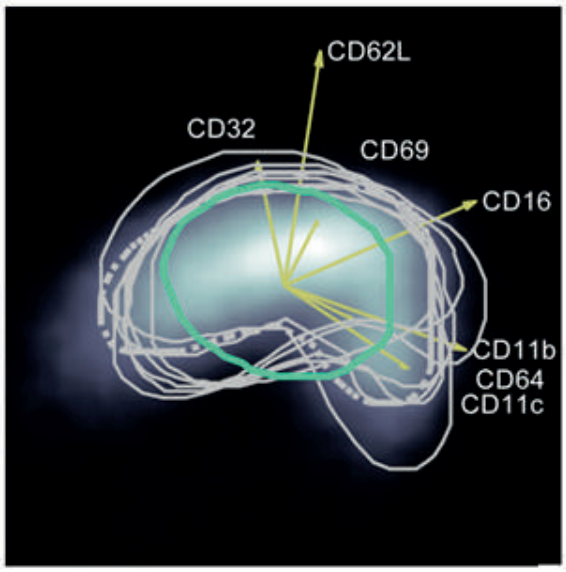

Figure 3: Control model biplot for a typical LPS–challenged individual.

Bright white areas indicate high cell densities. Yellow arrows represent surface marker loadings. The dotted white line indicates the 80% confidence interval (CI) for this individual. Solid white lines indicate 80% CIs for other LPS challenged individuals. The solid cyan line represents the 80% CI across all unchallenged individuals.

LPS response-specific surface marker information for each cell. The Response loadings of markers CD11b and specifically CD11c are relatively small, which indicates that there is relatively little variability in the abundance of these markers upon LPS challenge that was not captured in the Control space. The Control loading of surface marker CD69 is much smaller than the Response loading, which indicates that it varies only slightly among cells of unchallenged individuals but considerably more upon LPS response. The vertical orientation of the loading indicates that it is mostly related to an increase in abundance of this surface marker on cells projecting in the left lobe of the bean shape.

The Response loadings of markers CD62L and CD16 are oppositely directed, which implies that their abundances are negatively correlated among cells of challenged individuals. This negative correlation is in contrast to the more positive correlation among both surface markers in the Control space. The positive correlation in the Control model added to the negative correlation in the Response model results in the mutually independent expression of both surface markers observed in the bivariate scatter plot after LPS (**Figure 2B**). The Response loadings, specifically when interpreted together with their Control counterparts, provide advanced insight in the correlations among surface markers that emerge, disappear and otherwise change upon LPS response.

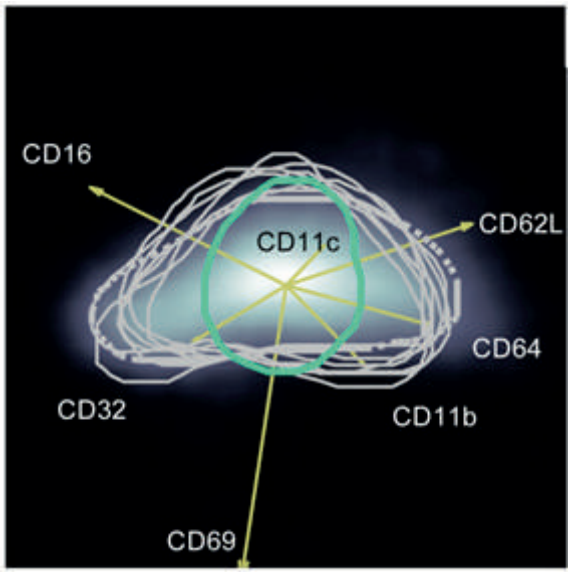

Figure 4: Response model biplot for a typical LPS–challenged individual.

Including individual-specific 80% CI for all LPS-challenged individuals (solid white lines, the dotted line corresponds to the typical individual). All LPS-challenged individuals exceed the Response benchmark (solid cyan line) defined by all unchallenged individuals. They have a consistent shape..

The projection of the cells from challenged individuals compared to the Response benchmark of unchallenged individuals indicates that the single-cell heterogeneity in challenged individuals increases upon response. Many cells lie outside this benchmark and are, therefore, likely LPS response-related. Note that the shape of the Control benchmark of the unchallenged individuals is also circular. Again, the Response scores of challenged individuals lie outside the Response benchmark for the unchallenged individuals, in two directions. Whether these two protruding groups consist of the same cells as those identified in the Control space can be investigated by gating the scores within both spaces.

Cross–validation and Gating

Figure 5 shows the FLOOD model results of one challenged individual in the Control space (**Figure 5.1**) and in the Response space (**Figure 5.3**). All results were obtained by a leave-one-out cross-validation protocol; the data of the plotted individual were excluded before generation of the FLOOD model. The Control scores of the challenged individual may be divided into three separate gates. The green cells are enclosed by the Control benchmark in a central gate ***b***. Those cells that lie outside the Control benchmark yet inside the 80%

contour line that encloses the cell scores of that individual are assigned to two additional gates (***a*** and ***c*** in **Figure 5.1**). The average surface marker expression of the cells within these three gates differs for almost all seven surface markers (**Figure 5.2**). In the Response model, the cells also score into three distinct gates (**Figure 5.3**).

The supplementary material (Section II, **Table S1**) shows the results of projection of the data of each individual, analogous to **Figures 5.1** and **5.3**. The projections in the Response space were obtained according to the leave-one-out method described above. The median surface marker expression of the cells within the three gates shown in **Table S1** of that Section are shown in **Table S2** of the same Supplementary Material section. Comparison of these results for the Control model shows that the differences between surface marker intensities of the gates are highly consistent across individuals. A similar observation is made for the results of gating from the projections in the Response model. Thus, **Tables S1** and **S2** in Supplementary Material II present the cross-validation of the method. A further comparison of the patterns in **Table S2** between the Control and the Response model also shows the close association between the gates defined in both models. These gates therefore contain highly similar sub-populations.

Gate ***b*** in **Figure 5.3** encloses the cells within the Response benchmark and contains 68% of all cells measured for that individual. These cells have surface marker profiles that are indistinguishable from cells of unchallenged individuals and are therefore likely fully mature neutrophils. Gates ***a*** and ***c*** each contain 10-12% of the neutrophils of that same individual.

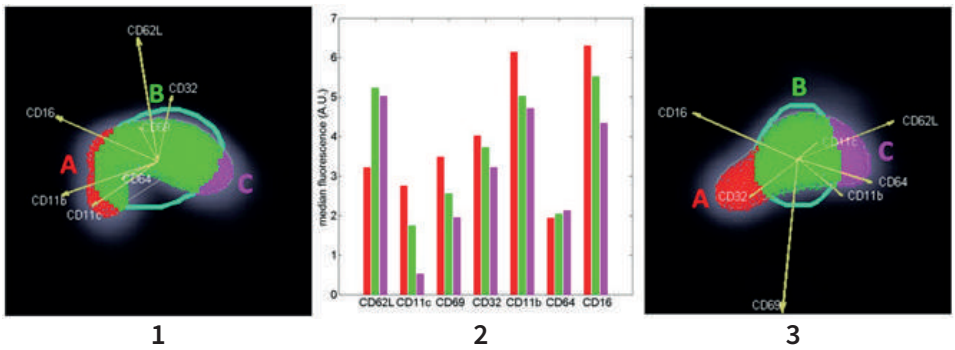

Figure 5: Gating based on FLOOD.

Panel 1: Gating of the Control scores of a typical challenged individual into three distinct regions: B (green, enclosing the Control benchmark), A coloured in red and containing the cells outside the Control benchmark, but inside the 80% CI of that individual and to the left of the loading origin and C (purple) the region outside the benchmark, inside the 80% CI of that individual and to the right of the loading origin. **Panel 2: the median surface marker expression of the cells within each of the gates** defined in panel 1. In each cluster of three bars, the first bar (red) is the median single marker intensity of the cells within gate A; the second, green one is for gate B; the third purple one is for gate C. **Panel 3: Gating of the Response scores**. Gates were determined similar to the gating in Panel 1, but using the Response benchmark instead of the Control benchmark.

The remaining cells lie outside the threshold that encloses 80% of the cell-to-cell variability of that individual. **Table S3** of Supplementary section II, also obtained by cross-validating both challenged and unchallenged individuals, shows that these numbers are highly similar for all individuals. The cross-validated scores, specifically the percentages of cells that lie within gate ***a*** and ***c***, are highly similar between individuals and are much lower for unchallenged individuals.

The percentage of cells that lie outside the unchallenged benchmark, both in the Control and Response models, may serve as quantitative indicator of challenge or disease (**Figure 6**). FLOOD indicates that individuals of which many cells score outside the Control benchmark exhibit an immune response. Note that the Control and Response benchmarks have been determined on all unchallenged individuals simultaneously, which does not imply that 20% of cells in each unchallenged individual should score outside these benchmarks. When a high percentage of cells also scores outside the Response benchmark, this indicates a response specifically to LPS.

For all challenged individuals, in comparison with the unchallenged individuals, a relatively high percentage of cells scores outside the 80% Confidence Interval serving as the Control benchmark (**Figure 6A**). In the Response model the difference in the percentages of cells scoring outside the benchmark between challenged and unchallenged individuals is even larger (**Figure 6B**). Unchallenged individual #5 has a relatively large percentage of cells that score outside the Control benchmark. This indicates that this individual exhibits an immune response (Supplementary Material II, **Figure S2**). However, the percentage of cells in this unchallenged individual that scores outside the Response benchmark is not exceptionally high compared to other unchallenged individuals and certainly not as high as in challenged individuals. This indicates that individual #5 does not suffer from a response to LPS, but might respond to an unknown non-experimental factor.

We have performed a sensitivity analysis in order to determine the effect of the level of the Confidence Interval on the diagnosis performance. A considerably lower confidence interval for the Control and Response benchmarks (50%) still allows diagnosis of an immune response that distinguishes challenged individuals from those that did not receive LPS (Supplementary Material II, **Table S4**). This diagnosis is however based on only a very limited subset of the measured cells for each individual, such that much of the cell-to-cell variability remains unused (Supplementary Material II, **Table S5**). Including almost all cells in the benchmarks (99% threshold) does not allow identification of an immune response using the Control or Response models (Supplementary **Table S4**). This is attributed to a large fraction of cells from challenged individuals being included in the both benchmarks (Supplementary **Table S5**). The 80% threshold on the Confidence Interval both allows disease diagnosis and makes use of most variability within the cell surface marker profiles. Clearly, further optimization of the benchmark percentage and gating strategy could be considered for the development of a routine diagnosis tool, but goes beyond the scope of this investigation.

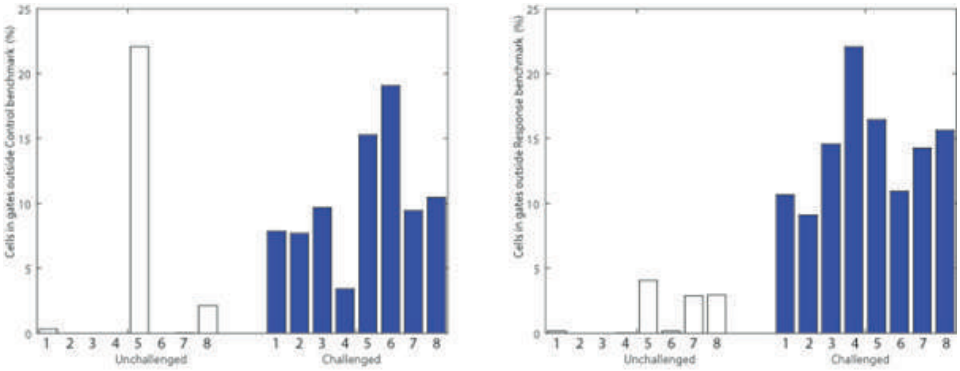

Figure 6: Gating for Diagnosis.

A. Percentage of cells that score outside the Control benchmark, yet inside the 80% CI established on the Control scores of that individual. Percentages are given for unchallenged (white bars, left) and for challenged individuals (blue bars, right). A higher percentage of cells of all challenged individuals score outside the benchmark compared to unchallenged individuals, except for unchallenged individual #5 **B. Percentage of cells that score outside the Response benchmark, yet inside the analogous 80% CI on the Response scores.**

Chapter 3.2 in this thesis contains a concise report on an application of FLOOD to study the effects of anaerobic exercise on elite sportsmen. It shows that the method can also be applied to study and quantify changes in surface marker profiles that are the result of subtle, non-pathogenic challenges.

Immunological interpretation

As the scores of all cells form a continuum, their sub-division in 'populations' is artificial and merely helps to point out the dynamics of the challenge. The defined gates distinguish themselves prominently on markers CD16 and CD62L, which corresponds with earlier findings [40]. However, the specifically multivariate view on the cellular populations that FLOOD provides indicates additional involvement of for example CD69 in the LPS response. This surface marker has been described as an activation marker *in vitro* after incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon-α or Interferon-γ [41] and differs along with CD16 between the gates. The orthogonal orientation of the Response loading vectors of CD62L and CD69 shows that their expression on individual cells is not directly related in the LPS-specific cell-to-cell variability.

Neutrophils in gate ***a*** exhibit a surface marker profile with elevated expression of integrin-chains CD11c, CD11b and activation marker CD69, which overall indicates a more 'activated' surface marker pattern, corresponding to immune suppressive cells with hypersegmented nuclei [40]. In homeostasis, peripheral blood neutrophils do not express CD69, although CD69 is upregulated after overnight incubation with IFN-γ [41]. Work by De Kleijn *et al.* [42] indicates

that neutrophils with the $CD62L^{dim}/CD16^{bright}$ profile show an IFN−γ transcriptome signature. These two combined findings support the observation of elevated CD69 expression within this specific ($CD62L^{dim}/CD16^{bright}$) subpopulation upon LPS challenge. Note that the loading of CD69 in the Response space (**Figure 5.3**) is large and indeed points in the direction of the gate ***a***, whereas its Control loading is relatively small and therefore contributes only little to the cell variation described by the Control space (**Figure 5.3**). The variability of markers CD11c and CD11b was already identified in the Control model as more prominent in challenged than in unchallenged individuals. The expression of these markers in gate ***a*** of the Control model exceeds those in gates ***b*** and ***c***. Thus, the FLOOD loadings indicate that the $CD11c^{bright}/CD11b^{bright}$ profile of the neutrophils in gate ***a*** (**Figure 5**) is challenge-related. Their positive correlation is however not specific to the challenge, because CD11c contributes only little to the Response loadings. The neutrophils within gate ***a*** defined in the Response model are $CD62L^{dim}$, although some variability of its expression within in the gates defined in the Control model exists between individuals (Supplementary material II, **Table S2**). The orientation of the CD62L Response loading away from gate ***a*** confirms this. Gate ***c*** neutrophils are characterized by lower expression of most markers compared to neutrophils in both other gates. Especially the surface markers CD16, CD11b, CD11c and CD69 are expressed less than in both other gates, which fits with the concept of neutrophils with 'banded nuclei' [40] that are in the process of maturation into fully differentiated neutrophils. This shows how FLOOD facilitates identification and description of subpopulations that are formed as a result of a challenge, in terms of correlations that already exist in unchallenged individuals (CD11b and CD11b) or that emerge or disappear upon response (CD16 and CD62L).

Discussion

Multicolour Flow Cytometry (MFC) technology provides three very promising capabilities for clinical practice: (1) diagnosis of disease responses at a targeted and personalized level, (2) insight in correlations between surface markers that are typical of such a response and (3) the identification of individual cells with a surface marker expression characteristic of the response. An analysis of the resulting data should therefore provide a comprehensive view on the cell marker profiles of specific cells observed on disease response.

FLOOD enables the exploitation of these capabilities for multivariate data on large numbers of cells. The shapes of the regions spanned by the Control and Response scores are used for diagnosis: a multi-cellular sample obtained from an LPS-responding individual will produce a score patterns in the Control and Response spaces that differ substantially from the shape of the Control and Response benchmarks, respectively. Cells that score outside the latter benchmark are likely response-related.

The cell-to-cell variability that underlies the response to LPS can now be very well revealed by FLOOD. The biplot representation we have introduced here for MFC data allows direct association of 'unusual' cells to all relevant surface markers and their mutual correlations that occur without challenge (Control model) and upon response.

The non-linear distances used by viSNE and FINE do not allow such intuitive geometric interpretation of the correlations between surface markers that is facilitated by the FLOOD biplots. SPADE neither explicitly models the correlations between surface markers in such an insightful manner. For all of these methods, the correlations between surface markers and their expression within specific groups of cells need to be investigated *post hoc*. We have included and commented an analysis of the same dataset with SPADE in Supplementary Material section III.

The main advantage of FLOOD over the abovementioned existing methods for MFC data analysis is that it specifically highlights the cell-to-cell variability that is associated with a challenge. The resulting contributions to the variability can be separately described by the Control and Response spaces. This provides a focus on the response to LPS challenge. The cell-to-cell variability within these spaces can be explored with PCA-based biplots, which allows unprecedented interpretation of the surface marker correlations that result from the response.

The cells with changed L-selectin (CD62L) and FcγRIII (CD16) expressions become very prominent in LPS challenged individuals compared to unchallenged controls. The large number of cells that lie outside the Control and Response benchmarks identifies many cells that are not present in the blood under the homeostatic conditions prior to challenge. It is to be expected that any disease associated with LPS-induced signals (*e.g.* trauma and sepsis) will evoke similar cellular response patterns [40]. The Control and Response benchmarks and biplots allow direct comparison and interpretation of the expression surface marker patterns between challenged and unchallenged individuals. This capability is eminently lacking in ViSNE and FINE, because these methods do not allow projection of data from novel individuals onto an existing model.

The Control and Response benchmarks serve another highly advantageous feature, as individual cells that express surface marker combinations unobserved in unchallenged individuals lie outside these benchmarks and can be gated as 'response-specific'. Integration of the FLOOD-based gating with Fluorescence-Assisted Cell Sorting (FACS) will allow immediate and automated in-line isolation of response specific cells following flow cytometric analysis. Not only can this method be used in context of human cells before and after challenge, it also has potential for *in vitro* stimulation assays with cell lines or for example to observe cell marker changes between two time points.

In conclusion, we demonstrated the performance of FLOOD on a seven-marker Flow Cytometric dataset. As the method is strongly rooted in PCA, application could extend to the simultaneous analysis of a virtually unlimited number of markers. Therefore, the method can be applied to seventeen fluorescent markers [7] or the even higher numbers of potential markers in CyTOF analysis [31,32]. This makes FLOOD a very powerful data analysis method for multivariate datasets of MFC and related techniques that has considerable implications for both research and disease diagnosis.

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Supplementary Material I: Mathematical Description of the FLOOD algorithm

As the FLow cytometric Orthogonal Orientation for Diagnosis (FLOOD) approach makes extensive use of PCA methodology, we first review this method and subsequently extend it to dedicated disease diagnosis with MFC data.

Principal Component Analysis

Cells collected from an individual and analysed by MFC, indicated by $1_i \ldots n_i \ldots N_i$ can be arranged into $(N_i \times J)$ individualized surface-marker profile matrices $\mathbf{X}_i$ for individuals $1 \ldots i \ldots I$ and surface markers $1 \ldots j \ldots J$. These surface-marker profiles can then be subjected to Principal Component Analysis—modified specifically for multi-cellular individualized profiles—according to Eq. (1) .

$$\mathbf{S}^{-1}\left(\mathbf{X}_i - 1_{N_i}\mathbf{m}_i^{\mathrm{T}}\right) = \mathbf{T}_i\mathbf{P}^{\mathbf{T}} + \mathbf{E}_i \qquad (1)$$

where S^{-1} is a diagonal matrix of size J that contains the standard deviation of each surface marker among all cells within unchallenged individuals, vector $\mathbf{m}_i$ of length J contains the average surface marker expression of all cells in individual i; matrix $\mathbf{T}_i$ of dimensions $(N_i \times A)$ contain the single-cell scores of individual i and matrix P $(J \times A)$ contain the Control loadings; E_i contains the PCA residuals for individual i; R is the selected number of PCA components.

The PCA model makes the multi-cellular surface marker profile insightful, as the scores T_i describe as much variability among the single cells as possible in R dimensions, where each incremental dimension describes as much of the surface marker variability as possible and the orthogonality of the model loadings $\mathrm{P}^{\mathrm{T}}\mathrm{P}=\mathrm{I}_A$ (where I_A is the identity matrix) ensures that surface marker variation described in one dimension does not occur in another.

This variability is expressed with respect to the average surface marker composition in vector $\mathbf{m}_i$. This 'centering' to the individual mean is the main distinction of this PCA model to specifically describe the multi-cellular surface marker profiles for different individuals, and is known as Simultaneous Component Analysis [43]. In this model, the linear combination of surface markers in loadings P indicates which markers are most important in the A dimensions and their mutual relationships. Thereby the model in Eq. (1) explicitly models the variability in all surface markers simultaneously. When A is chosen as two, the conventional bivariate histogram representation can be used, but each dimension will consist of mathematically defined contributions from all surface markers. These can be represented within the histogram in the 'biplot' representation [39,44]. This has, to the best of our knowledge, hitherto not been used in any MFC study. Note that, to optimally prepare for the subsequent FLOOD analysis, the loadings P and scaling constants S are determined for all relevant individuals simultaneously. This imposes identical relations between the surface markers for all individuals, which allows comparison between the scores of different individuals [38]. Further details on this model will be discussed in the next section.

FLow cytometric Orthogonal Orientation for Diagnosis

Rather than modelling the cell-to-cell variability simultaneously like PCA, FLOOD separately describes the cell-to-cell variability into multiple, well-defined and interpretable parts in a sequential multi-step approach. The first modelling step describes the surface marker variability between cells of unchallenged individuals, leading to the 'Control' model in Eq. 1. The sampled individuals are either unchallenged $1_u \ldots i_u \ldots I_u$ or challenged with LPS: $1_c \ldots i_c \ldots I_c$.

$$\mathbf{S}_C^{-1}\left(\mathbf{X}_{i_u} - 1_{N_{i_u}}\mathbf{m}_{i_u}^T\right) = \mathbf{T}_{C,i_u}\mathbf{P}_C^T + \mathbf{Y}_{i_u} \quad (2)$$

$\mathbf{S}_C^{-1}$ is a diagonalmatrix of size J that contains the standard deviation of each surface marker among all cells within unchallenged individuals with respect to $\mathbf{m}_{i_u}^T$, vectormiu of length J contains the average surfacemarker expression of all cells in individual i_u; matrix $\mathbf{T}_{C,i_u}$ of dimensions $(N_{i_u} \times A_C)$ contains the scores of individual i_u and matrix $\mathbf{P}_C$ $(J \times A_C)$ contains the Control loadings; $\mathbf{Y}_{i_u}$ contains the residuals of this model for each individual; $1_c \ldots a_c \ldots A_c$ indicate the components of the Control model.

The variability in the surface marker profile of each unchallenged individual X_{i_u} is expressed by scores $\mathbf{T}_{C,i_u}$. These are different from the PCA scores in Eq. (1), because the Control model is fitted on the unchallenged individuals alone. This is also reflected in the Control loadings $\mathbf{P}_C$, that now exclusively describe relationships between surface markers exhibited by unchallenged individuals. Note however that they do not explicitly exclude relations in challenged individuals if they are also exhibited by unchallenged individuals. One aspect that is not explicit in Eq. (2) is that we fit the model such, that each individual may contribute an equal amount of information to the model. It thereby averages between all individuals rather than between all cells. This is done by weighing the data of each individual with the corresponding cell count N_{i_u} according to a method adapted from multi-block PCA [45].

The Control model employs the same individual-based centering as the PCA model presented before, which centers the surface marker profiles in X_{i_u} on the average surface marker expression of *each unchallenged individual*. This important modelling choice results in $\mathbf{T}_{C,i_u}$ describing the surface marker variability *per* individual, which means that absolute score values may not be directly compared *between individuals*, but their mutual variability may. This choice results from consistent 'offset' differences between surface marker intensities we have empirically observed, that are not related to the challenge and therefore distract from the cell-to-cell variability within the same individual that is of primary interest. Instead of the cell-to-cell variability, the surface marker profiles are scaled here by $\mathbf{S}_C$, that contains the standard deviation among all cells of unchallenged individuals. The Control model therefore is exclusively based the information in unchallenged individuals.

As a result, the Control loadings do not describe correlations between the surface markers that are related to LPS challenge. How the Control model then describes the surface marker profiles of challenged individuals, can be found from their orthogonal projection onto these loadings as a second step of the FLOOD approach. This operation leads to the model in Eq. (3).

$$\mathbf{S}_{\mathrm{C}}^{-1}\left(\mathbf{X}_{i_{\mathrm{c}}}-1_{\boldsymbol{N}_{i_{\mathrm{c}}}}\mathbf{m}_{i_{\mathrm{c}}}^{\mathrm{T}}\right)=\mathbf{T}_{\mathrm{C},i_{\mathrm{c}}}\mathbf{P}_{\mathrm{C}}^{\mathrm{T}}+\mathbf{Y}_{i_{\mathrm{c}}} \tag{3}$$

where $\mathrm{T}_{\mathrm{C},i_{\mathrm{c}}}$ of dimensions $(N_{i_{\mathrm{c}}}\times A_{\mathrm{C}})$ contains the scores of a challenged individual expressed on the Control loadings and $Y_{i_{\mathrm{c}}}$ contains the residuals of the projection.

The scores $\mathrm{T}_{\mathrm{C},i_{\mathrm{c}}}$ that result fromthis projection nowdescribe the cell-to-cell variability in challenged individuals expressed on the relations between surface markers exhibited by unchallenged individuals. Note that the surface marker intensities have been scaled with the same parameters in S_{C}, such that the cell-to-cell variability in the Control model can be compared between unchallenged and challenged individuals.

The scores of challenged individuals may nowbe compared to those from unchallenged individuals obtained from Eq. (2), which reveals how LPS alters the cell-to-cell variability within the same individual through the specific combinations between surface markers exhibited by unchallenged individuals. Deviations from these correlations may be reflected in a different shape of the projection of the cells *per* individual. However, this representation does not involve processes that involve relationships between surface markers *not* exhibited by unchallenged individuals,which implies therewill be a considerable systematic mis-fit of the challenged surface marker profiles on the Control loadings. This information will end up in the projection residuals $Y_{i_{\mathrm{c}}}$, which are explored in the third step of the approach: a PCA model that fits this systematic information related to processes that do not occur in unchallenged circumstances, given in the 'Response' model in Eq. (4).

$$\mathbf{Y}_{i_{\mathrm{c}}}=\mathbf{T}_{\mathrm{R},i_{\mathrm{c}}}\mathbf{P}_{\mathrm{R}}^{\mathrm{T}}+\mathbf{E}_{i_{\mathrm{c}}} \tag{4}$$

where matrices $\mathrm{T}_{\mathrm{R},i_{\mathrm{c}}}$ $(N_{i_{\mathrm{c}}}\times A_{\mathrm{R}})$ contain cell scores of individuals i_{c} and P_{R} $(J\times A_{\mathrm{R}})$ the corresponding loadings on principal components $1\ldots a_{\mathrm{R}}\ldots A_{\mathrm{R}}$; $E_{i_{\mathrm{c}}}$ contain the model residuals.

Scores $\mathrm{T}_{\mathrm{R},i_{\mathrm{c}}}$ contain the cell-to-cell variability after LPS challenge, obtained without prior biological assumptions and the corresponding loadings P_{R} contain the relations among surface markers that occur in challenged, but not in unchallenged individuals and are therefore response-specific. Together thesemodel results forma *personalized* surface marker-profile that can be compared between challenged individuals: also the scores and loadings from Eq. (4) can be represented in a bi-plot — a single bivariate figure if R_{R} is two.

However, the scores of $\mathrm{T}_{\mathrm{R},i_{\mathrm{c}}}$ and their variability within challenged individuals can only be quantitatively evaluated against a benchmark of variability in unchallenged individuals. When R_{C} is chosen too low, systematic information shared by unchallenged and challenged individuals ends up in $Y_{i_{\mathrm{c}}}$, as it is not part of the orthogonal projection in Eq. (3) and therefore also becomes part of the scores $\mathrm{T}_{\mathrm{R},i_{\mathrm{c}}}$ through Eq. (4). This benchmark can be determined by an orthogonal projection of the Control model residuals of the unchallenged individuals ($Y_{i_{\mathrm{u}}}$, from Eq. (2)) onto the Response loadings from Eq. (4), leading to the fourth step in the FLOOD approach given in Eq. (5).

$$\mathbf{Y}_{i_{\mathrm{u}}}=\mathbf{T}_{\mathrm{R},i_{\mathrm{u}}}\mathbf{P}_{\mathrm{R}}^{\mathrm{T}}+\mathbf{E}_{i_{\mathrm{u}}} \tag{5}$$

where $\mathbf{T}_{\mathrm{R},i_u}$ of dimensions $(N_{i_u} \times A_R)$ contain the Response scores of unchallenged individuals and $\mathbf{E}_{i_u}$ the model residuals.

Scores $\mathbf{T}_{\mathrm{R},i_u}$ will not individually be of interest for interpretation as they are supposed to contain no information. However, if taken together for all unchallenged individuals they may be turned into a confidence interval. The cells of a challenged individual that fall inside this interval do not deviate more fromthe average $\mathbf{m}_{i_c}{}^{\mathrm{T}}$ than could be expected from the cell-to-cell variability in unchallenged individuals. The cells that exceed this interval are therefore of primary interest to the LPS response.

Both the Control and Response models are low-dimensional representations of high-dimensional MFC data. Several methods exist to determine appropriate dimensionality for PCA models [46]. However, when insufficient dimensions are selected for the Control model, the Response benchmark will contain systematic features (*i.e.* specific shape), which may serve as an additional, FLOOD-specific model validation.

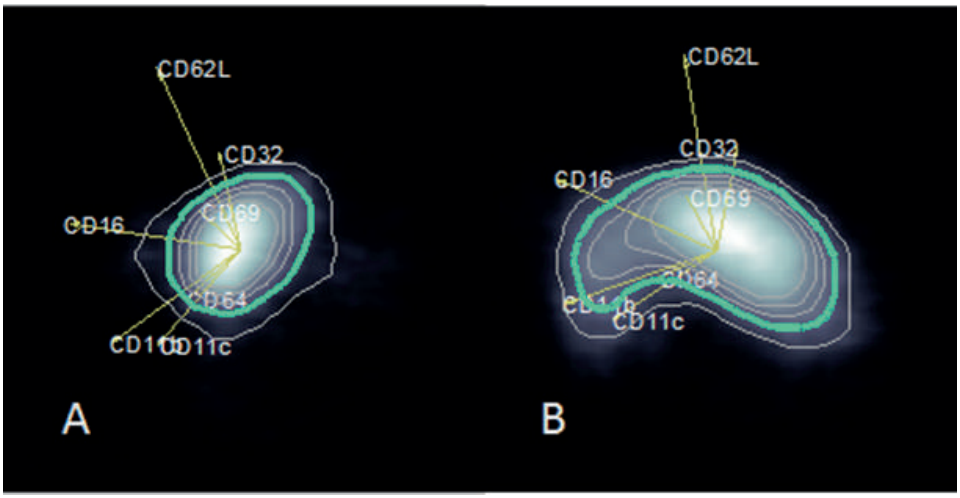
Cross–validation

The leave-one-out cross-validation conducted here to reproduce observations from the model with data that was not used to construct the model itself, emulating the situation that data of a new individual is expressed on the already existing model. This procedure consists of leaving all data of one individual out of the entire modeling sequence. The FLOOD model is then fitted on the seven remaining challenged and all unchallenged individuals. Then the projections described in Eqs. (3) and (5) are conducted on the data of this left-out individual, after which the resulting Control and Response scores can be interpreted. To perform cross-validation, this procedure is repeated for all individuals.

Data extraction from original .lmd files

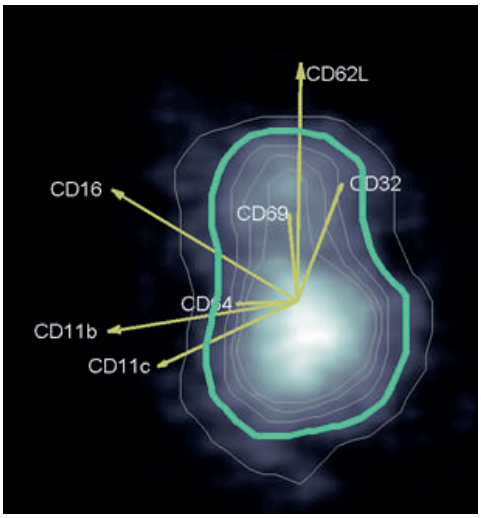
Using FCS express 3.0, neutrophils were gated using a manual gating strategy and .txt files were exported for analysis in Matlab 2013a. The fluorescence readouts of the selected cells did not contain zeroes or negative numbers and were, therefore, processed by an ordinary natural logarithmic transformation [47,48]. Because the amount of fluorescence emitted by different dyes varies, the ratio of the intensity of fluorescence of two dyes is not equal to the ratio of the abundance of both corresponding receptors, which warrants autoscaling of the fluorescence data to equalize the potential contribution of every surface marker to the models of reduced dimension.

Supplementary Material II:
Additional Results of the FLOOD algorithm for the LPS dataset



Supplementary Figure S1: Isolines that enclose specific percentages (50–90%) of cell scores in an unchallenged (A) and a challenged individual (B) for the Control models.

The isoline of 80% is highlighted in cyan.

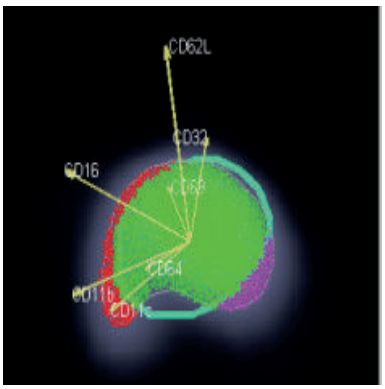
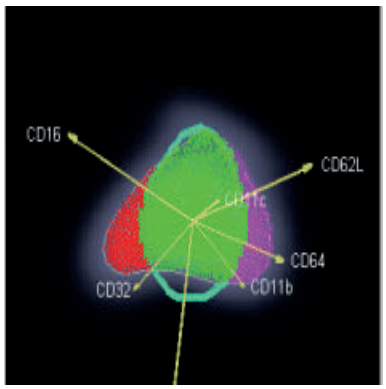
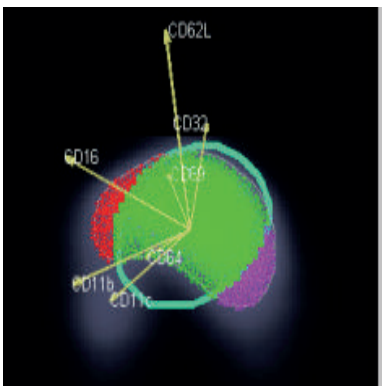
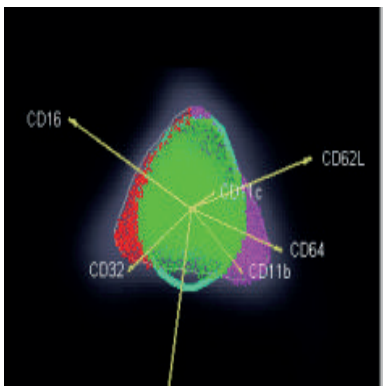
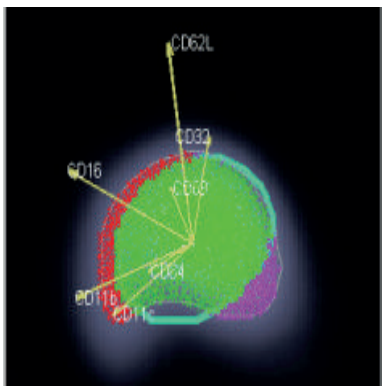
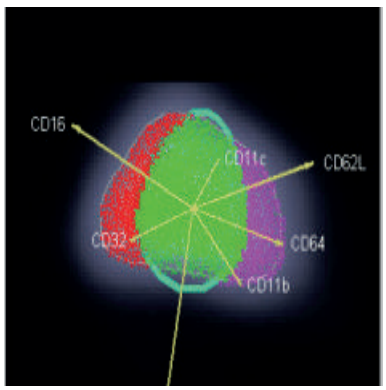


Supplementary Figure S2: Isolines constructed for the Control scores of unchallenged individual #5.

This individual exhibits variability among cells that deviates from that observed in other unchallenged individuals. The isoline of 80% is highlighted in cyan.

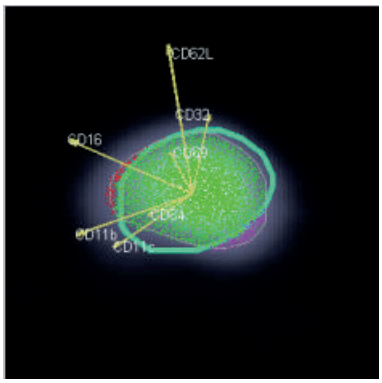
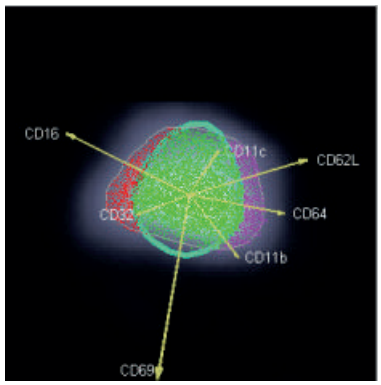
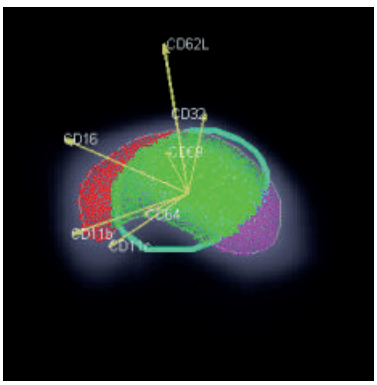
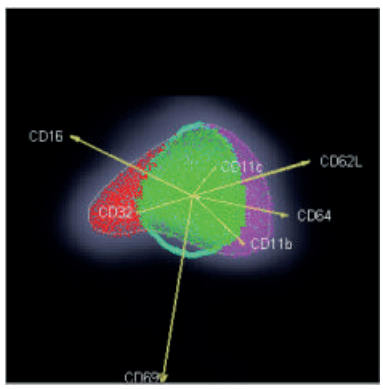
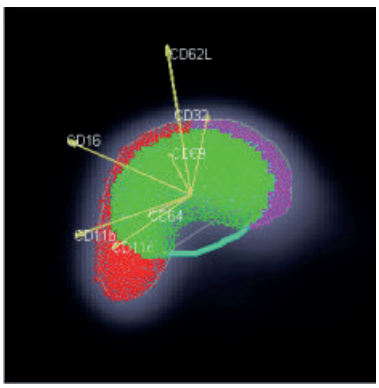
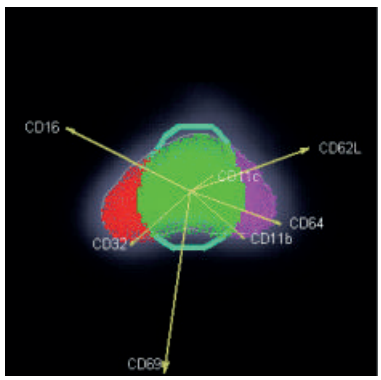
Supplementary Table S1: Gated cells for each challenged individual (number in first column), based on the Control (second column) and Response (third column) benchmarks

Gating of the scores into three distinct regions: a coloured in red and containing the cells outside the Control benchmark, but inside the 80% CI of that individual and to the left of the loading origin, b (green, enclosing the Control benchmark), and c (purple) the region outside the benchmark, inside the 80% CI of that individual and to the right of the loading origin. Yellow arrows represent the loadings of the surface markers.

#	Control model (challenge data)	Response model (challenge data)
1		
2		
3		

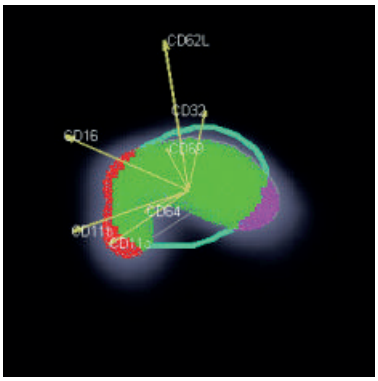
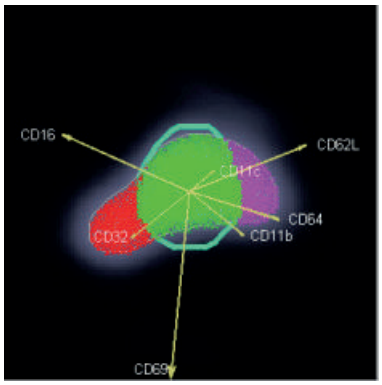
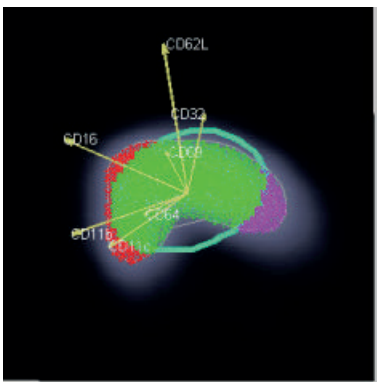
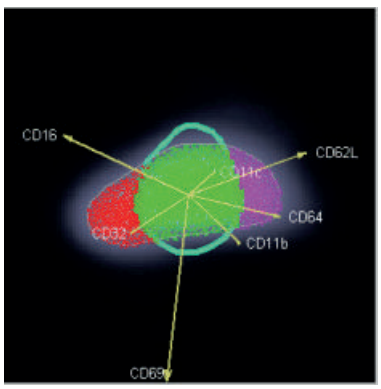
Supplementary Table S1 continues on next page.

Supplementary Table S1: ***Continued***

#	Control model (challenge data)	Response model (challenge data)
4		
5		
6		

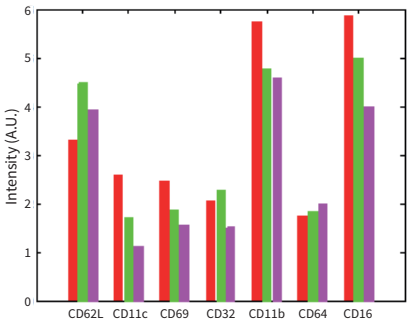
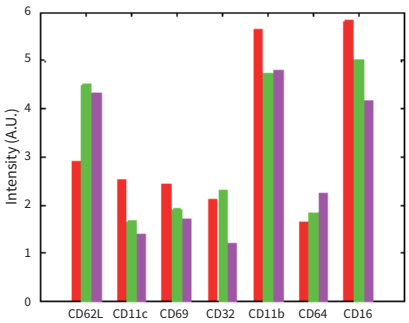
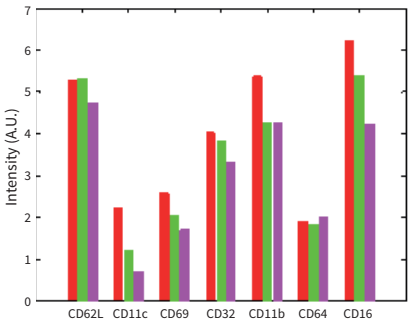
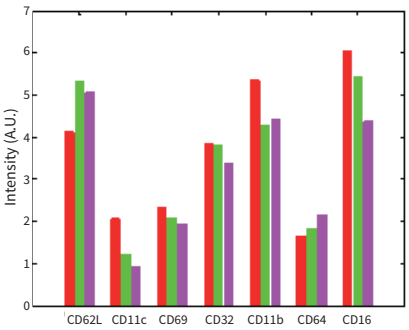
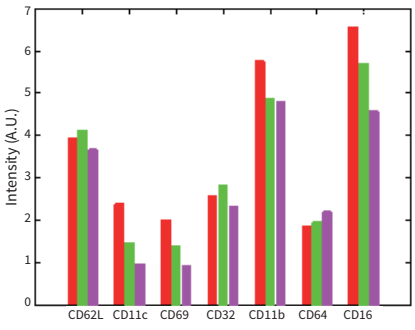
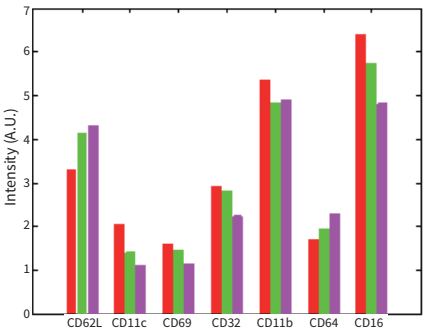
Supplementary Table S1 continues on next page.

Supplementary Table S1: ***Continued***

#	Control model (challenge data)	Response model (challenge data)
7		
8		

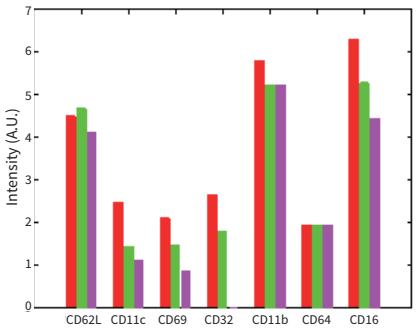
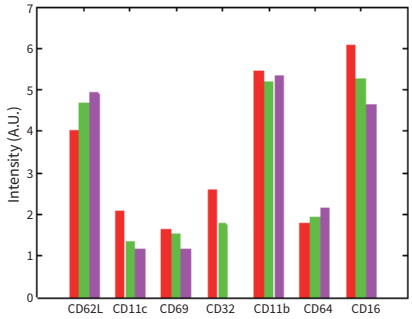
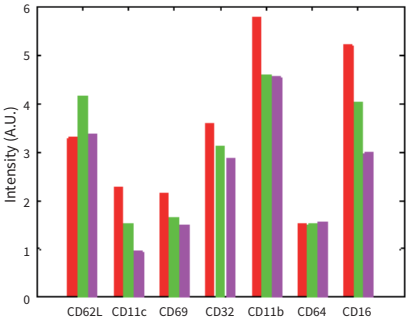
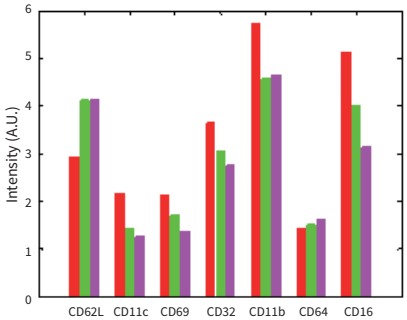
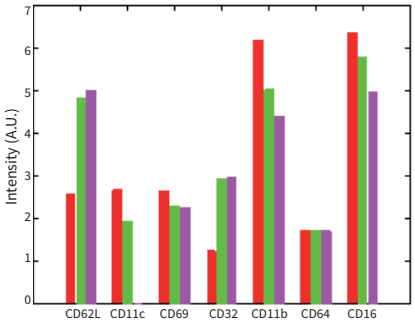
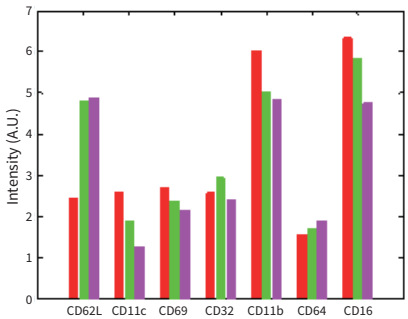
Supplementary Table S2: Median cell characteristics of the three gates for each individual

a (red, left), **b** (green, center) and **c** (purple, right) for each challenged individual. The results of individual #7 are also reported in Figure 5 of the main text.

#	Control model	Response model
1		
2		
3		

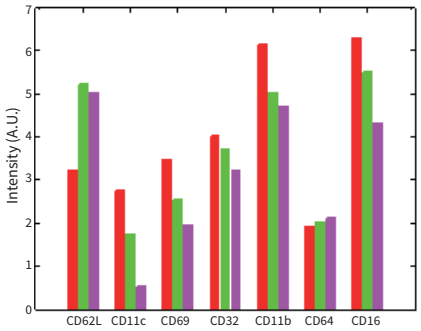
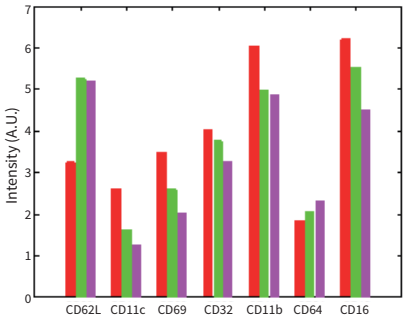
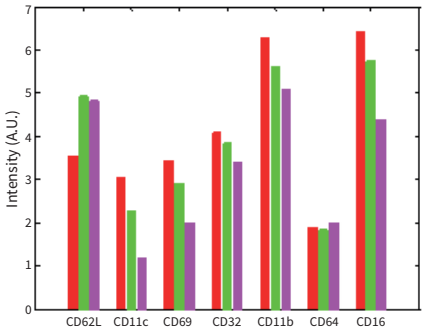
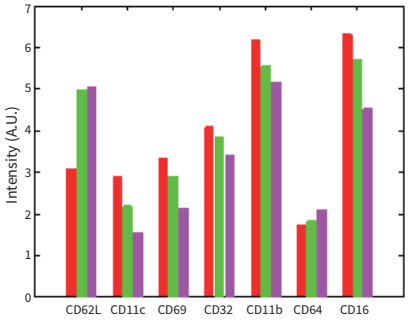
Supplementary Table S2 continues on next page.

Supplementary Table S2: *Continued*

#	Control model	Response model
4		
5		
6		

Supplementary Table S2 continues on next page.

Supplementary Table S2: *Continued*

#	Control model (challenge data)	Response model (challenge data)
7		
8		

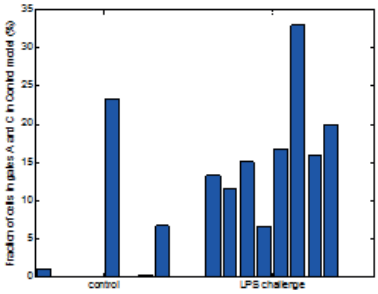
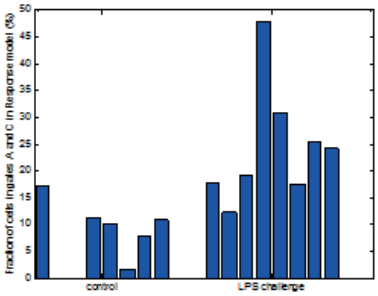
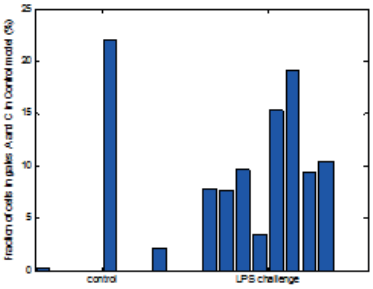
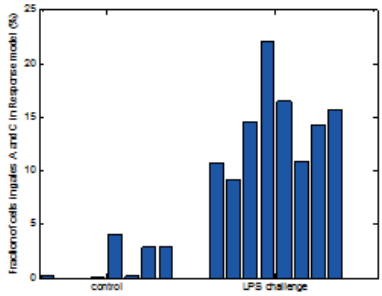
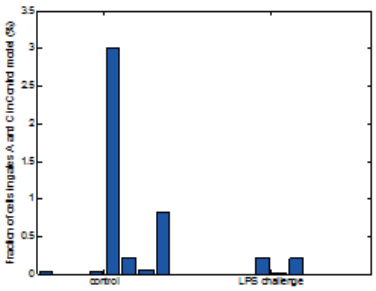
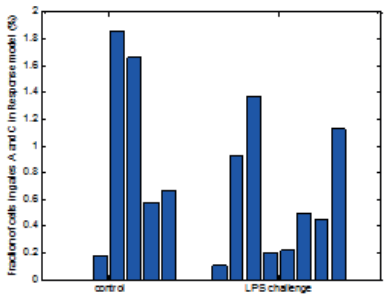
Supplementary Table S3: Percentage of cells in each gate for unchallenged and challenged individuals

Based on the distribution of scores established by cross-validation (*i.e.* projection of each individual onto the Control and Response spaces established for all other individuals). Gating of the scores into three distinct regions: ***a*** coloured in red in Table S1 and containing the cells outside the Control benchmark, but inside the 80% CI of that individual and to the left of the loading origin, ***b*** (green, enclosing the Control benchmark), and ***c*** (purple) the region outside the benchmark, inside the 80% CI of that individual and to the right of the loading origin. The remaining cells lay outside these gates and are not reported in this table. As the 80%-confidence intervals of single individuals may well be enclosed by the Control or Response scores, the percentage of cells enclosed by these benchmarks (gates ***b***) may exceed 80%. Similarly, the total percentage of cells reported per individual exceeds 80%.

		Control model			Response model		
		a	*b*	*c*	*a*	*b*	*c*
unchallenged	1	0.09	86.89	0.22	0.08	89.34	0.09
	2	0.00	87.81	0.00	0.00	86.69	0.00
	3	0.00	88.68	0.00	0.00	88.77	0.00
	4	0.00	85.48	0.00	0.02	87.05	0.00
	5	9.70	61.54	12.37	2.76	82.39	1.32
	6	0.00	87.78	0.00	0.10	88.35	0.06
	7	0.01	85.51	0.00	1.75	84.48	1.12
	8	0.41	81.80	1.74	1.86	81.30	1.08
challenged	1	5.05	76.07	2.82	7.45	70.87	6.55
	2	3.66	76.60	4.06	4.24	75.24	5.44
	3	5.55	75.20	4.14	12.75	63.71	9.85
	4	1.55	81.98	1.90	9.14	69.50	7.55
	5	8.71	68.85	6.59	10.78	63.79	10.08
	6	13.41	68.80	5.69	11.07	68.67	7.18
	7	5.52	76.21	3.95	12.87	63.66	10.59
	8	5.27	74.81	5.22	13.79	62.37	10.38

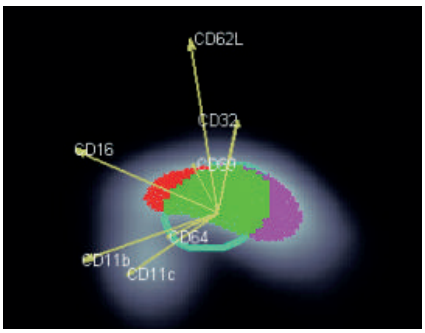
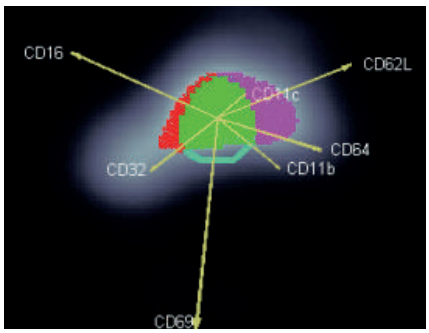
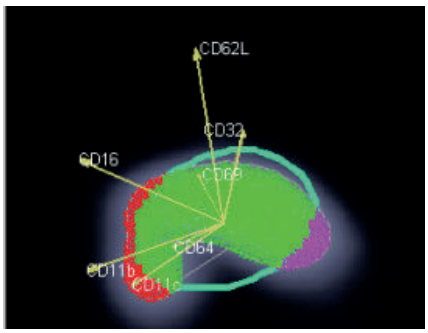
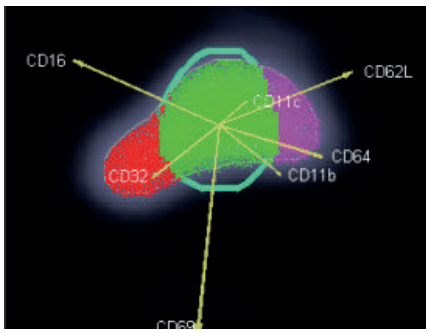
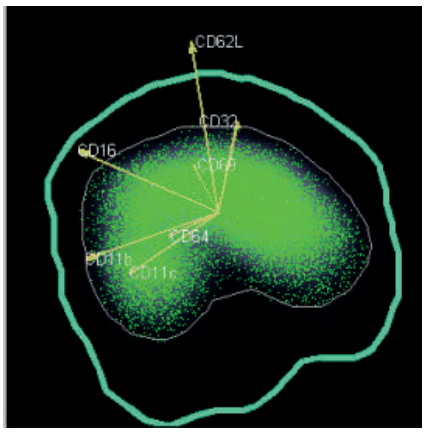
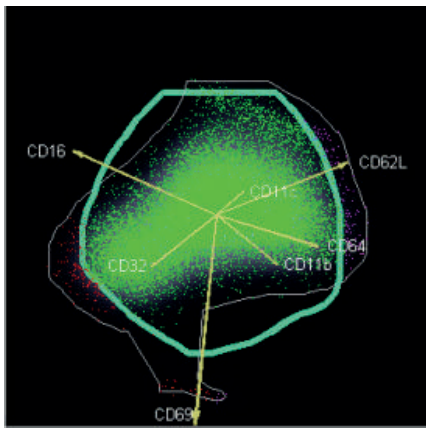
Supplementary Table S4: Sensitivity analysis of a low (50%) and high (99%) threshold for including cells in the gating for diagnosis

The graphs are analogous to the results in Figure 6 in the main text,(80%) which is also repeated in this table. A low threshold (50%) provides significant detection of an immune response in the Control model for most challenged individuals, like the 80% threshold used in throughout the chapter. Applying a high threshold (99%) does not allow distinction between unchallenged and challenged individuals.

CI	Control model	Response Model
50%		
	p=0.0067	*p*=0.0019
80%		
	p=0.0392, t-test	*p*=9.9e-7
99%		
	p=0.2282	*p*=0.9037

Supplementary Table S5: Gated cells of the challenged individual also presented in Figure 5 of the main text

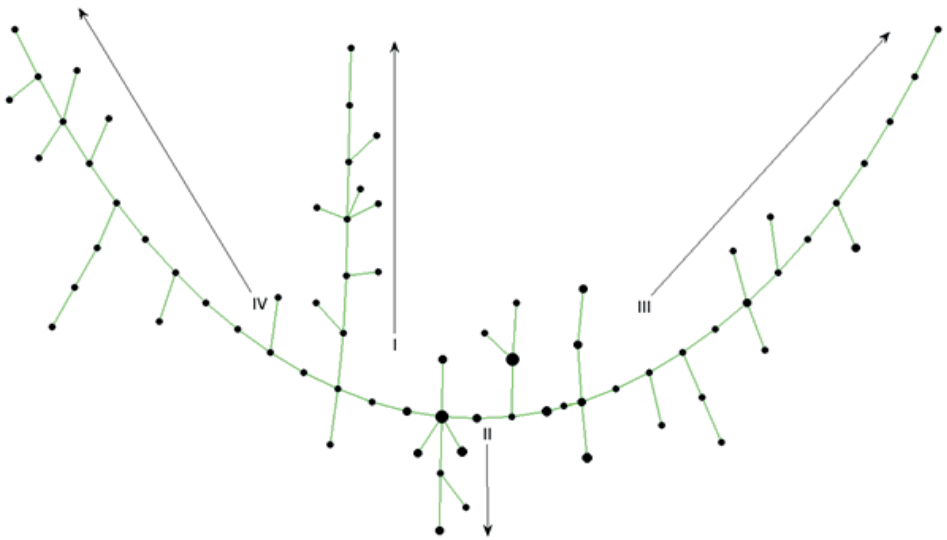
Showing a low (50%; top), intermediate (80%, middle) and a high (99%, bottom) threshold for including cells. Identical thresholds are imposed on the Control model (second column) and the Response model (third column). Although the 50% Confidence Interval threshold can be used for diagnosis (Supplementary Table S4), these results show that this diagnosis is based on only a very limited subset of the cells measured for that individual.

CI	Control model	Response Model
50%		
80%		
99%		

Supplementary Material III: Cytometry

A comparison between SPADE and FLOOD, with their results on the LPS dataset

We have conducted an analysis with SPADE on the data that was generated during the human LPS challenge study of which the details are explained in the main text. The data was pre-processed in an identical way to FLOOD, to optimally compare both results. The primary result of SPADE is a tree (**Figure S3**), in which the expression of each surface marker is color-coded for each cell [2].

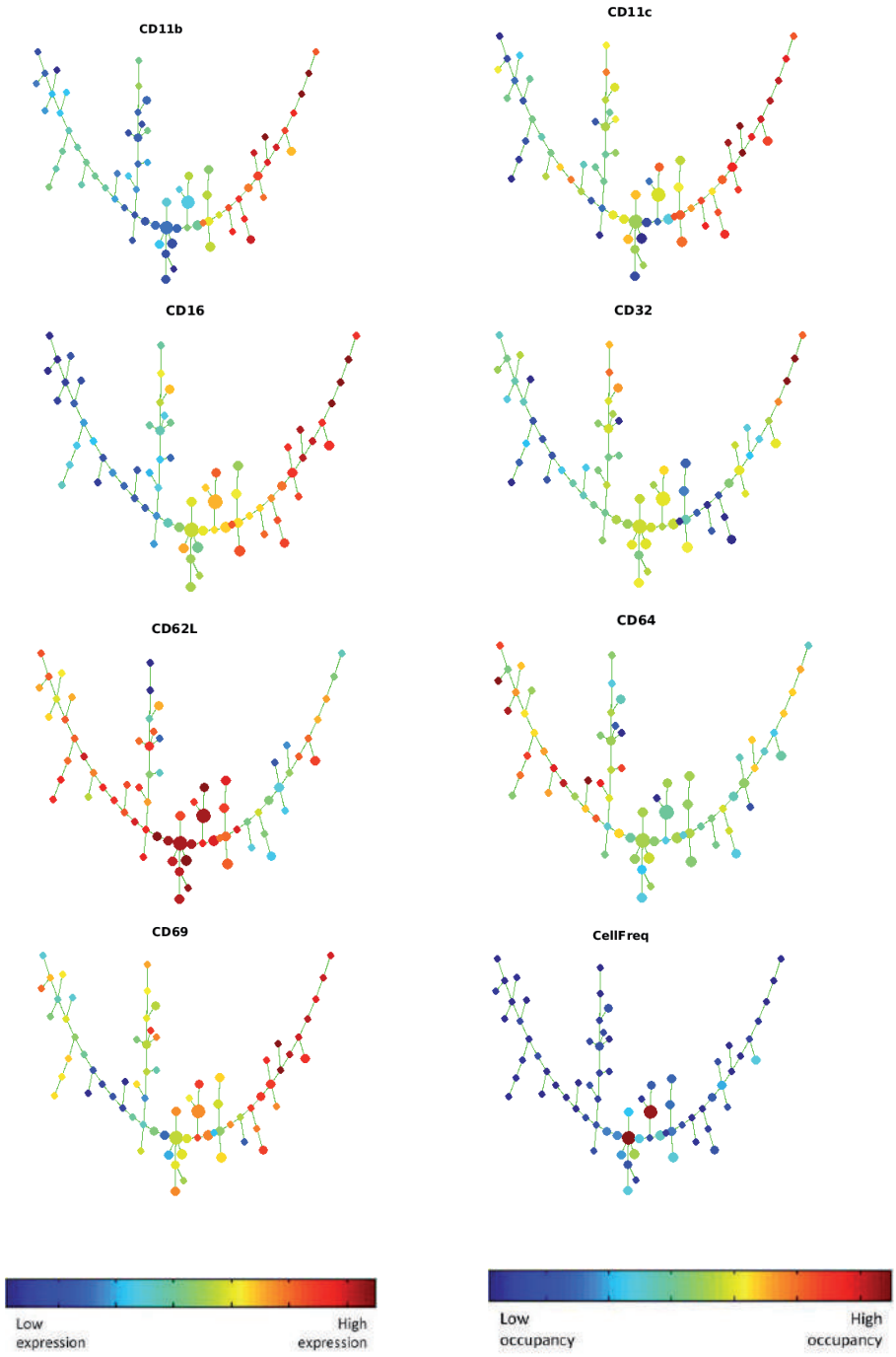


Supplementary Figure S3: SPADE tree.

Constructed from the LPS dataset, in which the most characteristic features are indicated by labels I-IV. The SPADE tree reveals a lineage of cells (branch I) that is expressing a mixture of several surface markers, as indicated by the marker-specific colouring of the SPADE trees that is provided on the next page. Another, shorter branch II contains cells that are mainly high in CD62L. The side-branch III contains cells that express great quantities of CD11c and CD11b. The left side-branch IV expresses CD62L and CD64.

Supplementary Figure S4: Average surface marker expression per node across individuals. →

The size of each node indicates the number of cells it contains, totaled across individuals: larger nodes contain more cells.

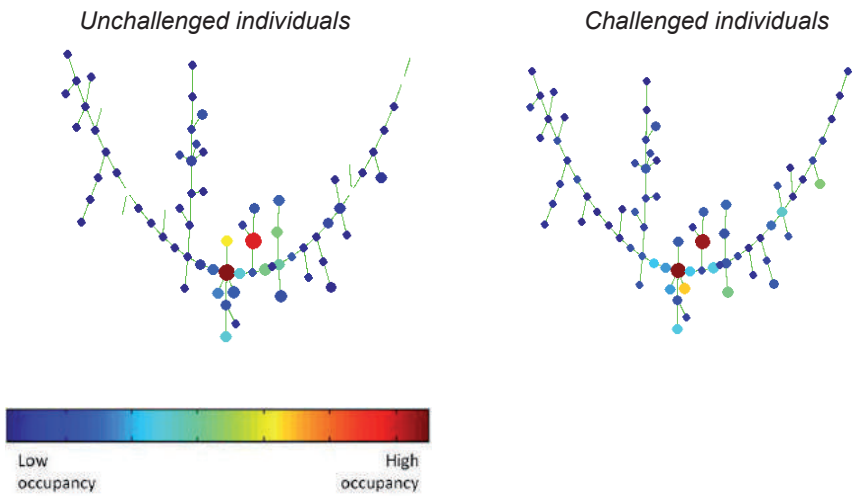


Colour bar indicating marker expression

Colour bar indicating cell frequency

Note that a comprehensive overview of the distribution of all surface markers across all nodes requires interpretation of seven SPADE figures. The two FLOOD biplots reveal correlations (that may signify biological relationships) among the surface markers that remain elusive in the SPADE trees.

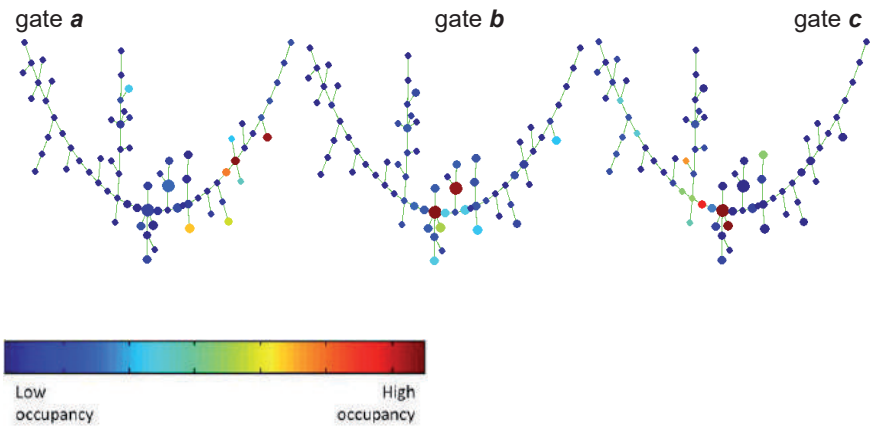
In a subsequent analysis, we have compared the number of cells that each SPADE tree node occupies. The average distribution all cells across the nodes is given below for both unchallenged and challenged individuals. Both unchallenged and challenged individuals occupy much the same nodes. Specifically branches I-IV of the tree that were identified before are sparsely and highly similarly occupied by cells, both for challenged and for unchallenged individuals. Thereby the population-specific branching constructed in the SPADE tree does not correspond to the changes in surface marker expression by all cells that LPS induces.



Supplementary Figure S5: Average distribution of cells across the clusters.

For unchallenged (left) and challenged (right) individuals. The node size again represents the occupancy of each node across all individuals.

As a final cross-method comparison we have superimposed the cells within the FLOOD gates ***a-c*** onto the SPADE tree. These results are given below and show that the cells from gate ***b*** occupy several different nodes, more to the right of the tree compared to those occupied by cells from the central gate ***a***. The cells from gate ***c*** occupy one node that is equal to those of the central gate, but also score distinctively in several nodes around that shared node. One SPADE node occupied by the central gate is not occupied by gate ***c***.



Supplementary Figure S6: Occupation of the nodes in the SPADE tree.

By cells from the three gates defined for the challenged individuals in the FLOOD response model.

The automatically gated cells identified by FLOOD can be only poorly distinguished in the SPADE tree. The branches that SPADE did identify cannot be associated to the immune response to the LPS challenge. This response could be much better revealed by FLOOD. The FLOOD model represents the relationships between all surface markers in two, rather than seven figures. It also isolates the cellular variability—and therefore the individual cells—that relate to the response in a way that can be quantified for diagnosis.

CHAPTER 3.2

Detailed analysis of the *in vivo* neutrophil response after anaerobic exercise in elite rowers

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Abstract

Rationale: *Exercise induces activation of the innate immune response in athletes. The response starts during exercise and lasts for approximately 24 hours. Athletes develop leukocytosis, and their systemic cytokine production is altered towards an inflammatory pattern. A detailed analysis of the expression of cell receptors is required to gain more insight into immune response in response to exercise. New tools to analyse multidimensional data can facilitate the analysis.*

Aim: *To determine the type and degree of the cellular response in peripheral blood of elite rowing athletes after anaerobic exercise.*

Methods: *16 healthy, non-asthmatic, rowing athletes, aged between 18 and 25 years, were included. The rowers performed an anaerobic maximum-effort test (3x1000m.) on an indoor rowing machine. Blood was withdrawn before the test (t=pre) and afterwards at time point t= 2 hours. White blood cells were isolated, stained with fluorescent antibodies CD35, TLR-4, CD62L, CD11c, CD49d, CD32, CBRM1/5, CD11b, CD64 and CD16 and measured by flow cytometry. FLOOD, a multidimensional flow cytometry data analysis method, was used to analyse the surface marker variation on leukocytes.*

Results: *A prominent rise in leukocyte count was observed in all but two rowers between the pre-exercise time-point versus the sample taken after two hours after exercise. Multidimensional analyses demonstrated that more heterogeneity in surface marker expression was present before exercise as compared to t=2h after exercise. A marked induction of neutrophilia was observed on exercise. Focusing on the 'new' neutrophils led to the finding that these cells were characterized by a 6-fold increase in $CD16^{dim}CD64^{dim}$ neutrophils that had a higher expression of CD11b, CD11c, CBRM1/5 and CD62L at t= 2h compared to pre-exercise.*

Conclusion: *Multidimensional analysis by FLOOD demonstrated that anaerobic exercise by elite rowers induces a clear systemic immune response with marked neutrophilia characterized by the appearance of a specific neutrophil phenotype in addition to an increase in 'homeostatic' neutrophils. These findings will help interpreting the changes in immune status of elite athletes during periods of intensive training.*

Introduction

The effect of exercise on the innate immune system in athletes has been subject of study because of their susceptibility to upper/lower respiratory infections [1]. The main changes in innate immune response are leukocytosis, altered receptor expression on leukocytes, and cytokine responses [2–4]. The leukocytosis generally follows a similar pattern in different studies. A representative study has been published by Kakanis *et al.* [5]. 10 male elite cyclists exercised for two hours at 90% of their ventilator threshold, blood was drawn at several time-points and analysed by a routine cell analyser. Striking is the 5-fold rise in neutrophil count in blood, which is quite similar to a response to bacterial infection or acute trauma [6,7]. The functional consequences of this leukocytosis are not well understood. Also detailed phenotypic information regarding individual neutrophils is lacking.

The innate immune response induced by exercise can have important consequences for athletes, particularly during episodes of high-intensity training. When the innate response is too extensive and/or aberrant, the response might develop into a systemic inflammatory response that is associated with direct damage of tissue, chronic inflammation such as found in the bronchial tissue of athletes with Exercise Induced Asthma (EIA) and/or impaired immune reactions to infections. Accurate prediction of a specific risk caused by tissue damage (occurrence of progenitor cells), acute inflammation (occurrence of immune-suppressive cells) and chronic inflammation (expression of priming markers on immune cells) is important for the diagnosis of exercise-associated pathology. Indications for each of these three mechanisms have been found in exercise studies of diverse types of athletes/asthma patients [8–10].

When exercise-induced musculoskeletal injury, bronchoconstriction and, on rare occasions, anaphylaxis are accompanied with a dysregulated inflammatory response the pathological response can be markedly enhanced even leading in rare cases to sudden death [11]. More insight in the 'inflammatory' response to exercise is, therefore, very important [12].

Neutrophilia can be induced by catecholamine release and is found not only after exercise but also during acute infections, sterile inflammation, and during [6,13,14] systemic inflammatory response syndrome (SIRS) [6,14,15]. SIRS can be evoked in normal volunteers by intravenous administration of Lipopolysaccharide (LPS) of E. Coli bacteria [15]. LPS triggers an innate stimulus by binding to TLR-4 receptor on target cells such as macrophages [29]. It leads to a cascade of events associated with acute inflammation such as a cytokine storm, fever and cardiovascular changes [15] . LPS is therefore used as a model to mimic the response to bacterial pathogens. Anaerobic exercise on the other hand, being part of routine training schemes, is a more physiological response without the presence of an extrinsic factor. It is, therefore, to be expected to cause much more subtle changes in the immune response compared to LPS challenge.

A multicolour antibody panel with 10 neutrophil markers was developed to study exercise-induced changes of neutrophil phenotype; TLR-4, CD35, CD16, CD32, CD64, CD62L,

CD11b, CBRM1/5, CD11c and CD49d. TLR-4, which is the receptor of LPS, and CD35 (CR1, a complement receptor/activation marker) were chosen to be able to compare neutrophils obtained during exercise-induced neutrophilia with neutrophils found in the blood after LPS-challenge [16]. The three Fcγ receptors CD16, CD32 and CD64 were included in the panel as they are generally linked to a response to bacterial infection [17]. CD62L (L-selectin) is an adhesion molecule required for neutrophil transmigration through endothelium and its expression is lowered upon neutrophil-endothelial cell interactions [18]. CD11b is the α-chain of the MAC-1 integrin (CD11b/CD18) or complement receptor 3 and has a function in leukocyte adhesion and migration [19]. In addition to this, CBRM1/5 is used because it only binds to an epitope present on the active conformation of the MAC-1 complex [20]. CD11c or alpha X is the α-chain of the p150/95 integrin (CD11c/CD18) is an integrin with a poorly understood function on neutrophils. CD11c is an important marker as it is clearly upregulated on neutrophils upon LPS challenge [6]. And finally, CD49d is the integrin alpha subunit of two integrins VLA4 (α4β1) or LPAM (α4β7) that was found to be positive on neutrophils in allergic patients [21].

This study describes a comparison in response of peripheral blood cells of elite rowers between time-point 0h (pre-exercise) and 2h after anaerobic exercise bout (peak of cellular response). FLow cytometric Orthogonal Orientation for Diagnosis (FLOOD, Chapter 3.1) was used for the data analysis of high-dimensional flow cytometry data [22] .

Methods

For this study ethical approval was obtained from the Medical Ethical Committee of the University Medical Center Utrecht, protocol nr. 13-031. It was registered at the international trial registry clinicaltrials.gov, code NCT01893762.

Subjects

The study population were healthy rowing athletes who underwent a training scheme of minimally 10 hours of training per week at rowing clubs Triton, Orca or Viking in Utrecht, The Netherlands. In order to be eligible for participation in this study, a subject had to meet all of the following criteria: Rowing for 12 months or longer, >6 times per week, aged 18-25 years, pretested maximum heart rate (by exercise test up to maximum effort), performed a sports medical examination test (required for competing on a national level by the Royal Dutch Rowing Association). Exclusion criteria were: (1) asthma, (2) current respiratory infection, sinusitis, otitis or any other sign of acute/ chronic inflammatory disease and (3) physical injury.

Before inclusion rowers were approached by their coaches to ask if they were interested in the study. If so, an independent researcher gave the subject information letter, made an appointment to answer additional questions and asked for informed consent.

Design

In this investigator driven, mono-center observational study, rowers were asked to perform a 3x1000m maximum exercise test on a rowing machine (Concept-II model D), with a 5 minute rest interval after each 1000m. Blood was obtained by venepuncture before exercise and 2 hours after exercise in a 9mL sodium-heparine tube and processed as described earlier in Chapter 3.1. Antibody staining of white blood cells was performed with a panel of fluorescently labelled antibodies (**Table 1**).

Table 1: The flow cytometry panel

FL1 FITC	FL2 PE	FL3 ECD	FL4 PeCy5.5	FL5 PeCy7	FL6 A647/ APC	FL7 A700	FL8 A750	Fl9 PB	FL10 KO
CD35	TLR-4	CD62L	CD11c	CD49d	CD32	CBRM1/5	CD11b	CD64	CD16

Methods of measurement & Analysis

The same method of flow cytometry measurements was used as described in Chapter 3.1 [22]. In short, samples were analysed using a BC Gallios™ flow cytometer (Beckman Coulter, Brea, California). FCM data was extracted using FCS express 3.0. FLOOD data analysis (algorithm in Matlab2013a) was used as described in Chapter 3.1, with time-point 2h after exercise used for the construction of the *Control* space and time-point 0h for the *Response* space.

All results of the FLOOD analysis were obtained by a leave-one-out cross-validation protocol; the data of the plotted individual (both pre- and two-hours-after exercise) were excluded before generation of the FLOOD model to exclude influence of the individual's data on the model that could change the output.

Results

Sixteen rowers agreed to participate, signed informed consent and performed a 3x1000 meter maximum effort test (See **Table 2** for the base-line characteristics of the participating rowers).

FLOOD–analysis

In order to highlight the difference between the homeostasis (pre-exercise) and the state in which a leukocytosis is observed, the two-hours after exercise data point was taken as reference in the comparison. **Table 3** presents the results of projection of the data in the Control space (for construction of Control and Response spaces see Chapter 3.1).

Table 2: Base–line characteristics

male (n=14), female (n=2)		
	Average	SD
Age (years)	20,6	1,9
Height (cm)	187	6,3
Body weight(kg)	78.2	8,3
BMI	22,2	1,5
Training (hours/week)	13,0	2,1
Average power (watt/kg)	4,4	0,4

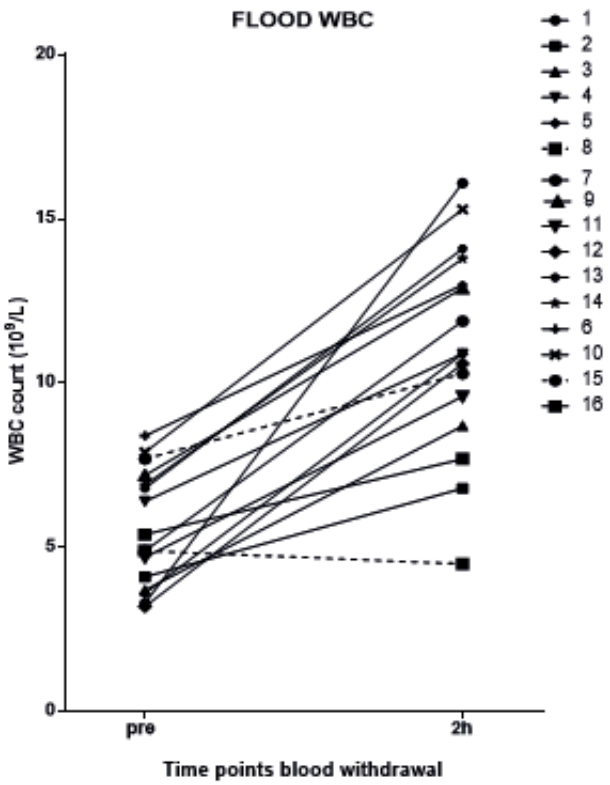


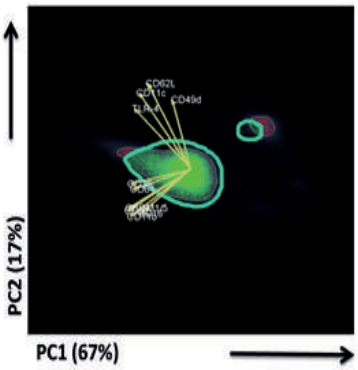
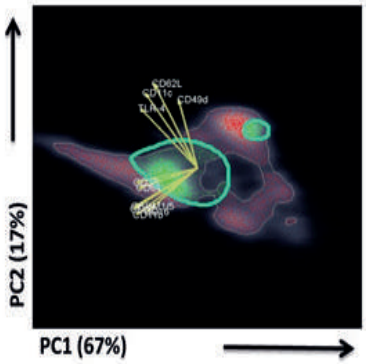
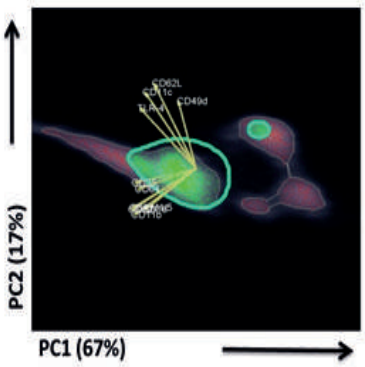
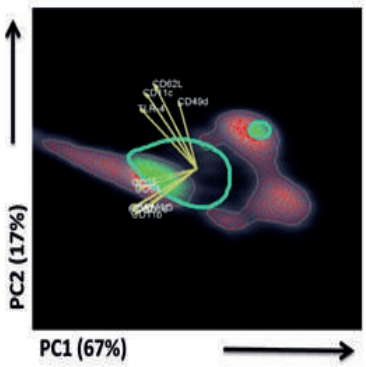
Figure 1: White blood–cell–counts (WBC) per individual, pre–exercise and 2h after exercise.

The two dotted lines belonging to subject 8 and 15 have the lowest increase of WBC in response to exercise compared with the others.

In the graphs in **Table 3**, showing the results for Rower 10, as a good representative of all except two individuals, and for Rower 8, as an example of the two deviant individuals (Rowers 8 and 15);

Table 3: Control model

Rower #10's two graphs (top row) are a representative example of the change in cell distributions observed in all rowers on exercise, except for #8 and #15. These latter two both show a very similar cell profile between post- and pre-exercise as shown by #8 (bottom row).

Number	Two-hours-after exercise	Pre exercise
Rower 10 (similar response in 14 out of 16 rowers)		
Rower 8 (similar response as Rower 15)		

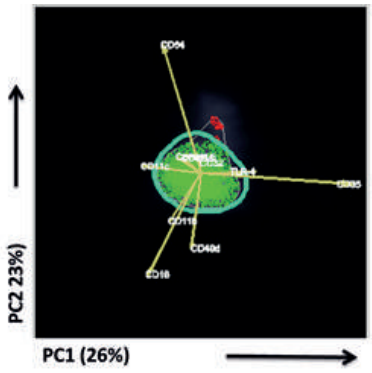
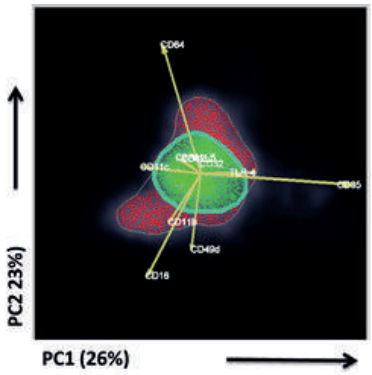
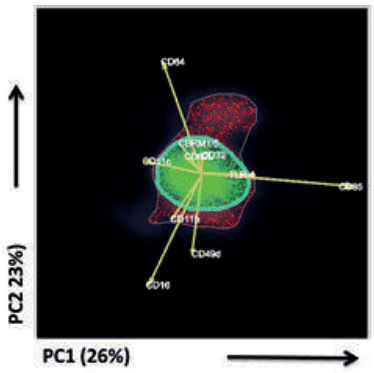
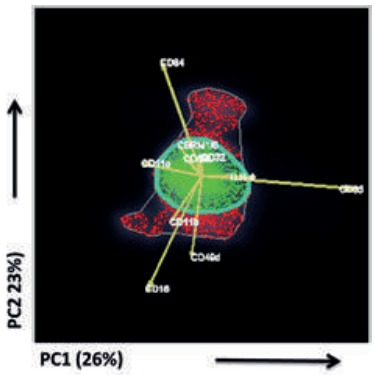
- the first PC is represented by the horizontal axis describing 67% of the 2h-after-exercise time point;
- the second PC is represented by the vertical axis describing 17% of the 2h-after-exercise time point;
- the cyan line indicates the Control benchmark, *i.e.* the 80% CI of cells taken from all individuals 2h after exercise;
- the grey line indicates the 80% CI of cells of the individual 2h after exercise (left-hand column) or pre-exercise (right-hand column);
- the yellow arrows are the loadings in the Control space;
- cells in gate ***a***, green cells, are enclosed by both the Control benchmark and the 80% CI of the individual (separately in grey).

The Control benchmark consists of a main cluster of cells that is egg shaped and a smaller 'island' to the upper right. The egg shaped islands are in fact neutrophils. The smaller island corresponds with the second most abundant population of cells in peripheral blood: lymphocytes. There are two responses in the group of rowers; 14 out of 16 rowers show a very similar response with a rise in neutrophils and the seeming disappearance of the other cell clusters at the right and left of the Control benchmark. The two other rowers (#8, #15) show a very comparable profile between the two time-points, with only a slight change in neutrophil characteristics and persistent clusters of lymphocytes and monocytes.

The projections in the Response space were also obtained according to the leave-one-out method. In the graphs in **Table 4**, showing the results for Rower 10, as a good representative

Table 4: Response model

Rower 10 (top row) is a representative example for all rowers except for rower 8 (bottom row) and rower 15. At t=pre cells in red plot outside the Control benchmark and at t=2h almost all cells plot within the benchmark. In rowers 8 and 15 the heterogeneity of t=pre persists at t=2h after exercise.

Number	Two-hours-after exercise	Pre exercise
Rower 10 (similar response in 14 out of 16 rowers)		
Response in #8 (similar response in #15)		

of all except two individuals, and Rower 8, as an example of the two discordant individuals (#8 and #15);

- the first PC is represented by the horizontal axis describing 26% of the variance of the pre-exercise time point;
- the second PC is represented by the vertical axis describing 23% of the variance of the of the pre-exercise time point;
- the cyan line indicates the Response benchmark, *i.e.* the 80% CI of cells taken from all individuals except the one left out, pre- exercise, identical in both columns;
- the grey line indicates the 80% CI of cells of the individual, 2h after exercise (left-hand column) or pre-exercise (right-hand column);
- the yellow arrows are the loadings in the Response space;
- cells in gate ***a***, green cells, are enclosed by both the Response benchmark and the 80% CI of the individual;
- cells in gate ***b***, red cells, plot outside the Response benchmark but inside the 80% CI of the individual.

Notably, the cells taken at t= 2h plot inside the Response benchmark. The cells taken from individuals before exercise partly plot outside the benchmark, often producing a projection that has two nodes or sub-clusters. This is attributed to the greater heterogeneity of the cell population before exercise as compared to t=2h after exercise, when neutrophilia has evidently occurred.

A more quantitative comparison of the observations is given in **Figure 2** and **Table S1** (Supplementary material). Combining the results of these models **Figure 2** shows the sum of the fractions of cells in the gates ***b*** in both models for each of the individuals, comparing 2h after exercise and before exercise. The figure also clearly shows that there are two strong deviations from the observation that after exercise a small fraction of cells plot in gate ***b***: these deviations occur in rowers #8 and #15. These individuals also have the lowest increase in White-Blood-cell Count two hours after exercise (see also **Figure 1**). This is interpreted as an absence of response to the exercise. **Table S1** confirms that cell populations taken from individuals before exercise have a higher fraction projecting inside gate b. This is evident in both the Control model and the Response model, confirming the observation in **Table 3** that the 2-h-after-exercise cell population is more homogeneous compared with the pre-exercise cell population.

Both the Control model and Response model seem to indicate that cell populations disappear upon exercise. To establish whether or not this phenomenon actually occurs all the cells were plotted in a combined density and dot plot in which single cells still are visible and no cells are lost in the periphery due to smoothing. **Figure 3** shows the result for Rower 10 as a representative example, comparing the t=pre time point (left) with 2h after exercise (right). At the t=2h time point the neutrophil population is visible just to the left of the origin of the vectors and the other populations are still visible to the right of the axis. Yet,

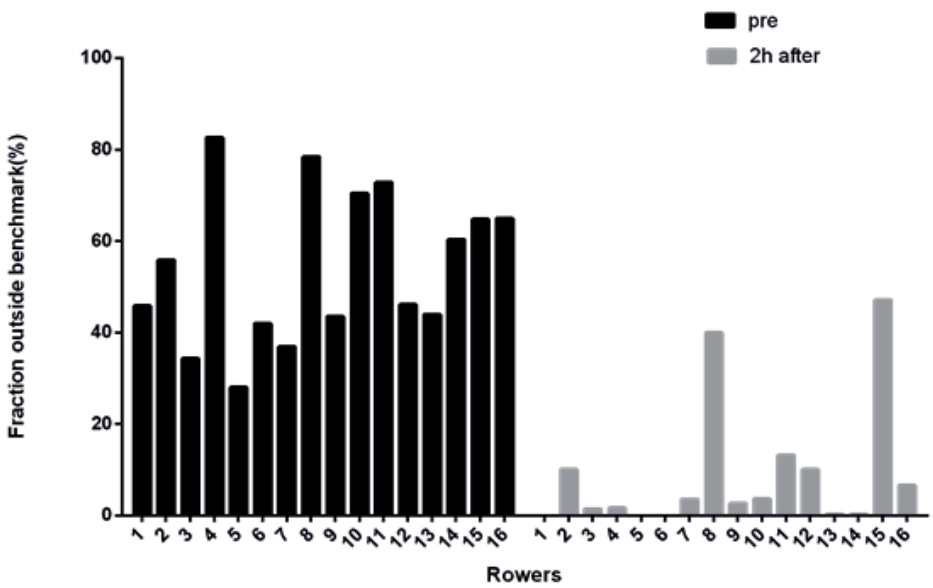


Figure 2: Fraction of cells outside the Response benchmark, pre-exercise (black) and 2h after exercise (grey).

the relative concentration of these populations compared to the neutrophils is far lower than at t=pre. The t=pre graph shows cells with higher variation in receptor expression and all cell populations are more clearly visible just right from origin of the vectors compared to t=2h.

This observation prompted a second analysis with a focus on neutrophils, which were gated again based on CD16 and CD62L expression and subsequently subjected to FLOOD analysis. The gating strategy is visualized in Chapter 3.1.

Neutrophils

Neutrophils at t=pre and t=2h were compared in two analyses: t=pre as control and t=2h as response and vice versa. The model with t=pre as control and t=2h as response was chosen based on an increase in receptor variation of neutrophils after exercise.

First of all, without looking at receptor variation and just at cell counts, the neutrophil count rises from ~3.5 million neutrophils/mL at time point t=pre to ~9 million neutrophils/mL 2h after exercise (**Figure 4**). Although the neutrophil count rises extensively, the receptor variation seems to change only slightly. This is best visualized in **Figure 5** with neutrophils of Rower 10 at time point 2h in the Response model. The pattern observed in neutrophils of Rower 10 is consistent for all rowers, including Rowers 8 and 15. The Response benchmark (80% CI, in red) 'floods' the Control benchmark (80% CI, in green) at the right-hand side of the origin of the loading vectors. Markers CD16 and CD64 have high loadings pointing to

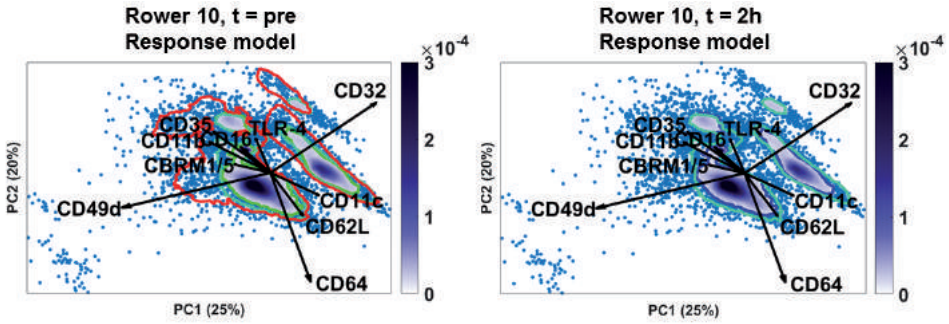
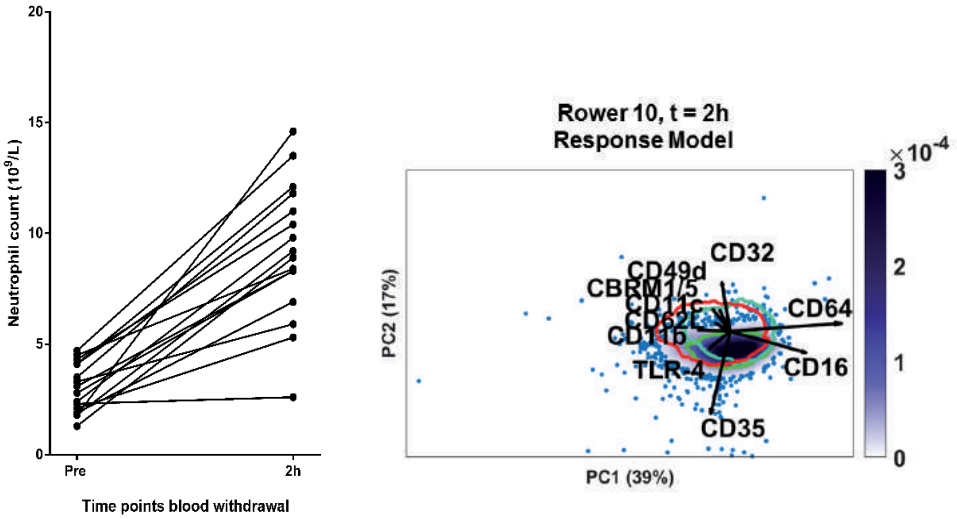


Figure 3: Response model as in Table D.

Rower 10 is a representative example for most rowers. The heterogeneity of the cell populations is high at t=pre and low at t=2h.The red line indicates the average Response benchmark for all rowers. The green line is the 80% CI of all rowers at t=2h. And the cyan line represents the 80% CI of Rower 10 within each graph. The increase in cellular density in the neutrophil population results in an overall more homogeneous peripheral blood profile at t=2h. The neutrophil population mobilised in exercise is less heterogeneous than the population present before exercise.



Left: Figure 4. Neutrophil counts at t=pre and t=2h. Right: Figure 5. Rower 10 as a representative example of all rowers.

The cyan line is the Control benchmark of the combined FCM data before exercise challenge, the red line is the 80% CI Response benchmark of all rowers and the green line is the 80% CI of rower 10 in the Response space. There is a shift towards most activation markers and away from CD64 and CD16, indicating a higher expression of CD49d, CBRM1/5, CD11b, CD11c and CD62L versus a lower expression of CD64 and CD16

the left-hand side and all other activation markers point more to the right. The shift of cells is mainly visible in PC1. In PC2 CD35 has a high loading. Yet, the shift of cells is more in the horizontal direction of PC1 with CD16 and CD64 as important loadings versus CBRM1/5, CD11b, CD11c and CD62L pointing in the opposite direction.

The FLOOD data was loaded into FCS express to determine the percentage of neutrophils 'flooding' the 80% CI Control benchmark at the right side after exercise and to scrutinize single parameter expression of this group of neutrophils by means of back-gating. **Figure 6A** shows that the Control gate contains 91% of the neutrophils of Rower 10 before exercise, while the red 'Response gate' contains a mere 7%. **Figure 6B**, on the other hand, shows that upon exercise (t=2h) 80% of the neutrophils of this rower are found in the Control gate while the percentage of cells in the Response gate has increased to 20%. Overall, analyzing all rowers before exercise, 90% (sd 5%) of neutrophils plot within the Control gate and 8% (sd 5%) plot in the "Response gate" (in case of t=pre this is not an actual response gate). After exercise 80% (sd 7%) of cells still plot within the Control gate and 20%(sd 6%) in the Response gate. Notably, as mentioned above, the total neutrophil count rises from around 3.5 million cells/mL to around 9 million cells/mL. All rowers, Rowers 8 and 15 included, were taken into account in this average overall rise. Thus, neutrophils present in the Response gate increase from a peripheral blood concentration of ~0.3 million cells/mL at time point t=pre to ~1.8 million cells/mL two hours after anaerobic exercise, a 6-fold increase.

Figure 7 shows the marker expression profiles of the 'exercise-associated' neutrophils of rower 10, *i.e.* those in the Response model obtained at t=2h with the Control gate in black and the Response gate in red. The fluorescence intensity plots reveal the subtle differences between the two time points, the black line representing the intensity at t = pre and the red line the intensity at t = 2h. CD64 expression and CD16 expression on neutrophils appear to be lower at t = 2h. This is observed for all rowers, and supports the observation in **Figure 5** where the loadings of CD16 and CD64 point into the opposite direction (left) of the population of neutrophils "flooding" the 80% CI of the Control benchmark (right). Other activation markers pointing into the right-hand direction in **Figure 5** are higher in the 2h time point with the highest increase in CD11b, CBRM1/5, CD11c and CD62L expression. Other markers as CD35, TLR-4, CD32 and CD49d also show a mild increase in the exercise-associated neutrophils. These subtle changes are not visible at an individual marker level and become only visible after performing FLOOD analysis. This is best visible at an individual marker level comparing the Control and Response gate in the Control model and in the Response model separately (**Figure S1**, supplementary material) and is identified only after multidimensional FLOOD analysis.

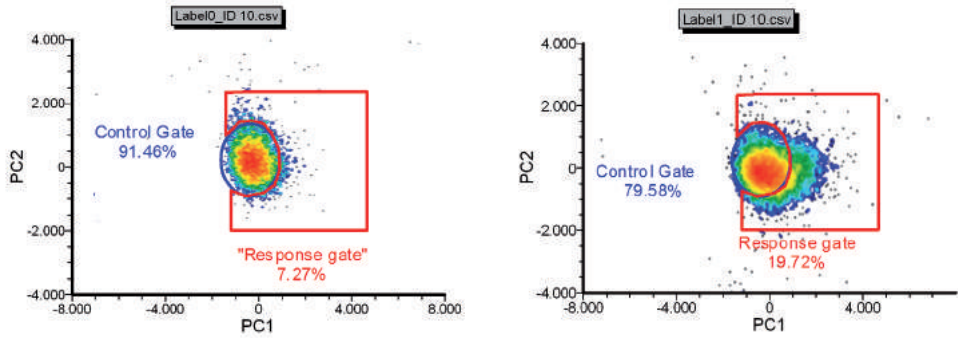


Figure 6: Manual back–gating strategy in FCS–express.

With as an example rower 10 **(A) before** and **(B) after exercise**. Neutrophils have the same shift towards the right as in Figure 5. The population flooding the Control benchmark at the right-hand side is gated in the Response gate at the right side of the graph. The blue Control gate was constructed by visual transfer of the Control benchmark at t=pre (80% CI in FLOOD) onto the representation in FCS Express.

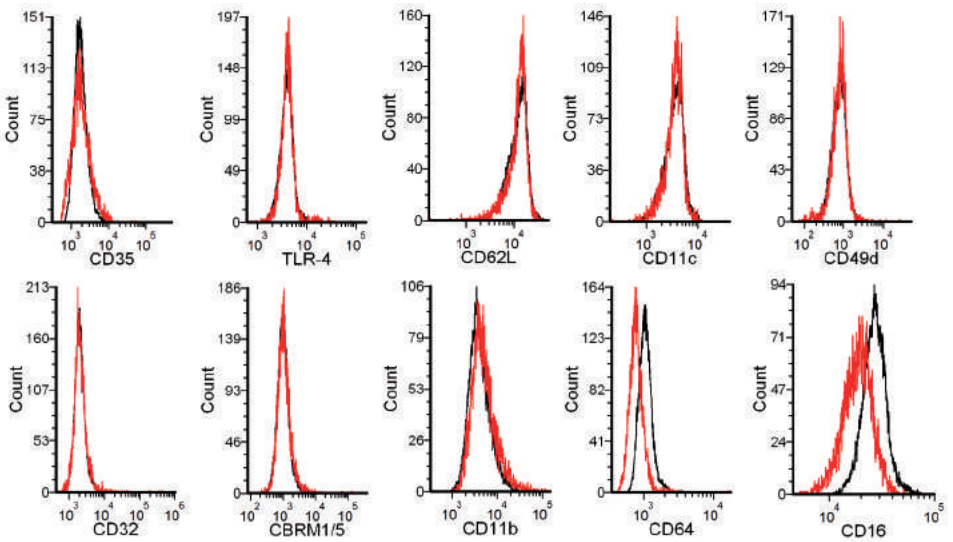


Figure 7: Fluorescence intensity in histograms per parameter.

On the vertical axis normalized cell counts are plotted and on the horizontal axis fluorescence intensity for a specific marker using a logarithmic scale. The population in the Control gate is plotted in black and in red the population in the Response gate (Figure 6). Again these are gated neutrophils from Rower 10 in the Response space.

Discussion

FLOOD analysis of MFC data from whole blood visualized that anaerobic exercise by elite rowers is accompanied by a leukocytosis at two hours post-exercise with an overall more homogeneous peripheral blood cell profile. The homogeneity of peripheral blood cells is mainly caused by neutrophilia. Subsequent FLOOD analysis on gated neutrophils indicated an increase of $CD16^{dim}$ and $CD64^{dim}$ neutrophils compared to neutrophils present before exercise. These $CD16^{dim}CD64^{dim}$ 'exercise-associated' neutrophils have an overall higher expression of CBRM1/5, CD11b, CD11c and CD62L compared to the pre-exercise neutrophils. In percentage of total neutrophils the 'exercise-associated' neutrophils increase from 8% to 20%. Corrected for an increase in total neutrophil count, this type of cell goes from $0.3x10^9$/L to $1.8x10^9$/L, which is a 6-fold increase.

The presence of neutrophils with an overall lower CD16 expression after intense exercise has been described by Peake *et al.* [23]. In this study a lower expression of CD11b was described in addition to lower CD16 expression after both moderate and intense exercise on a treadmill. In contrast, our study CD11b is more highly expressed after exercise as indicated by the loading of CD11b in **Figure 5** and a very subtle difference towards higher expression in the fluorescence intensity histogram in **Figure 7**. Eeden *et al.* also described an upregulation of CD11b on neutrophils 30min after acute maximal exercise to exhaustion on a cycle ergometer [24]. Beside the use of FLOOD analysis for interpretation of FCM data, another factor that might contribute to variation in findings is difference in stimulus. In the study of Eeden *et al.* athletes performed a more comparable anaerobic exercise test which lasted minutes while in the study of Peake *et al.* they stayed below the anaerobic threshold in an exercise test of 60 minutes. A second explanation of the varying results is a difference in time points of blood withdrawal after exercise which was at 30min (Peake *et al.*), 1h (Eeden *et al.*) and 2h after exercise (this study). In summary, the lower overall expression of CD16 in the neutrophil population after exercise has been observed earlier. The expression of CD11b, on the other hand, is higher in the exercise-associated neutrophils in our study and in the study of Eeden *et al.*

The exact phenotype of 'exercise-associated' neutrophils has not been described earlier with $CD16^{dim}CD64^{dim}$ expression and a higher expression of CBRM1/5, CD11b, CD11c and CD62L. It is challenging to relate the findings in our study to other studies due to the use of FLOOD analysis and the high number of markers in one panel. The increased amount of fluorescent markers that can be measured simultaneously on one cell led to the development of multiple new analysis methods of FCM data [25–27]. FLOOD is one of these novel analysis methods and facilitates the identification of 'exercise-associated' neutrophils by grouping them together and by identifying the difference in surface marker expression profile compared to the pre-exercise state. The use of benchmarks makes it possible to precisely compare a pre-exercise cell profile to a post-exercise cell profile. This is a unique approach that can only broadly relate to conventional single marker expression profiles on cell populations.

The implication of an 'exercise-induced' change in neutrophil phenotype is unclear. However, the data can be compared to the results of neutrophil analysis after LPS challenge. The three phenotypes that appear after LPS challenge in humans are characterized by $CD16^{dim}CD62L^{bright}$ cells with a banded nuclear phenotype, $CD16^{bright}CD62L^{bright}$ cells with a normal nuclear phenotype and $CD16^{bright}CD62L^{dim}$ cells with on average more nuclear segments [6]. Based on the receptor characteristics 'exercise-induced' neutrophils show similarities to the banded neutrophils detected after LPS challenge which are CD16dimCD62Lbright. On the other hand, the phenotype of cells obtained after exercise is different, this is illustrated by CD11b expression. The expression of CD11b is lower on the banded population after LPS challenge compared to mature neutrophils, but slightly higher in the 'exercise-associated' neutrophils compared to neutrophils before exercise. The function of young/banded neutrophils is yet unclear. However, unpublished results by P. Leliefeld (Koenderman group) indicate that these cells are superior in killing of bacteria compared to the other neutrophil phenotypes. Little is known regarding the exercise-induced neutrophils that are within the Response benchmark. Therefore, it is important to sort the different populations of cells after exercise and study their functionality in terms of both killing micro-organisms as well as immune regulation.

The difference in immune response in rowers can result in differences in cell counts and in expression of surface markers on cells. Two rowers did not respond to the anaerobic challenge by means of a leukocytosis. These same rowers had almost no change in variation of surface marker expression on all leukocytes despite the exercise challenge (**Table 3**). An explanation for this difference could not be related to their performance as both rowers delivered adequate power compared to the others and followed a similar training scheme. After analysing the neutrophils of these rowers (#8 and #15), there was a similar pattern in receptor expression upon exercise compared to the other rowers. Interestingly the leukocytosis was absent while the response in receptor variation did occur in the neutrophils. The rise in neutrophil count and the response in receptor variation were discordant in these two rowers compared to the others. Overtraining might be an explanation for the discordant response in this group of elite athletes following high-intensity training schemes, although there is no current evidence for alterations in immuno-phenotypes in overtraining syndrome [28]. Unfortunately our study was not designed to correlate cellular characteristics with parameters of overtraining.

In summary, anaerobic rowing exercise induces a leukocytosis characterized by a more homogeneous surface marker profile compared to a pre-exercise state. Neutrophils are the main cell type responsible for the leukocytosis and detailed analysis of neutrophils by FLOOD analysis shows there is a change in receptor variation towards lower expression of CD16 and CD64 and higher CD11c, CD11b, CBRM1/5 and CD62L expression. FLOOD analysis was required to identify and characterize exercise-associated neutrophils that would not have been identified by conventional biplots of MFC data. As visualised by FLOOD the overall response to exercise is very consistent among rowers, except for two rowers who did not develop a leukocytosis upon exercise but did have a neutrophil response by means of

receptor expression similar to the other rowers. The function and role of exercise-associated neutrophils is still unclear, future experiments including functional assays with sorted cells identified by FLOOD analysis will provide more insight in the innate immune response to exercise.

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Supplementary Material

Supplementary Table S1: Quantitative comparison of the number of cells

In gates ***a*** (inside Control benchmark) and ***b*** (outside Control benchmark) per individual for both the Control and Response model both 2h after exercise (top) and before exercise (bottom).

2h after	Control model		Response model	
	gate *b*	gate *a*	gate *b*	gate *a*
Individual	(%)	(%)	(%)	(%)
1	0.0	84.2	0.0	83.0
2	10.1	73.5	0.0	82.6
3	0.0	83.2	1.4	82.4
4	1.6	81.1	0.0	82.9
5	0.0	83.0	0.0	82.9
6	0.0	83.0	0.0	82.9
7	3.5	80.0	0.0	82.4
8	25.7	58.2	14.2	67.7
9	1.5	80.8	1.1	80.5
10	2.9	79.3	0.7	80.9
11	7.3	75.7	5.8	76.3
12	8.5	75.4	1.5	81.0
13	0.3	83.2	0.0	82.8
14	0.2	82.6	0.0	84.7
15	32.7	49.6	14.4	66.9
16	6.5	76.7	0.0	83.3

Supplementary Table S1 continues on next page.

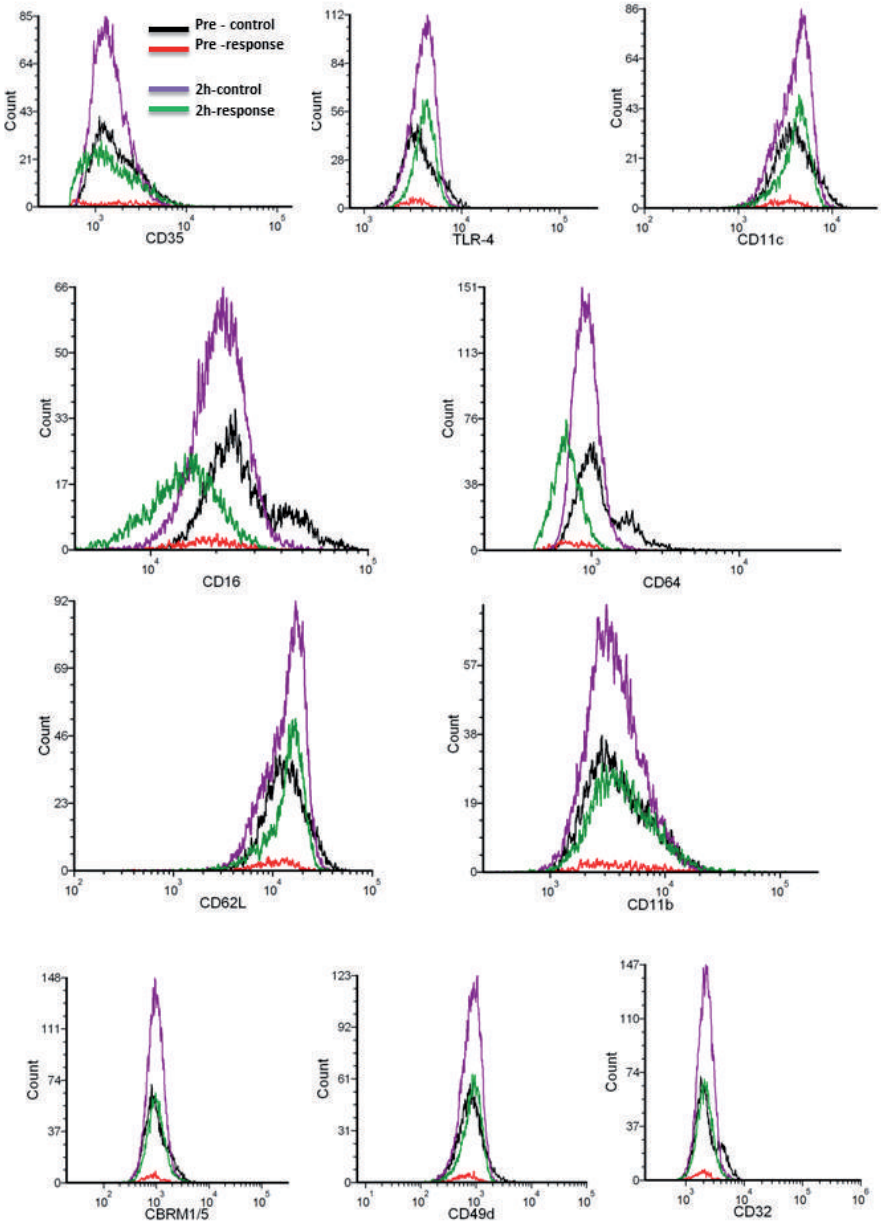
Supplementary Table S1: ***Continued***

2h after	Control model		Response model	
	gate *b*	gate *a*	gate *b*	gate *a*
Individual	(%)	(%)	(%)	(%)
1	34.0	49.7	11.8	70.1
2	42.6	41.1	13.2	68.6
3	23.8	60.5	10.5	71.3
4	61.6	22.2	21.0	61.0
5	19.0	64.4	9.0	72.8
6	21.1	62.4	20.8	61.2
7	24.7	58.4	12.2	70.1
8	53.3	31.2	25.1	56.9
9	29.9	52.6	13.6	68.4
10	46.0	37.5	24.4	57.4
11	32.7	50.6	40.1	43.0
12	29.0	54.2	17.1	65.6
13	35.0	48.2	8.9	72.8
14	48.3	35.2	12.0	69.8
15	45.1	37.8	19.6	62.6
16	58.4	24.9	6.5	75.3

Supplementary Table S2: Percentages of cells in the Control gate and Response gate before and after exercise

The gates were set manually transferring the FLOOD Control benchmark to the FACS Express gate. (Figure 6).

	t=pre-exercise		t =2h after exercise	
Rower	Control gate (%)	Response gate	Control gate	Response gate
1	90.5	7.0	68.9	29.1
2	87.8	11.5	84.9	15.4
3	82.0	16.5	85.4	14.8
4	83.8	16.0	86.9	13.1
5	96.7	2.9	63.7	36.7
6	86.1	13.0	79.8	19.7
7	87.2	12.4	76.3	21.8
8	97.1	2.3	80.6	20.2
9	96.0	2.9	85.6	15.1
10	91.5	7.3	79.6	19.8
11	93.6	4.9	76.1	24.2
12	90.9	5.0	82.9	17.1
13	85.5	15.1	85.7	14.3
14	93.1	6.7	73.4	25.7
15	92.2	7.7	77.6	22.2
16	92.4	7.0	86.3	15.0
Average	**90.7 (sd 5%)**	**8.4 (sd 5%)**	**80.1 (sd 7%)**	**19.8 (sd 6%)**



Supplementary Figure S1: Gated neutrophils of Rower 1 from the FLOOD Response Model from t=pre and t=2h and for each time–point a Control gate and a Response gate.

On the vertical axis normalized cell counts are plotted and on the horizontal axis fluorescence intensity for a specific marker using a logarithmic scale. It resulted in four histograms for each of the labels. In **Black**: Control gate for t=pre, **Red**: Response gate for t=pre, **Purple**: Control gate for t=2h and **Green**: Response gate for t=2h. The highest neutrophil count is in the t=2h Control gate and in this rower the t=2h Response gate contains even more cells than the Control gate for t=pre.

CHAPTER 3.3

Multidimensional and unbiased analysis of peripheral blood cells reveals new inflammatory patterns in severe asthma patients

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To be submitted.

Abstract

Rationale: *Identification of a raised peripheral blood eosinophil count is a first important step in selecting a specific group of asthma patients that are responsive to anti-IL5 treatment. However, more detailed peripheral blood analysis using flow cytometry measurements (FCM) is necessary to further specify asthma phenotypes and predict treatment effects.*

Hypothesis: *Automated multi-colour flow cytometry (FCM) data analysis of blood leukocytes leads to the discovery of inflammatory patterns in asthma and improves diagnostic work-up of asthma phenotypes.*

Method: *11 healthy controls and 24 asthma patients were included. Five asthma patients had mild/moderate asthma and 19 severe asthma according to the British Thorasic Society (BTS) guidelines (2015). All participants received a full asthma-diagnostic work-up including sputum induction, FeNO measurement, asthma questionnaires and Radioallergosorbent (RAST) tests. Blood was taken and whole blood was stained with the following antibodies CD193, CD3, CD4, CD8, CD123, CD14, CD16 and CRTH2. Red blood cells were lysed and white blood cells were measured by FCM. Data analysis was performed using DAMACY, a multivariate analysis strategy that combines Principal Component Analysis (PCA) and Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA).*

Results: *Characteristic cell populations for eosinophilic asthma patients were eosinophils, basophils, Th2 cells and $CD3^{+}CD8^{+}CRTH2^{+}$ cells (Tc2 cells). The overall higher level of CD8 expression on $CD3^{+}CD8^{+}$ cells of non-eosinophilic asthma patients versus healthy controls also contributed to the separation of the two groups. This was strengthened by the observation of the presence of $CD3^{+}CD8^{++}$ cells in non-eosinophilic asthma versus eosinophilic asthma. A typical observation in the controls versus asthma patients is a larger variation in level of CD16 expression in neutrophils. Based on blood profiles, asthma patients were separated from healthy controls with a diagnostic accuracy of 90%.*

Conclusion: *Detailed analysis of white blood cells in asthma patients reveals specific inflammatory patterns not observed in healthy controls. Cellular patterns associated with severe eosinophilic asthma were increased numbers of eosinophils, basophils, Th2 cells and Tc2 cells. Unbiased multi-dimensional analysis of flow cytometry data is a promising tool to identify inflammatory patterns in peripheral blood and to facilitate the discrimination of different inflammatory phenotypes in human asthma.*

Introduction

Asthma is a highly prevalent chronic inflammatory airway disease that affects 5-10% of all adults and children [1]. Within this large population of patients, a total of ~300 million people worldwide, an estimated 4% suffer from severe refractory asthma defined as difficult-to-control asthma despite adherence to treatment and a correct inhalation technique [2]. This vulnerable subgroup suffers from substantially higher morbidity and mortality and accounts for more than half of the asthma-related health and economic impact [1,3,4]. Regardless of maximal treatment and correct medication use, these patients suffer from two or more asthma attacks per year, requiring oral prednisolone treatment and/or hospitalization. There is a strong need to identify patients with severe refractory asthma in an early stage and start personalized treatment as quickly as possible. Standard practice is treatment with oral glucocorticoids; upcoming therapeutic options for this group are biologicals such as anti-IL-5, anti-IL-4-alpha and anti-IgE [5–7].

Deep disease phenotyping is key to determine which treatment is appropriate for an individual asthma patient. Up to this date, there is no perfect test to predict the course of disease in asthma nor to predict treatment responses to steroids or biologicals. Various biomarkers measured in sputum, exhaled breath, peripheral blood and urine have been studied and were proven to associate with specific disease characteristics and some with treatment responses [8]. In the past sputum eosinophilia has been extensively studied and proven to be valuable to titrate treatment with oral glucocorticoids thereby reducing exacerbations [9–11]. Unfortunately this test has been proven to be suitable only for specialized asthma centers and fails in 10-30% of the procedures for various reasons ranging from coughing technique to the absence of sputum at a particular time point of measuring. Less time consuming and less laborious measurements such as Fraction of exhaled nitric oxide (FeNO) and blood eosinophilia have been studied to predict a reduction in exacerbation frequency in treatment with steroids and Mepolizumab respectively [12–16].

The FeNO based treatment strategy was tested in primary care settings and reduces ICS use, was cost-effective and had a small effect on exacerbation reduction [17]. FeNO was also proven to have an only weak association with sputum eosinophilia [18]. High levels of FeNO, blood eosinophil count and periostin levels in blood proved to have a positive predictive value for a reduction in exacerbation frequency in response to anti-IgE [5]. In an overall more severe hospital population with allergic asthma, forty-eight months of anti-IgE treatment reduced the exacerbation frequency for patients with a high FeNO (≥19.5 ppb) and/or with high periostin (≥50ng/mL). In summary FeNO might be important for asthma in primary care setting and in severe allergic asthma, but the value of FeNO for making treatment decisions in severe refractory asthma is yet unclear.

Blood eosinophilia above $0.15x10^9$/L was found to have significant positive predictive value for a reduction of exacerbations by anti-IL-5 (Mepolizumab) in asthma [19,20]. In a *post hoc* analysis of the MENSA and DREAM studies, patients with a blood eosinophil count of at least $0.15x10^9$/L in the past year had 39% and 30% less exacerbations, respectively, when

given Mepolizumab [21]. These cut-off values corresponded with a higher relative risk of suffering from severe exacerbations above 0.25x10^9/L in a UK primary-care population. In this population there also was an increasing relative risk of exacerbations with increasing blood eosinophil levels [22].

However, the relative risk (RR) of blood eosinophilia >0.25x10^9/L on having an asthma exacerbation is only 1.2. And although Mepolizumab brings blood eosinophil levels down quickly, resident airway eosinophils can still be measured up to a year after commencing treatment and in similar numbers compared to placebo treatment [23]. Full understanding of the function of the effect of biologicals in asthma is lacking. The first trials with IL-5 failed due to broad inclusion criteria [24]. An effect of treatment with anti-IL-5 on exacerbation reduction has been proven in RCTs only after selecting patients with eosinophilic disease [16,25,26]. Yet, it is very likely that more accurate phenotyping could lead to larger treatment effects of registered biologicals such as anti-IL-5. Therefore, together with upcoming biologicals there is a strong need for better patient selection.

An attractive approach to improve asthma phenotyping is to study inflammation associated profiles of blood leukocytes of severe asthma patients into more detail. Multi-Color Flow Cytometry (MFC) measurements contain data with detailed cellular profiles of more generic cells such as neutrophils and monocytes and more asthma associated cells such as eosinophils, basophils, and Th2 cells. These MFC data files not only consist of multiple cell types each with a certain count, but also contain relative frequencies to other cell types. In addition, the degree of expression of single markers on single cell and their interactions are part of the obtained information. In summary, MFC datasets contain information on four hierarchical levels for interpretation (**Table 1**). The high-dimensional datasets from MFC measurements are usually analyzed by biplots and miss the other hierarchical levels. We developed methods to integrate all four levels [27] (and unpublished results by Tinnevelt *et al*., Buydens group).

This study was initiated to study asthma associated cellular profiles in an ongoing cohort study in a tertiary severe-asthma clinic referred by primary and secondary care physicians. Detailed blood measurements were performed in combination with known phenotypical characteristics such as sputum analysis, FeNO and lung function. We hypothesized that peripheral blood eosinophilia and/or airway eosinophilia would be associated with specific patterns in other asthma associated cells such as basophils, Th2 cells and in case of severe inflammation, neutrophils. Therefore, we compared:

I the overall asthma population to healthy controls
II asthma patients with blood eosinophilia and/or sputum eosinophilia regardless of severity, to healthy controls
III 'non-eosinophilic' patients to healthy controls
IV eosinophilic asthma to non-eosinophilic asthma
V eosinophilic asthma defined by blood and sputum eosinophilia to non-eosinophilic asthma

Table 1: Hierarchical levels of MFC data–analysis

Hierarchical levels	
1	The multivariate (co)-expression of markers on single cells
2	Aggregation into cell populations with similar marker expression
3	Representation of cell populations in a specific individual/subject
4	The cellular representation in a specific (clinical) phenotype

By studying these cellular patterns we aim to unravel specific inflammatory patterns in peripheral blood associated with eosinophilic and non-eosinophilic asthma.

Methods

Patients aged 18-80 years visiting the outpatient clinic of the Oxford University Hospital with a diagnosis of asthma were approached by their Respiratory Specialist to participate in a long term observational cohort study that was approved by a local Institutional Review Board (IRB). Patients were provided with study information and on a second visit research nurses obtained written informed consent. Asthma was diagnosed according to GINA guidelines [28]. Exclusion criteria were a smoking history of >10 pack years, current smoking or a current airway infection. After taking informed consent research nurses collected data on demographics, BMI, medical history, medication use and filled out questionnaires (ACQ-7, MARS and AQLQ). The inclusion criteria for the DAMACY sub-analysis in this study were patients not treated with oral steroids. Eosinophilic asthma was defined by >3% eosinophils in sputum and/or peripheral blood eosinophilia $\geq 0.27 \times 10^9$ cells/L and other patients were assumed to suffer from non-eosinophilic asthma. The cut-off of 0.27×10^9 cells/L was found by Wagener *et al*. to be the optimal cut-off for sputum eosinophilia with a positive predictive value of 79% and a negative predictive value of 91% [18]. Both markers of eosinophilia were explicitly chosen for patient selection instead of the subjective measure, severity. Importantly, asthma severity is linked to symptom expression and it is known that symptom expression does not correlate well with eosinophilic inflammation [29].

At the base-line visit patients donated blood and underwent FeNO measurement and sputum induction. The NIOX VERO® (Aerocrine, Solna, Sweden) was used for NO-measurements, with 10 seconds expiration time and a reported maximum of max 10% variability between measurements [30]. Sputum induction was performed according to the ERS guideline [31], using a DeVilbiss Ultraneb® for nebulisation of the salt solution. Cytospin slides of sputum cells were stained with May-Grünwald Giemsa and cells were differentiated and counted by an experienced and, for the outcome, blinded technician. A cut-off value of 3% eosinophils in sputum was used to classify patients with eosinophilic or non-eosinophilic inflammation.

Venous blood samples were collected in two sodium-heparin tubes, whole-blood cell count was performed at the routine hospital laboratory and the rest of the blood and sputum samples were transported on ice and processed within an hour at the Respiratory Laboratory of the Nuffield Department of Medicine.

In a polystyrene tube 50µL of whole blood was stained with CD193 (eBioscience, APC, clone 5E8-G9-B4), CD3 (eBioscience, PerCP, SK7), CD4 (Biolegend, Alexa 700, OKT4), CD8 (Biolegend, Pacific Blue, SK1), CD123 (Biolegend, PE-Cy7, 6H6), CD14 (Elnitrogen, PE-Texas Red, MHCD1417), CD16 (eBioscience, FITC, CB16) and CRTH2 (Miltenyi Biotec, PE, BM16). After staining red blood cells were lysed and fixed using BD Phosflow™ lyse/fix buffer according to manufacturer instructions. Subsequently cells were spun down with 500g during 5 min and washed with 2mL of phosphate buffered saline (PBS), again spun down with 500g during 5 min and finally resuspended in 250µL PBS. Fluorescence intensities of antibody-fluorophore bound white blood cells were measured at the.

Statistical analysis

The measured flow cytometry data files were fully loaded into Mathworks' MATLAB without gating cell populations or setting new thresholds in Forward Scatter or Side Scatter. Data analysis was performed using DAMACY (Tinnevelt *et al*., unpublished results), a multivariate analysis strategy that combines Principal Component Analysis (PCA) and Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA). DAMACY stands for Discriminant Analysis of MultiAspect CYtometry (DAMACY), a multivariate method that first maps the leukocyte composition of multiple individuals by using PCA biplots. Subsequently, the method finds correlation between specific cells to discriminate between clinical phenotypes. This results in a hematological map where regions of cells are coloured based on whether the cells are more or less represented in the clinical phenotype. In such plots (*v.i.*) arrows indicate relative marker expression related to the phenotypes. Based on the map the method can predict whether an individual belongs to a specific clinical phenotype. Thereby, DAMACY can integrate all four levels of MFC information (**Table 1**) and in this way differentiate between immunological phenotypes based on quantified co-expression of multiple markers on different cell populations.

More specifically, MFC data from the BD LSRFortessa™ was loaded into MATLAB. Due to background corrections this data contains negative values. We assumed that negative fluorescence does not exist and therefore removed outliers in the negative part [32]. Then from every column the minimum value was subtracted and 1 was added in order to perform common log transformation. From this point the data was split into a training and validation set for the cross validation. Next the data was centered using the median followed by scaling using the standard deviation. Principal Component Analysis was performed on the entire training dataset and the test samples were projected into the same space. Histograms were made based on the scores of Principal Component 1 and 3 and the histograms were smoothed [33]. Then the histograms were unfolded so that each row contains an individual and each column is a histogram bin, a location within the PCA space. Then the training set

was split into a test set and a training set. The data was then mean centered and an OPLS-DA model was built were the Y dummy variable would be -1 if the sample belonged to the one class and +1 for the other class. Based on the prediction of the discriminant analysis, the number of latent variables was determined. Then a final OPLS-DA model was built using the optimal number of latent variables and class was predicted for the validation set. A double cross-validation was performed to test the model until every subject had been selected. Double cross validation was leaving a control and a test subject out as a validation set while the rest functioned as calibration set. The final prediction plotted was the mean of the predicted class. Five comparisons were made:

- Healthy controls were compared to asthma patients as a group (analysis I)
- Eosinophilic asthma was compared to healthy controls (analysis II)
- Non-eosinophilic asthma was compared to healthy controls (analysis III)
- Non-eosinophilic asthma was compared to eosinophilic asthma (analysis IV)
- Eosinophilic asthma defined by blood and sputum eosinophilia was compared to non-eosinophilic asthma (analysis V)

Interpretation of DAMACY output (Figure 1)

Each analysis resulted in a figure with diagnostic score (**Figure 1A**) and a cellular heat map (**Figure 1B**). **Figure 1A** shows the prediction score with a certain diagnostic accuracy of the OPLS-DA model with asthma patients (blue crosses) and healthy controls (red rounds). The threshold is set by the model and separates asthma patients and controls with a certain diagnostic accuracy.

The cellular heat map (**Figure 1B**) shows all cells that have different characteristics between patients and controls; cells that did not contribute to the model are not plotted. Cells with characteristics particularly found in patients are found in red areas in **Figure 1B**; cells characteristic of controls are found in blue areas. The cellular markers on which this discrimination is based are represented by vectors. The direction of a vector indicates higher receptor expression of this specific marker and the length of the vector indicates overall marker variation. For example a cell high in CD8 appears high up 'north' of the origin of this plot.

By saving the scores of each cell in different Principal Components and reloading them into FCS express 5 (De NOVO software, Glendale, CA) we could back-gate the cell populations that were observed in the DAMACY Cellular Heat maps (**Table 2**). The back-gating functioned to cross-validate the populations present on the maps. These populations were studied for receptor expression using conventional gating to ensure the cell type. This step was necessary to assess the characteristics of cell populations identified by DAMACY.

Results

Thirty-five participants were included for analysis, 24 asthma patients and 11 healthy controls. Based on the presence of sputum eosinophilia (≥3 % of non-squamous cells) and/or blood eosinophilia (≥0.27x10^6/ml) 14 patients were diagnosed with eosinophilic asthma and 10 patients with non-eosinophilic asthma (**Table 3**, demographics at base-line).

Table 2: Result of back–gating in FCS Express 5.0 of DAMACY in Figure 1

Back-gated cell populations	Cell type (based on receptor profile)
$CD3^+CD8^{++}$	high CD8 expression
$CD3^+CD8^+$	$CD8^+$ T-lymphocytes
$CD3^+CD8^+CRTH2^+$	Tc2 cells
$CD16^+CD193^+CRTH2^+$	eosinophils
$CD16^+$	neutrophils
$CD123^+CD193^+CRTH2^+$	basophils
$CD3^+CD4^+CRTH2^+$	Th2 cells
$CD14^+$	monocytes

Table 3: Base–line table with eosinophilic/non–eosinophilic patients and healthy controls

Asthma patients n = 47	Eosinophilic n = 14	Non-eosinophilic n = 10	Controls n = 11
Age in years (mean)	57	57	39
Gender (M/F)	6/8	5/5	2/9
BMI, kg/m^2	30	31	19
ACQ-7	2.28	2.25	N/A
FeNO (ppb)	48	17	28
% predicted FEV_1 (L)	81	68	N/A
Total eosinophil count in PB (* 10^9/L)	0.47	0.10	0.16
IgE (kU/L)	384	208	23
Aspirin Sensitivity (yes/no)	5/9	2/8	0/11
Nasal Polyposis (yes/no)	7/7	0/10	0/11
GINA classification			N/A
GINA 1	1	1	-
GINA 2	0	2	-
GINA 3	1	0	-
GINA 4	12	7	-
GINA 5	0	0	-

Analysis I

DAMACY on eosinophilic asthma (blue) versus healthy controls (red) (Figure 1)

Based on similarities and differences between groups asthma patients could be separated from controls with an accuracy of 90% (**Figure 1**). The overall representation shows two characteristic features:

1. The neutrophil population (most abundant cell type at the center) has less marker variation in asthma patients. In controls two additional red neutrophil populations appear next to the high-density blue population of asthma patients. The variation of CD16 expression is the most important factor in this difference.

2. 5 distinct blue populations are more typical of asthma patients
   a. $CD123^{+}CD193^{+}CRTH2^{+}$ cells (basophils)
   b. $CD3^{+}CD4^{+}CRTH2^{+}$ (Th2 cells)
   c. $CD16^{+}CD193^{+}CRTH2^{+}$ cells (eosinophils)
   d. $CD3^{+}CD8^{+}CRTH2^{+}$ (Tc2 cells)
   e. $CD3^{+}CD8^{++}$ cells

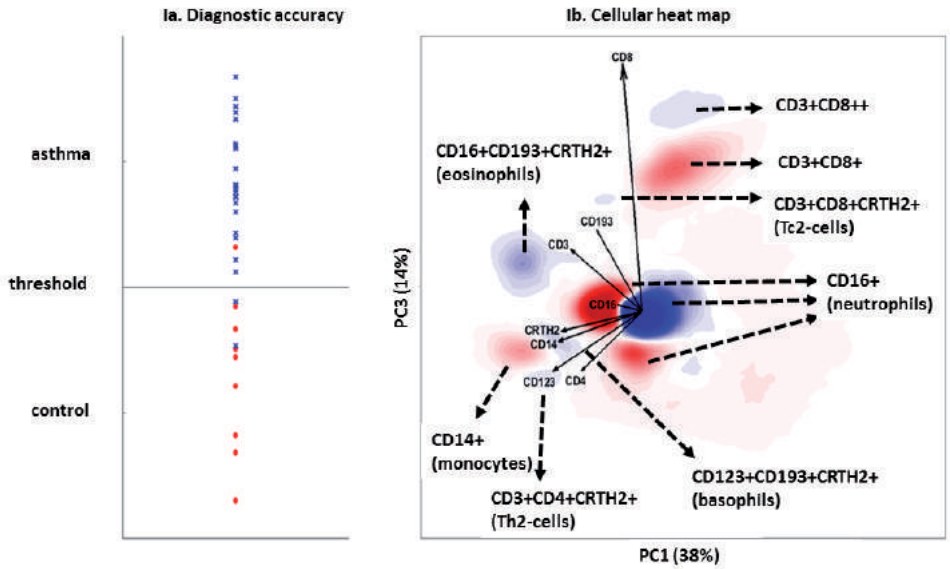

Figure 1: Comparison between asthma patients (eosinophilic and non–eosinophilic) and healthy controls.

In the overall cohort eosinophils (blue, left upper corner) are typical of asthma patients and also basophils form a small blue population (left lower corner). Neutrophils overall show a larger variation in marker expression in healthy controls with two red additional neutrophil populations next to the high-density blue population of homogeneous neutrophils in asthma patients. Two other observations can be made: there is a population with higher CD8 expression at the top of the figure (blue) and there is a small blue population next to the bigger red CD8+ population that is CD3+CD8+CRTH2+. The latter cells are Tc2-cells.

Analysis II

DAMACY on eosinophilic asthma (blue) versus healthy controls (red)

In this comparison (**Figure 2**) groups could be separated with an accuracy of 71%. The aggregation of cell populations with similar marker expressions (**Figure 2B**) is as follows:

1. Eosinophils are grouping together (the left upper corner of **Figure 2B**), notably eosinophils were also part of the group selection of eosinophilic asthma (≥0.27*10^6/ml eosinophils in peripheral blood)
2. In the eosinophilic asthma patients other cells such as $CD3^+CD8^+CRTH2^+$ cells (Tc2 cells) form a population, this is the population next to the $CD3^+CD8^+$ cells from controls.
3. $CD123^+CD193^+CRTH2^+$ (basophils) are present in the eosinophilic asthma population and in lower concentrations in healthy controls
4. Importantly, two blue populations are missing in eosinophilic asthma patients compared to **Figure 1B**. The first population consists of $CD3^+CD4^+CRTH2^+$ cells (Th2 cells), which merged with basophils (verified by back-gating) and the second population are the $CD3^+CD8^{++}$ cells

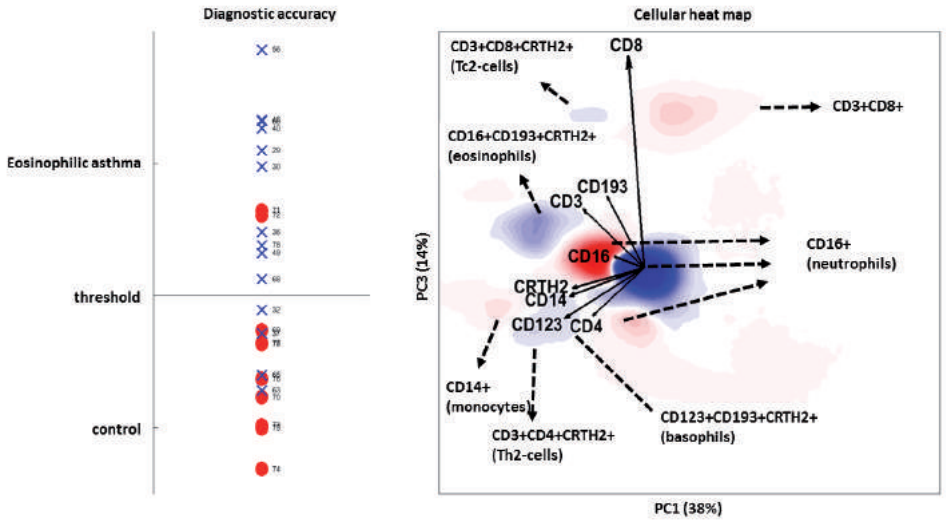

Figure 2: Controls versus severe eosinophilic asthma (eosinophils >0.25x10^9/L and/or sputum eosinophilia.

The patients with severe eosinophilic asthma are characterized by a large population of eosinophils, a smaller population of basophils, and a very small population of Tc2-cells. Notably controls have typically more variation in the expression of CD16.

Analysis III

DAMACY on control (red) versus non–eosinophilic asthma (blue)

To identify typical characteristics of non-eosinophilic asthma, patients were compared to healthy controls (**Figure 3**). The diagnostic accuracy of this classification was 80%. The following observations were made:

1. Neutrophils show less marker variation in non-eosinophilic asthma compared to controls, this is similar to eosinophilic asthma.
2. There are more $CD3^{+}CD8^{++}$ cells in non-eosinophilic asthma.
3. Although very small, the eosinophil population (left upper quadrant, blue) is still present as a population more typical of non-eosinophilic asthma compared to healthy controls.

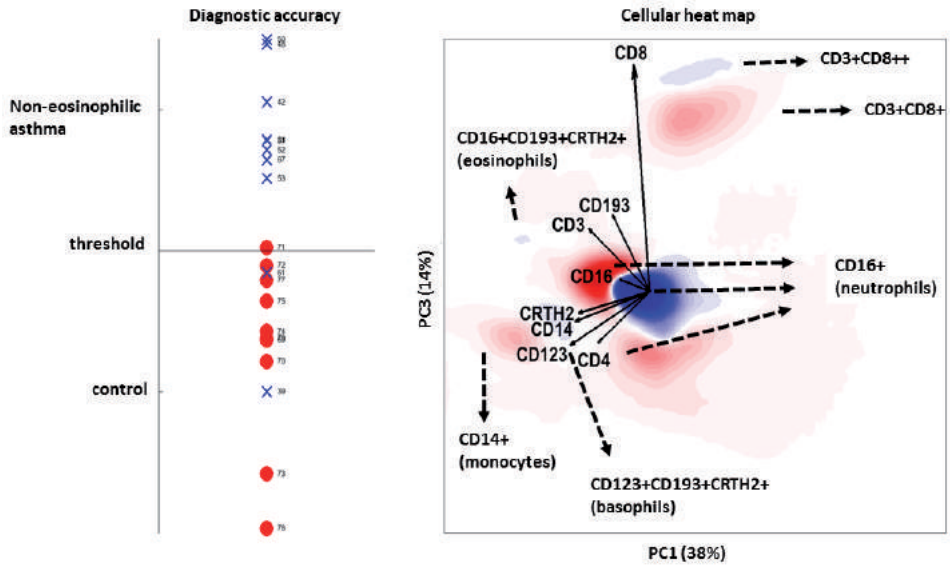

Figure 3: DAMACY on control (red) versus non–eosinophilic (blue).

In controls there are two neutrophil populations visible, one higher and the other lower in CD16-expression. Monocytes are present in higher numbers in controls (lower left). In the non-eosinophilic group the overall expression of CD8 on CD3+CD8+ cells is higher.

Analysis IV

DAMACY on eosinophilic asthma (blue) versus non–eosinophilic asthma (red)

As noticeable in **Figure 4A**, 'diagnostic accuracy', patients are not well separated. The diagnostic accuracy was random. Strikingly there are two patients with non-eosinophilic asthma that are very similar in blood profile compared to patients with eosinophilic asthma. Vice versa there are 5 patients with eosinophilic asthma that resemble the blood profile of patients with non-eosinophilic asthma. There is a marked difference between the two selected groups and their blood profiles; the 5 patients with eosinophilic asthma either had blood eosinophilia or sputum eosinophilia, while the rest of the group actually had both except for one. Based on this observation, we more strictly selected eosinophilic patients by selecting them both on sputum eosinophilia as well as blood eosinophilia. This new classification was used to run DAMACY again (Analysis V).

The observations from the cellular heat map in **Figure 4** are that non-eosinophilic patients' neutrophils are overall higher in CD16 expression, as indicated by the red population which lies in the direction of the CD16 vector. The second observation is that the $CD8^+$ population has higher CD8 expression in non-eosinophilic asthma, which is in line with Analysis III. Characteristic cells for eosinophilic asthma are eosinophils, basophils and Tc2-cells. This is similar to Analysis II.

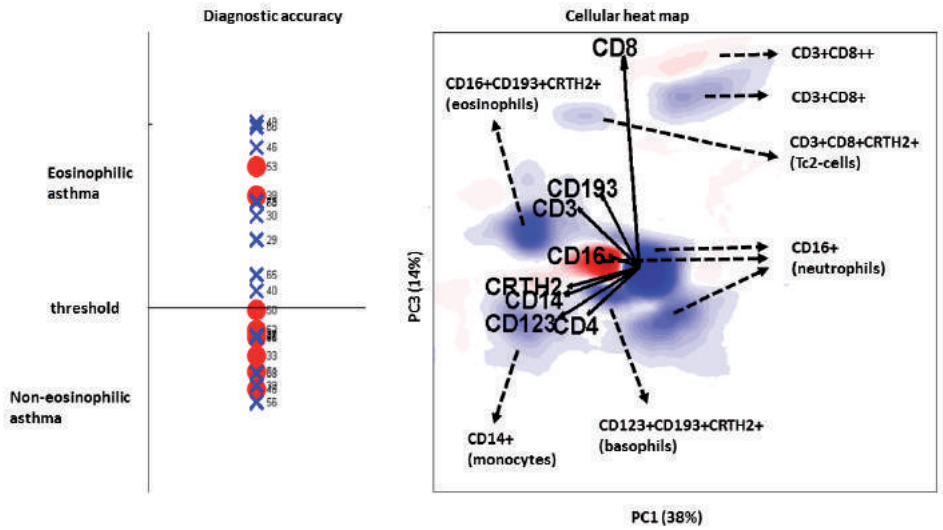

Figure 4: Eosinophilic asthma (blue) versus non–eosinophilic asthma (red).

Comparing two extremes, non-eosinophilic patients' neutrophils are overall higher in CD16 expression. Again eosinophils, basophils and Tc2 cells appear as characteristic cells for eosinophilic asthma. In the non-eosinophilic group the CD8+ population has the highest CD8 expression.

Analysis V

"Second run" of DAMACY with more strictly defined eosinophilic asthma (blue) versus non–eosinophilic asthma (red).

Eosinophilic asthma is now defined as blood eosinophilia >0.27x10^9/L *and* sputum eosinophilia ≥3% of total cells. The diagnostic accuracy of eosinophilic versus non-eosinophilic asthma by this comparison is 79%, while Analysis IV led to a non-significant accuracy between the two groups. Compared to **Figure 4**, the cellular heat map of **Figure 5** shows:

1. An intense neutrophil population at the origin of the vectors with next to it two smaller blue populations with neutrophils more typical of patients with eosinophilic asthma, indicating more heterogeneity in the neutrophil population. There is more homogeneity in the neutrophilic population of non-eosinophilic asthma patients compared to patients with eosinophilic asthma.
2. A large $CD3^+CD8^{++}$ population 'north' of the origin.
3. Focussing on eosinophilic asthma: Tc2 cells (blue, far left upper corner), eosinophils (left of the origin of the vectors) and basophils (small blue population lower left of the origin) were more prominent in eosinophilic asthma.

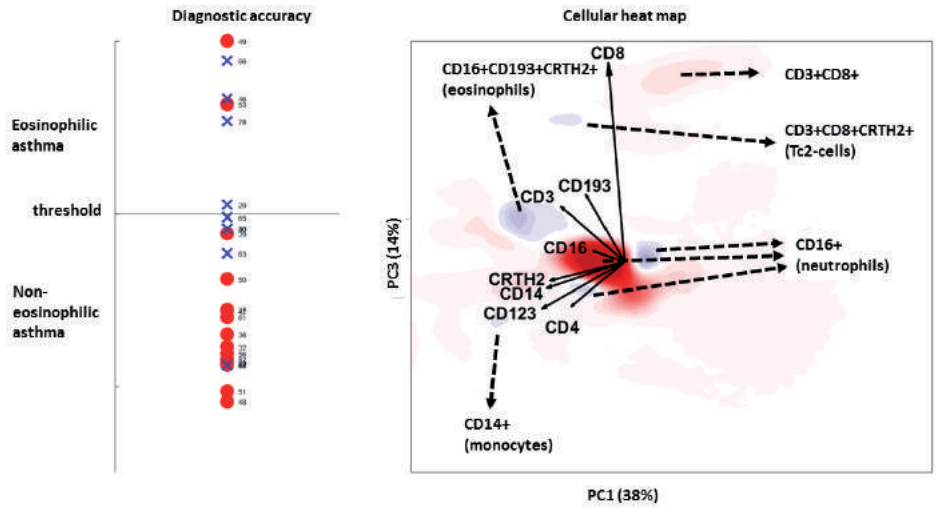

Figure 5: Second analysis of more strictly defined eosinophilic asthma (blue) versus non–eosinophilic asthma (red).

Eosinophilic asthma is now defined as blood eosinophilia >0.27x109/L and sputum eosinophilia ≥3% of total cells. In this graph the non-eosinophilic group is more prominently present compared to Figure 4, characterized by a high intensity neutrophil population with more homogeneity in surface marker expression compared to eosinophilic asthma (two small blue populations right next to the high density red population at the origin of the vectors). In comparison with analysis IV there is better diagnostic accuracy, 79%.

The two other comparisons from the second run of DAMACY are non-eosinophilic asthma versus controls (**Figure S1**, supplementary material) and eosinophilic asthma versus healthy controls (**Figure S2**, supplementary material). **Figure S1** of the supplementary material is very similar to **Figure S3**, although with an improved diagnostic accuracy of 84%. **Figure S2** (supplementary material) is almost identical to **Figure 2**, yet the diagnostic accuracy dropped to 63%. This is most likely due to a decrease in power with only 10 eosinophilic patients having both sputum and blood eosinophilia at the time of inclusion. The diagnostic accuracy of DAMACY is negatively influenced by low subject numbers.

Discussion

In this study specific cellular patterns were found by studying immune profiles of leukocytes in peripheral blood of severe-asthma patients and healthy controls applying multi-color flow cytometry (MFC) in combination with a novel data analysis approach (DAMACY) (unpublished results by Tinnevelt *et al*. see attached manuscript submitted for publication). In addition to peripheral blood eosinophilia, severe eosinophilic asthma was characterized by larger populations of peripheral blood basophils and $CD3^+CD8^+CRTH2^+$ (Tc2) cells compared to healthy controls. The eosinophilic profile was even more profound in the comparison between eosinophilic asthma and non-eosinophilic asthma (Analysis IV) with the characteristic presence of eosinophils, basophils, Th2 cells and Tc2 cells. Typical of non-eosinophilic asthma are a higher expression of CD8 in the $CD3^+CD8^+$ population and, in neutrophils, a higher CD16 expression with overall a more homogeneous surface marker profile compared to controls (supplementary **Figure S1**) and to non-eosinophilic asthma (**Figure 5**).

Peripheral blood basophils, Tc2 cells and Th2 cells were associated with eosinophilic asthma. Basophils are known to be present in higher levels in peripheral blood of asthma patients [34]. The specific cell population with receptor profile $CD3^+CD8^+CRTH2^+$ (Tc2 cells) have not been identified as markers of eosinophil asthma before, but were present in higher numbers in peripheral blood of these patients. They have been linked to airway inflammation in asthma [35]. In earlier studies Th2 cells were measured in higher concentrations in the airways, but were not found in higher concentrations in peripheral blood [36]. Interestingly, our analysis identified a population of $CD3^+CD4^+CRTH2^+$ cells that was higher in eosinophilic asthma patients compared to healthy controls. This population is likely consisting of Th2 cells, but receptor positivity is formally not the same as type-2 cytokine producing cells (T-helper 2 cells). There is evidence to suggest that $CD4^+CRTH2^+$ T-cells are in fact Th2 cells [37].

Overall the asthma patients are well separated by DAMACY from healthy controls (90% accuracy). Separating eosinophilic asthma from controls resulted in 71% accuracy and non-eosinophilic asthma from controls in 80% accuracy. The explanation of a lower accuracy in eosinophilic asthma versus the overall asthma population is likely due to the low numbers of patients included in the analysis. The diagnostic accuracy of DAMACY will improve with a higher number of patients leading to a more accurate model and threshold for diagnosis.

An important note is that determination of the diagnostic accuracy of DAMACY analysis was not the primary goal of our study, nor did we validate accuracy by testing an external cohort of patients.

The fourth comparison, *i.e.* between eosinophilic and non-eosinophilic asthma, did not result in a significant accuracy. Looking into more detail at the diagnostic accuracy plot of **Figure 4**, three non-eosinophilic patients have a blood profile which resembles eosinophilic asthma and vice versa five eosinophilic patients have a non-eosinophilic blood profile. This implies that our biomarker selection approach using sputum and/or blood eosinophilia does not result in clear peripheral blood phenotypes. As presented in the Results section, a second analysis by DAMACY with a more strictly selected group having both sputum eosinophilia and blood eosinophilia led to a better classification with 79% accuracy to discriminate between non-eosinophilic and eosinophilic asthma (**Figure 5**). In **Figure 5** the classical eosinophilic asthma associated cells (eosinophils, Tc2 cells and basophils) are plotted in blue and in red the populations characteristic of non-eosinophilic asthma with a homogeneous neutrophil population and the presence of $CD3^{+}CD8^{++}$ cells.

MFC has been used extensively in clinical studies in the past years to measure specific cell populations in peripheral blood. Generally, MFC data is visualized through 'bivariate' scatter plots with fluorescence intensities of two cell surface-bound epitopes for each cell within a sample followed by multiple gating to study the cell population of interest [38]. This strategy cannot be used to compare intensities of more than two surface markers simultaneously (**Table 1**, levels 1 and 2). Another missing information criterion in flow cytometry data analysis is relative frequencies of cells in one subject compared to another (**Table 1**, level 3). A third factor of importance can be the height of surface marker expressions at a single cell level compared between subject groups (**Table 1**, level 4). DAMACY on the other hand, uses relative frequencies, height of receptor expression per cell, correlations between receptor expression, and adds to this the testing of differences between subject in pre-set groups *e.g.* asthma and non-eosinophilic asthma. It results in a diagnostic score and discriminate populations of cells typical of each of the two comparative groups. DAMACY requires non-gated flow cytometry data of two pre-selected groups and is, thereby, unbiased in cell analysis but influenced by group selection. Only at the end of the analysis process conventional back gating was used to understand which populations distinguish between asthma phenotypes. This specific concept is unique to the field.

The peripheral blood samples were stained with a fixed antibody-panel: CD3, CD4, CD8, CD14, CD16, CD123, CD193, and CRTH2. The antibody-panel discriminates between white-blood-cell types and has specific markers to measure Type-2 cells such as basophils, Th2 cells, Tc2 cells and eosinophils. The panel was designed to be suitable for peripheral blood measurements in asthma patients. A fixed panel is necessary to compare samples between subjects and, on the other hand, implies a restriction because not all relevant cell types can be measured using this antibody-panel. For example, innate lymphoid cells, NK-cells and other T-cell subsets could not be measured. In future experiments additional and/or larger panels could add to the performance of DAMACY analysis.

Confirmation of the findings of this study requires replication and testing in treatment conditions to search for treatable traits [39]. At this point in time our study is a proof-of-principle analysis of blood samples from a single center, using uniform pre-processing protocols and one flow cytometer. To obtain comparable data in multiple centers DAMACY requires the same pre-processing and well-calibrated flow cytometers with the same measurement features as used originally.

The advantage of using Principal Component Analysis in DAMACY is the representation of multidimensional data in two or three principal components while maintaining almost all the information in the data [40]. The method is unsupervised and does not require any additional input. The goal of the PCA is to map the differentiation of the cells, which may not lie in the first two PCs. In this case the best differentiation was obtained with PC1 and PC3. PCA is thus very suitable to identify trends in the data which would otherwise by missed by conventional two-dimensional sequential gating.

The clinical implications of this study are (1) hypothesis generation by testing relevant surface markers to analyse a variety of cells, (2) improved diagnosis by assessing a more detailed cellular pattern in peripheral blood of asthma patients, and (3) the potential for monitoring success of treatment. All of these implications are not biased by a human intervention in the analysis (*e.g.* choice of gates etc.).

Overall, our study confirms previous findings illustrating the power of this unbiased technique. It also brings new insight into peripheral blood profiles such as the identification of the new association of $CD3^+CD8^+CRTH2^+$ cells (Tc2 cells) with eosinophilic asthma. It also showed an association of the presence of a high-density homogeneous neutrophil population and the presence of more $CD3^+CD8^{++}$ cells with patients suffering from non-eosinophilic asthma. DAMACY both led to the identification of inflammatory patterns in peripheral blood of severe-asthma patients and seems a promising diagnostic technique which might ultimately lead to a better understanding of the pathophysiology of asthma and to an improved diagnosis of severe asthma.

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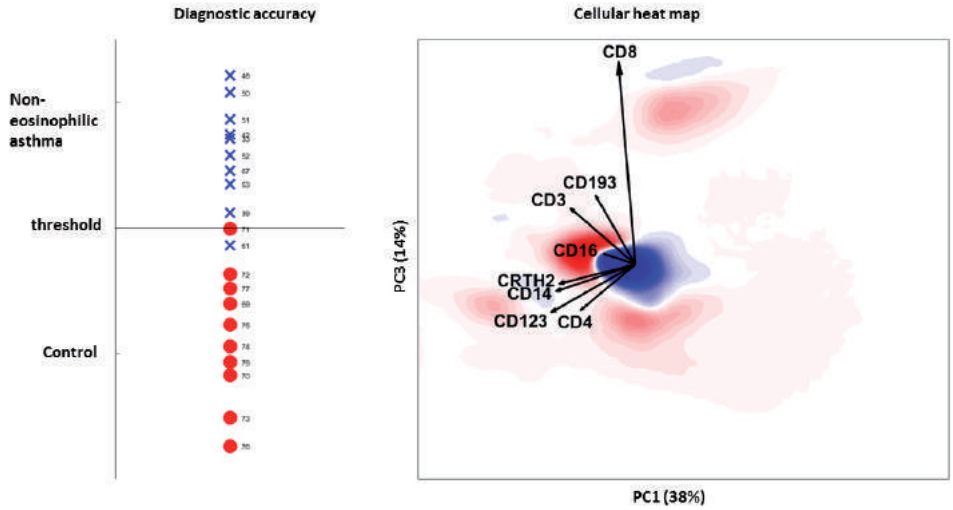
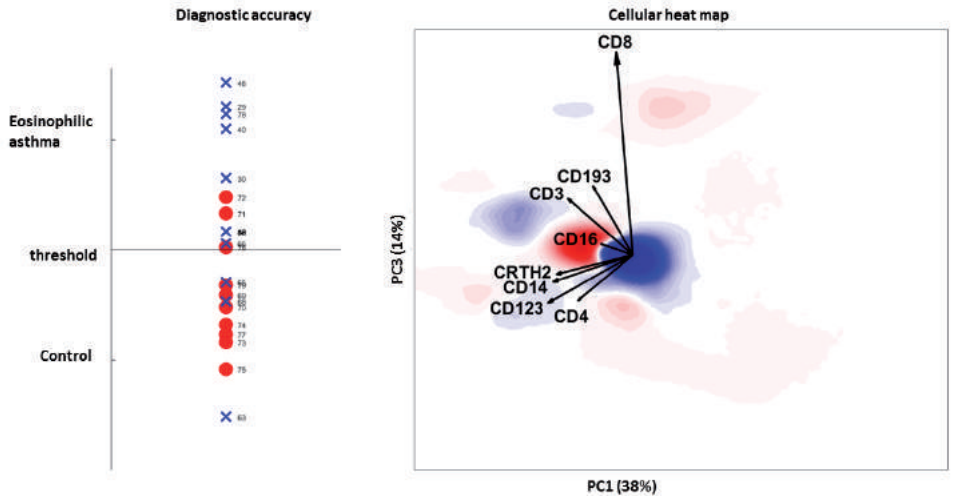
Supplementary Material



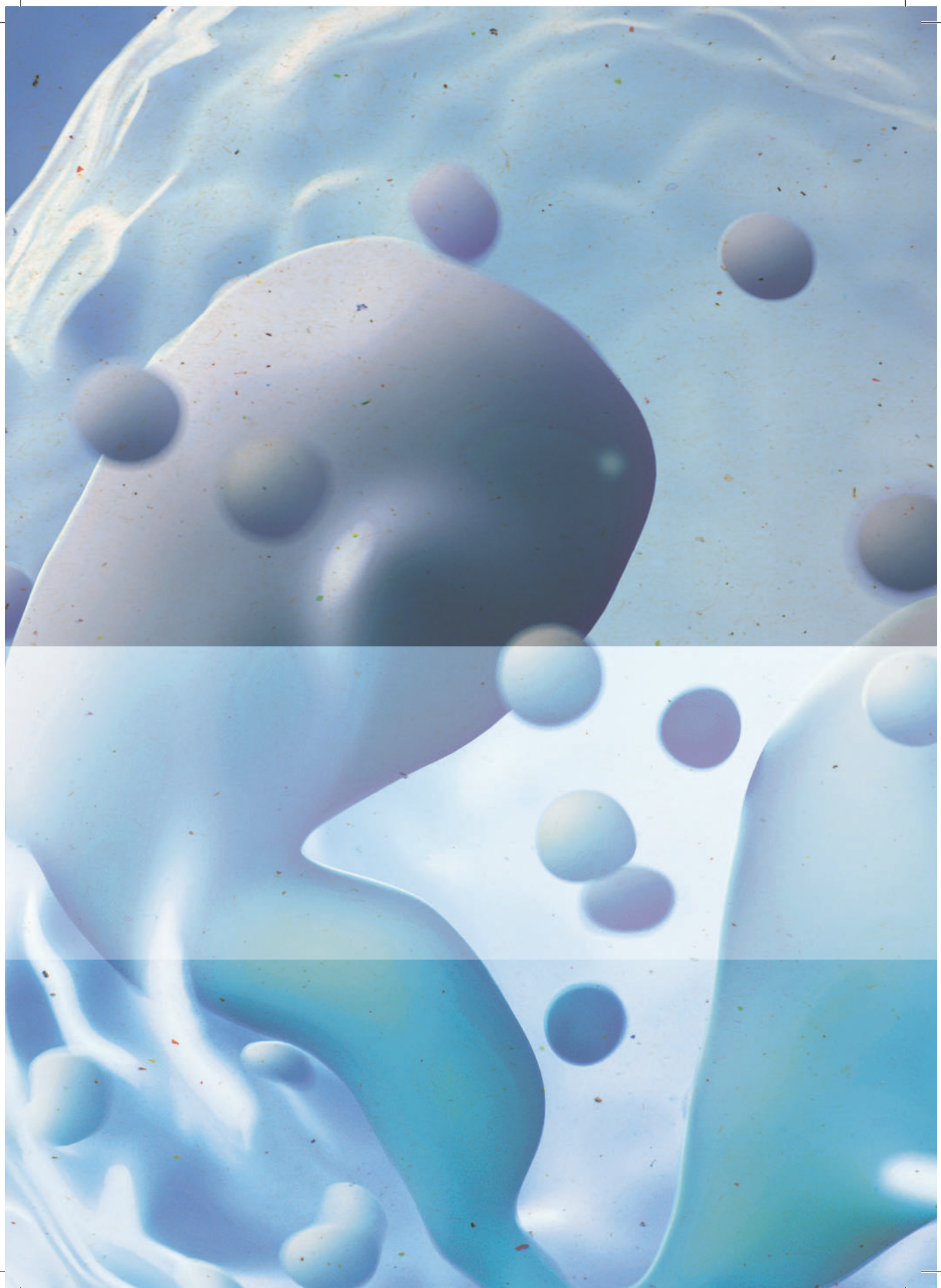
Supplementary Figure S1: Analysis V, second run of DAMACY.

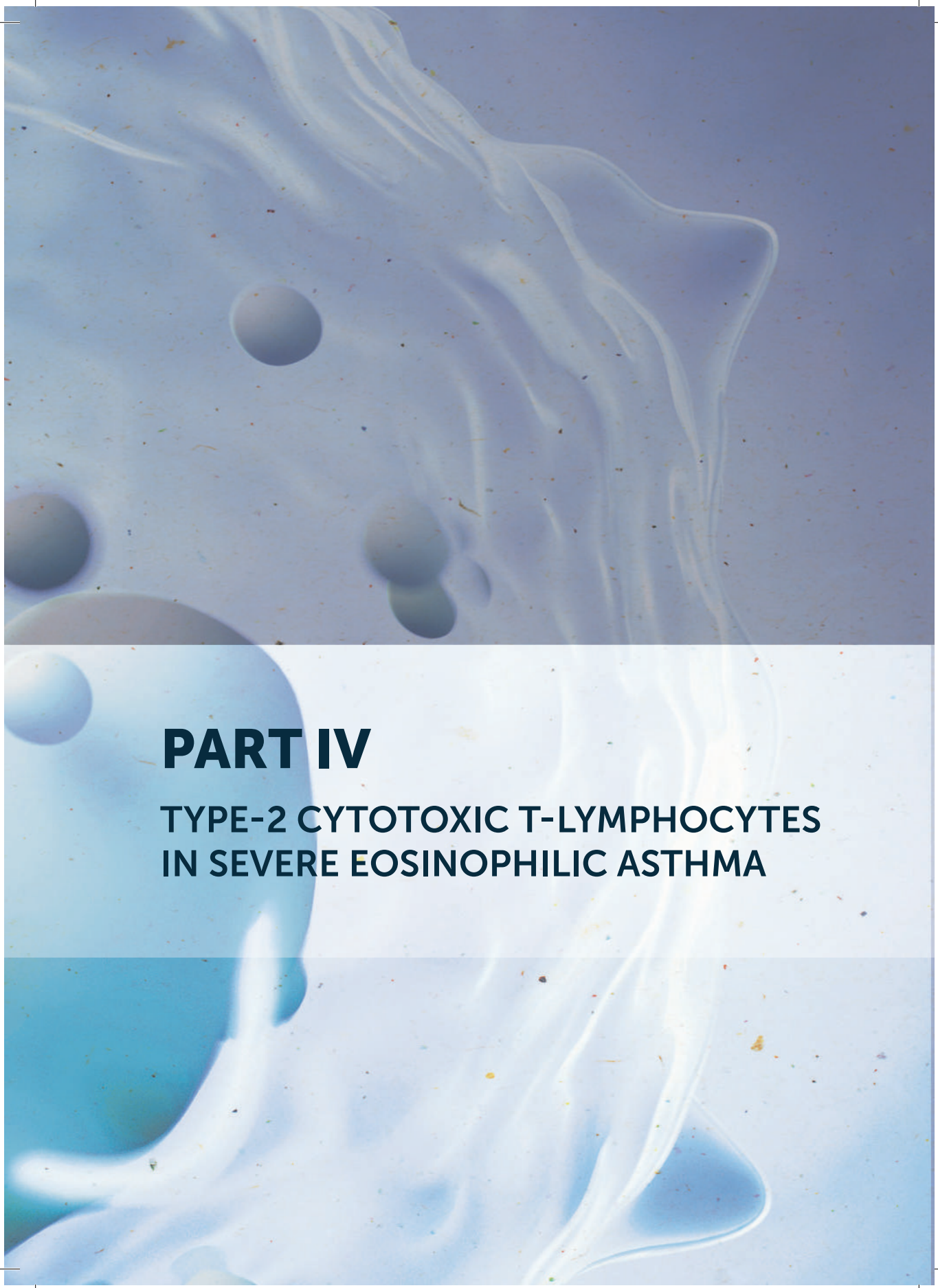
Non-eosinophilic patients versus healthy controls.



Supplementary Figure S2: Analysis V, second run of DAMACY.

Eosinophilic patients versus healthy controls.





PART IV

TYPE-2 CYTOTOXIC T-LYMPHOCYTES IN SEVERE EOSINOPHILIC ASTHMA

CHAPTER 4

Type-2 cytotoxic T-lymphocytes responsive to PGD_2 and LTE_4 promote inflammation in severe eosinophilic asthma

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Submitted.

Abstract

The severe eosinophilic asthma phenotype is associated with a high risk of exacerbation, persistent airway eosinophilia despite high-intensity inhaled corticosteroids and responsiveness to type-2 cytokine-targeted therapies. In two independent patient cohorts type-2 cytokine-secreting $CD8^+CRTH2^+$ Tc2 cells – rather than Th2 or ILC2 cells – and prostaglandin D_2 (PGD_2) and cysteinyl leukotriene E_4 (LTE_4) were enriched in blood and airways specifically in severe eosinophilic asthma. In vitro PGD_2 and LTE_4 functioned synergistically in Tc2 cell recruitment and activation. They regulated a diverse range of genes in Tc2 cells inducing type-2 cytokines and many other pro-inflammatory cytokines and chemokines which could contribute to eosinophilia in severe asthma. Thus, human Tc2 cells are important participants in severe eosinophilic asthma and constitute a potential target for therapeutic intervention.

Introduction

Asthma is a chronic inflammatory disease of the lower respiratory airways characterized by airway hyper-responsiveness (AHR), infiltration of pro-inflammatory cells into the airway, mucus hypersecretion and airway remodelling. It is a common condition, affecting 5-10% of the population and more than 300 million people worldwide [1]. Up to 10% of this population have severe disease despite high intensity treatment, with significant morbidity, mortality and health service burden. Severe asthma is now recognised as a heterogeneous syndrome encompassing a range of abnormalities of airway function and distinct cellular mechanisms [2,3].

One important phenotype is eosinophilic asthma, defined by the presence of increased numbers of eosinophils in the airway [2,4]. Eosinophils are important in defence against parasitic infection [5], but also significant players in the pathogenesis and severity of chronic inflammatory disorders of the airways including asthma [4]. Through release of pro-inflammatory mediators including granule-derived basic proteins, lipid mediators, cytokines and chemokines, activation of tissue eosinophils contributes to inflammation of the airways, leading to airway dysfunction and asthma exacerbations [6]. Blood and sputum eosinophilia are associated particularly with exacerbations in patients with eosinophilic asthma [7]. In a proportion of patients with severe asthma eosinophils persist in the airways despite taking high doses of inhaled corticosteroids (ICS), pointing to relative insensitivity to this standard asthma therapy [8]. This phenotype is commonly linked to co-morbid rhinosinusitis, nasal polyposis and aspirin-induced bronchoconstriction [9]. The pathogenic mechanisms of eosinophilic asthma are still unclear. Therefore understanding the main drivers of this phenotype is the key to further therapeutic advances.

Type-2 cytokines, including IL-4, IL-5 and IL-13, play crucial roles in orchestrating allergic inflammation including eosinophilic asthma. IL-4 drives the differentiation of $CD4^+$ precursors into Th2 cells. IL-4 and IL-13 are potent activators of B cell antibody production, particularly IgE. IL-5 stimulates eosinophil release from bone marrow, and is chemotactic for eosinophils. It also activates mature eosinophils and prolongs their survival. IL-13 has additional roles in mucus hypersecretion and regulation of airway hypersensitivity. Increased local concentrations of type-2 cytokines are detected in both airway and bronchoalveolar lavage fluids (BAL) from patients with asthma [10]. Anti-type-2 cytokines have been considered as new approaches to treat asthma [11]. Notably Mepolizumab, an anti-IL-5 antibody, has shown clinical benefit in severe eosinophilic asthma by inducing significant reduction in circulating eosinophils, as well as asthma exacerbations [12,13]. The major sources of type 2 cytokines recognised in the human immune system so far are type-2 $CD4^+$ T-helper cells (Th2), type-2 $CD8^+$ cytotoxic T-cells (Tc2) and group-2 innate lymphoid cells (ILC2). Eosinophilic asthma is commonly considered as a Th2 disorder [14], as in mild to moderate asthma, increased numbers of Th2 cells are found in BAL and mucosal biopsies, and levels correlate with the degree of airway eosinophilia [15]. In mouse models of asthma driven by ovalbumin (OVA), the genetic or antibody-mediated depletion of $CD4^+$ T-cells abolished key

features of asthma, whereas the adoptive transfer of Th2 cells from mice with transgenic expression of an OVA peptide-specific T-cell receptor leads to the induction of asthma features [16,17]. In recent years, numerous studies have identified a novel lineage negative type-2 cytokine-producing cell population, ILC2, in mice and human subjects [18-20], and established the importance of ILC2s in the initiation of airway eosinophilic inflammation [21]. It has also been reported that peripheral blood ILC2 are increased in asthma patients [22]. However, most studies performed thusfar on the role of type-2 immunity in allergic inflammation or asthma have focused on Th2 and ILC2 cells, while the role of Tc2 cells in human disease is largely unexplored.

All these three types of type-2 cytokine-producing cells highly express chemo-attractant receptor-homologous molecule expressed on Th2 cells (CRTH2), a receptor for mast cell lipid mediator prostaglandin D_2 (PGD_2) [20,23]. It has been well established that, through CRTH2, PGD_2 elicits chemotaxis, stimulates type-2 cytokine production, and suppresses apoptosis in Th2 and ILC2s [24-27]. CRTH2 antagonists have been shown to have mixed effects on symptoms and lung function in asthma with efficacy most apparent in patients with eosinophilic asthma [28] and particularly patients with severe eosinophilic asthma [29]. We have previously shown synergistic enhancement of PGD_2 with other mast cell lipid mediators cysteinyl leukotrienes (cysLTs) including leukotriene C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) in the activation of human Th2 and ILC2 cells [30,31]. The role of these lipid mediators and their receptors in Tc2 cells has not been properly studied.

To investigate the immunopathogenesis of severe eosinophilic asthma, we conducted a cross-sectional comparison of immune-cell profiles in blood, BAL and bronchial biopsy in patients with mild (requiring β2-agonists only), moderate (controlled on inhaled corticosteroids) and severe (as defined by ATS/ERS taskforce on severe asthma) asthma [8]. Patients were further stratified as eosinophilic asthma, defined as asthma in association with an induced sputum eosinophil count of >3% [32]. Interestingly, we found that Tc2 but not Th2 cells were significantly enriched in both peripheral blood and lung tissues in eosinophilic asthma. Such enrichment was also observed to be absent in ILC2 population in blood. To further understand the role of Tc2 cells in the disease, we also evaluated whether the airway environment was conducive to their activation via CRTH2 by measuring airway levels of PGD_2 and LTE_4, the interaction of Tc2 cells with these lipid mediators, and the transcriptional and functional responses of such stimulation. Furthermore, we investigated whether the activation of Tc2 cells could drive airway eosinophilia. These data provide compelling evidence for a role for Tc2 cells in severe eosinophilic asthma and additionally suggest a mechanism by which they may contribute to the abnormal type-2 cytokine production and eosinophil levels seen in this condition.

Results

Tc2 cells enriched in eosinophilic asthma

CRTH2 is highly expressed on type-2 cytokine-producing human peripheral blood $CD8^+$ Tc2 cells (**Figure S1**). Our cross-sectional comparison of the immune-cell profiles between asthma phenotypes in a cohort of 51 participants from Oxford, UK showed that Tc2 cells were significantly enriched in the blood of patients with severe eosinophilic asthma (**Figure 1**). The numbers of peripheral blood Tc2 cells detected with CD3, CD8 and CRTH2 biomarkers (**Figure 1A**) were substantially higher in patients with severe eosinophilic asthma (~21.5±4.2x10^6/L, n=24) than in severe non-eosinophilic asthma (~7.4±2 x10^6/L, n=12, p=0.001) and healthy controls (~5±0.6 x10^6/L, n=15, p=0.001) (**Figure 1B**, **Table 1**). In contrast, the numbers of $CD3^+CD4^+CRTH2^+$ Th2 cells and $Lin^-CD45^{high}CD127^+CRTH2^+$ ILC2s (**Figure S2**) did not differ significantly between groups (**Figure 1B**). Analysis of functional IL-5 and IL-13 producing $CD8^+$ T-cells *ex vivo* detected with PrimeFlow assay also supported the finding of Tc2 enrichment in patients with severe eosinophilic asthma (**Figure 1C**).

In a second cohort of 74 participants from Southampton, UK (**Table 1**) [33] we used intracellular cytokine staining to measure $CD3^+CD8^+IL^-13^+$ (Tc2) cells in blood and airway tissues. These cells were also found to be increased in peripheral blood in asthmatic patients (~0.3% of $CD8^+$ T-cells, n=47) when compared with healthy donors (~0.05%, n=19, p=0.04) (**Figure 1D panel 1**), and this increase correlated with asthma severity (**Figure 1D panel 2**, p=0.01) and was associated with the presence of nasal polyps (**Figure 1D panel 3**, p=0.008) and with a history of ever having smoked (**Figure 1D panel 4**, p=0.008), comorbidities known to be associated with severe asthma. By contrast frequencies of $CD3^+CD4^+IL^-13^+$ (Th2) cells were significantly increased in mild (steroid-naïve) asthma (0.5%, n=14) compared with health (0.19% n=22, p<0.01) but not in steroid-treated moderate or severe asthma (**Figure S3**). Interestingly, expression of the type-2 cytokine IL-4 in sputum T-cells was associated positively with peripheral blood Tc2 frequencies (r_s=0.537, p=0.006), but negatively associated with peripheral blood Th2 frequencies (r_s= -0.442, p=0.03) (**Figure S4A**).

Table 1: Study subject details. Mean ± SD

	Oxford Cohort				Southampton Cohort			
	Control	Severe asthma			Control	Asthma		
Characteristic	(n=15)	Non-eosinophilic (n=12)	Eosinophilic (n=24)	*P*-value	(n=22)	Non-eosinophilic (n=42)	Eosinophilic (n=10)	*P*-value
Age (y)	36.8 ± 11.7	60.8 ± 8.9	53.3 ± 14	0.0002	31.7 ± 11.9	38.4 ± 15.1	43.8 ± 15.4	0.1
Sex (male %)	15	67	50		64	38	80	0.03
Atopy (%)	23	33	58		0	81	100	<0.0001
BMI	22 ± 2.9	28.1 ± 3.4	32.1 ± 7.5	0.0003	25.5 ± 4.9	29.2 ± 7.8	28.12 ± 5.2	0.1
FEV1 (% pred)	104.2 ± 11.6	79.8 ± 22.4	73.9 ± 21.9	0.004	107.4 ± 13.8	88.6 ± 22.0	69.6 ± 22.3	<0.0001
Sputum eosinophils (%)	0.25 ± 0.12	1.4 ± 0.8	30.1 ± 25.0	<0.0001	0.25 ± 0.65	0.72 ± 0.67	10.4 ± 10.2	<0.0001
FeNO (ppb)	19 ± 6.6	23 ± 9.2	37.1 ± 38	0.5	17 ± 7.1	42 ± 45	63 ±48	0.001
Blood eosinophils (10^9/l)	0.13 ± 0.08	0.25 ± 0.29	0.59 ± 0.3	<0.0001	0.15 ± 0.07	0.21 ± 0.15	0.37 ± 0.28	<0.05
Serum IgE (IU/ml)	33 ± 53	319 ± 382	546 ± 860	0.0006	37 ± 30	251 ± 401	541 ± 698	0.0002
ICS dose	0	1367 ± 637	1758 ± 486	<0.0001	0	762 ± 794	1452 ± 1172	<0.0001

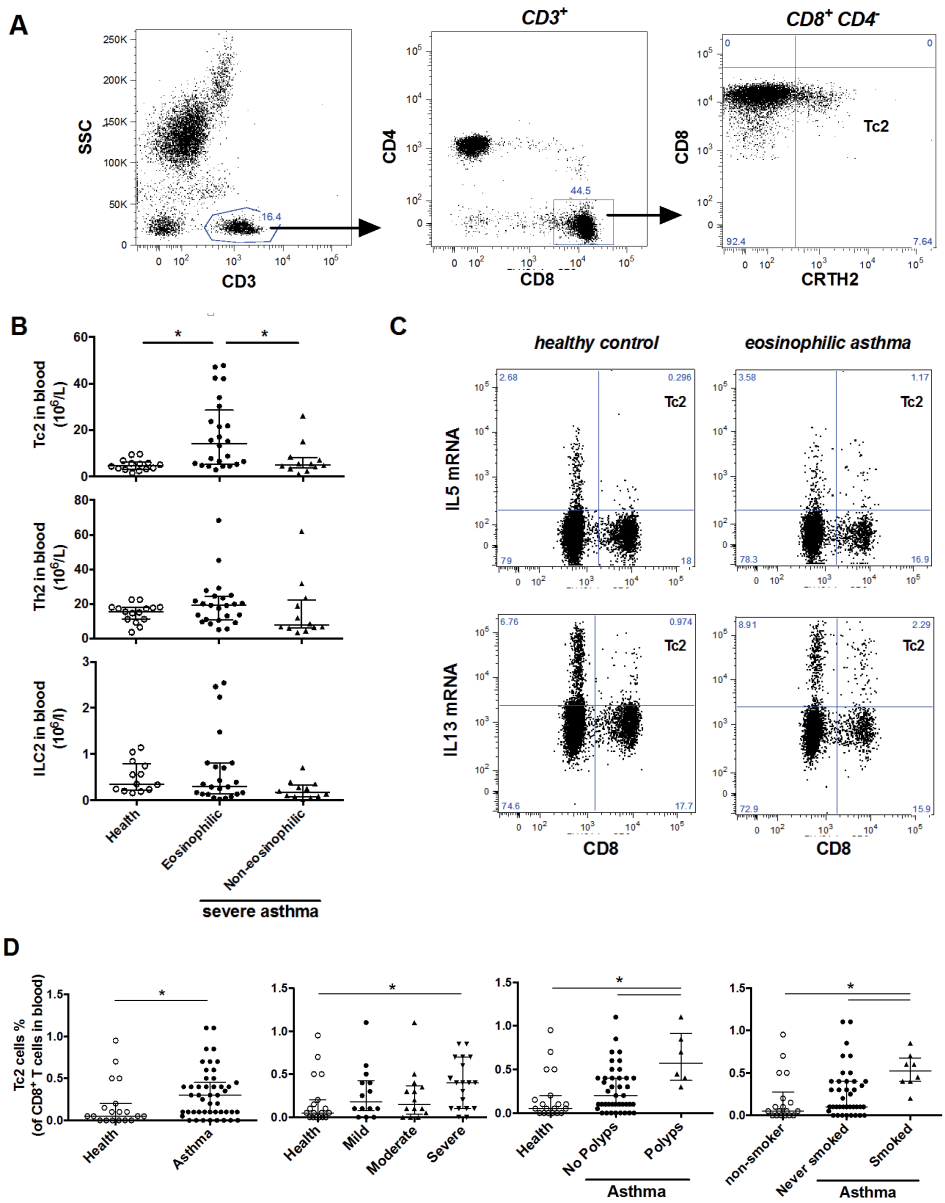


Figure 1: Tc2 cells are enriched in peripheral blood from patients with severe eosinophilic asthma.

(A) Tc2 cell gating strategy: human whole blood was stained with a mixture of antibodies against immune-cell surface markers and analysed with flow cytometry. $CD3^+CD8^+CD4^-CRTH2^+$ cells were gated as Tc2 cells. **(B)** Numbers of $CD3^+CD8^+CRTH2^+$ Tc2, $CD3^+CD4^+CRTH2^+$ Th2 and $Lin^-CD45^{high}IL\text{-}7R\alpha^+CRTH2^+$ ILC2 cells in peripheral blood were compared between healthy controls (n=15) and severe asthma patient groups (n=24 for eosinophilic and n=12 for non-eosinophilic, demographics in Table 1) in the Oxford cohort. Tc2 but not Th2 or ILC2 cells were enriched significantly in blood from the patients with severe eosinophilic asthma. **(C)** IL-5- and IL-13-producing Tc2 cells detected with PrimeFlow were increased in blood from patients with severe eosinophilic asthma.

Tc2 cells were found in the BAL fluid and sputum from the lungs of patients with severe eosinophilic asthma (**Figure 2A**). CD8 T-cells were detected abundantly in sections of bronchial biopsies (BB) from the same group of patients (**Figure 2B**). Consistent with the findings in peripheral blood (**Figure 1**), we observed a striking increase of $CD3^+CD8^+IL^-13^+$ cells in BB and BAL fluid in eosinophilic asthma (~2.05%, n=8 for BB; ~1.5%, n=9 for BAL) compared with non-eosinophilic asthma (~0.1%, n=24 for BB; ~0.2%, n=26 for BAL) and healthy controls (~0.2%, n=13 for BB; ~0.2%, n=17 for BAL; $p<0.005$) in the Southampton cohort (**Figure 2C** and **2D** upper panels). Again, by contrast, Th2 cell frequencies in both BB and BAL were increased equally but non-significantly in eosinophilic and non-eosinophilic forms (**Figures 2C** and **2D** bottom panels). When we investigated clinical correlates of Tc2 inflammation, we observed that amongst asthmatics, high frequencies of BB Tc2 cells were associated with high bronchodilator reversibility (Figure S4B, $p<0.05$).

Stimulatory eicosanoid receptors and mediators enriched in eosinophilic asthma

Since Tc2 cells highly express CRTH2 and $CysLT_1$, a leukotriene receptor (**Figures S1** and **S5**), we also compared the expression level of CRTH2 and $CysLT_1$ in the cells and the levels of their ligands in lung between asthma phenotypes (**Figures 2E-H**). In the Southampton cohort when CRTH2 expression was measured by microarray on sorted populations of $CD3^+$ T-cells from induced sputum, we observed an increased expression in eosinophilic (median relative expression 1.10, n=6) compared with non-eosinophilic disease (0.984, n=25, $p<0.01$) (**Figure 2E** upper panel), although these differences were not significant in blood Tc2 measured by flow cytometry in the Oxford cohort (**Figure 2E** bottom panel). No significant difference in the expression of $CysLT_1$ at gene level in sputum-derived $CD3^+$ T-cells (**Figure 2F** upper panel) and protein level in blood Tc2 cells (**Figure 2F** bottom panel) was detected. The concentration of PGD_2 in the airways assessed in induced sputum supernatant from asthma patients was significantly increased compared with healthy controls (~2.76±0.54 ng/g sputum, n=6, $p<0.01$) although no significant difference between eosinophilic (~5.4±0.59 ng/g sputum, n=11) and non-eosinophilic groups (~7.2±1.46 ng/g sputum, n=6, $p=0.14$) was detected (**Figure 2G**). LTE_4 was only significantly increased in the lung from the patients with severe eosinophilic asthma (~164.5±52 ng/g sputum, n=10) but not in non-eosinophilic patients (~1.84±0.65 ng/g sputum, n=8) compared with that in healthy controls (~0.34±0.15 ng/g sputum, n=5, $p=0.1$) (**Figure 2H**).

Figure 1: *Continued.*

(D) Frequencies of IL-13 secreting $CD3^+CD8^+$ T-cells in peripheral blood determined by flow cytometry with intracellular cytokine staining were increased in asthmatic patients from the Southampton cohort (left panel), which was associated with asthma severity according to global physician assessment (left middle panel), history of nasal polyposis (right middle panel, Jonckhere-Terpstra test for linear trend) and history of smoking (right panel). *$p<0.05$, (Data in C are representative of 3 independent experiments).

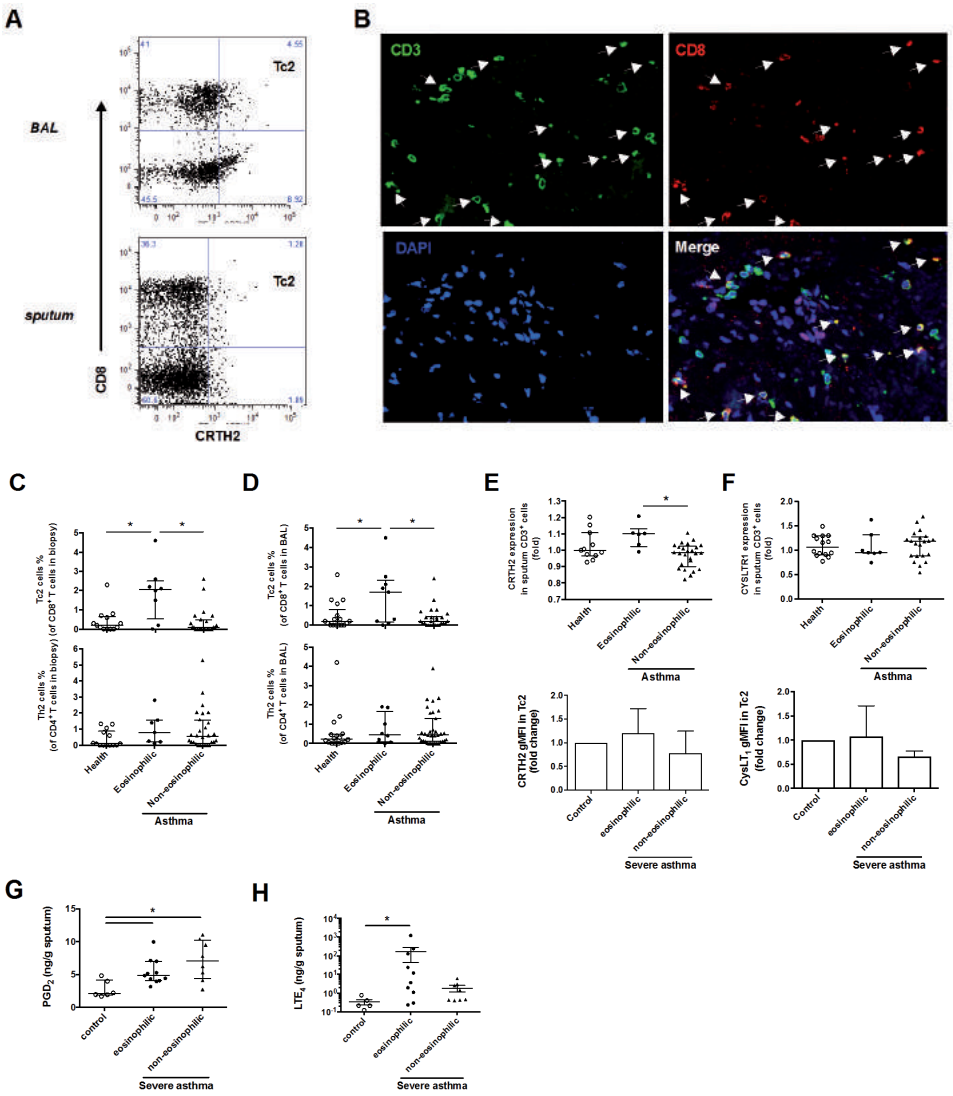


Figure 2: Tc2 cells, eicosanoid mediators and their receptors are increased in the lung from patients with eosinophilic asthma.

(A) Tc2 cells were detected in the BAL and sputum from severe asthma patients by flow cytometry. **(B)** $CD3^+CD8^+$ T cells (arrows) were detected in bronchial biopsies by using immunohistochemistry. **(C-D)** Frequencies of $CD3^+CD8^+IL\text{-}13^+$ but not $CD3^+CD4^+IL\text{-}13^+$ T-cells in bronchial biopsies **(C)** and BAL fluid **(D)** were increased in eosinophilic but not in non-eosinophilic asthma compared with healthy individuals determined with intracellular cytokine staining. **(E)** Relative expression of CRTH2 in T-cells, measured by microarray in FACS-sorted $CD3^+$ cells from induced sputum from the Southampton cohort, was up-regulated in eosinophilic (n=6) but not in non-eosinophilic (n=25) asthma compared with healthy controls (n=11) (upper panel). However, the up-regulation of CRTH2 by blood Tc2 cells determined by paired comparison between healthy controls (n=9) and severe asthma groups (n=4 for eosinophilic and n=5 for non-eosinophilic) from the Oxford cohort with flow cytometry was not significant (bottom panel). Healthy controls were used to define 1-fold.

Tc2 cell migration induced by PGD2 and LTE4

To explore the potential role of Tc2 cells and the lipid mediators enriched in eosinophilic asthma in the pathogenesis of lung inflammation in eosinophilic asthma, we isolated Tc2 cells from human blood and cultured them for further *in vitro* investigation (**Figure S6A**). In culture, these cells showed at baseline higher type-2 gene expression signatures (*IL17RB*, *GPR44*, *CLECL1*, *IL9R*, *NAMPT*, *AF208111*, *HPGDS*, *P2RY14*, *RG4*, *IRS2* and *GATA3*) but lower type-1 (*IFNG*, *AIF1*, *LTA*, *TXK* and *IL18RAP*) and killer cell gene signatures (*KIR2DL1*, *KIR2DL4*, *KIR2DL5A*, *KLRF1*, *CD160* and *TYPOBP*) compared with other $CD8^+$ cells (**Figure S6B**).

PGD_2 and cysLTs are strong chemotactic agents for Th2 cells and many other types of immune cells [27,30,34]. To investigate the potential mechanism of recruitment of Tc2 cell to inflamed airways, we examined the effect of these lipids on Tc2 migration. In a standard chemotaxis assay (**Figure 3**) both lipids caused cell migration in a typical bell-shaped dose-dependent manner, peaking at ~30 nM for PGD_2 and ~10 nM for LTE_4. The maximum response induced by PGD_2 was higher (2.4 fold) than that induced by LTE_4 (**Figure 3A**). Cell migration was synergistically enhanced by combined stimulation (**Figure 3B**). The contribution of PGD_2 and LTE_4 on the cell migration was blocked by the CRTH2 antagonist TM30089 and the $CysLT_1$ antagonist montelukast.

Enhancement of type–2 cytokine production in Tc2 cells by PGD2 and LTE4

Type-2 cytokines (IL-4, IL-5, IL-9 and IL-13) are important biomarkers associated with biological function of Tc2 cells. We investigated the effects of PGD_2 or LTE_4 on type-2 cytokine production in Tc2 cells (**Figure 4**). After treatment with increasing concentrations of PGD_2 or LTE_4 for 3 hours, IL-5 and IL-13 production was elevated at both transcriptional (**Figure 4A**) and translational (**Figure 4B**) levels in a dose-dependent manner with EC_{50}=17.3 nM at mRNA) or 17.9 nM at protein on IL-5, and 21.1 nM at mRNA or 16 nM at protein on IL-13 for PGD_2; and EC_{50}=4.5 nM at mRNA or 13.5 nM at protein on IL-5, and 7.4 nM at mRNA or 9 nM at protein on IL-13 for LTE_4. The cell responses to PGD_2 were significantly stronger than those to LTE_4. Compared with other type-2 immune cells (Th2 and ILC2),[25,27,35] the effect of PGD_2 on Tc2 cells is much more potent (**Figure 4C**). The effect of LTE_4 is also more potent in Tc2 cells than in Th2 cells (**Figure 4C**).

Figure 2: *Continued.*

(F) Expression of CysLT1 (CYSLTR1) in T cells from sputum measured by microarray did not differ between healthy controls (n=14) and asthmatic groups (n=7 for eosinophilic, n=21 for non-eosinophilic) from the Southampton cohort (upper panel). Expression of $CysLT_1$ in blood Tc2 detected by comparison paired between healthy controls (n=6) and severe asthma groups (n=6 for eosinophilic and n=2 for non-eosinophilic) from the Oxford cohort by flow cytometry did not differ significantly (bottom panel). **(G-H)** Levels of PGD_2 **(G)** and LTE_4 **(H)** in sputum from the Oxford cohort determined with ELISA were compared between healthy controls (n=6 for PGD_2 and n=5 for LTE_4) and severe asthma groups (n=10 for eosinophilic and n=8 for non-eosinophilic). *p<0.05. (Data in **A** are representative of >10 independent experiments; Data in **B** are representative of 3 independent experiments).

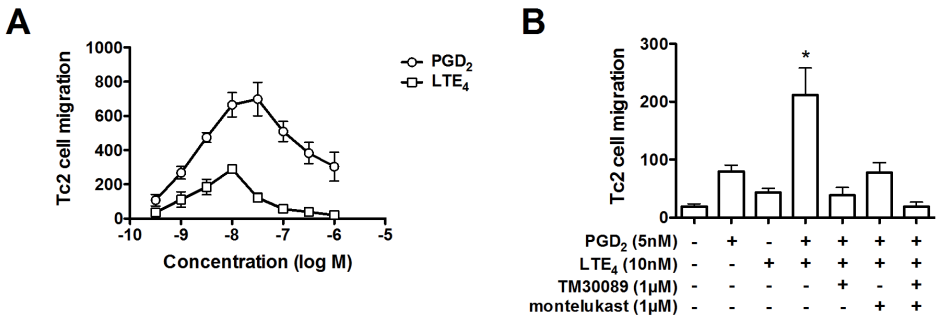


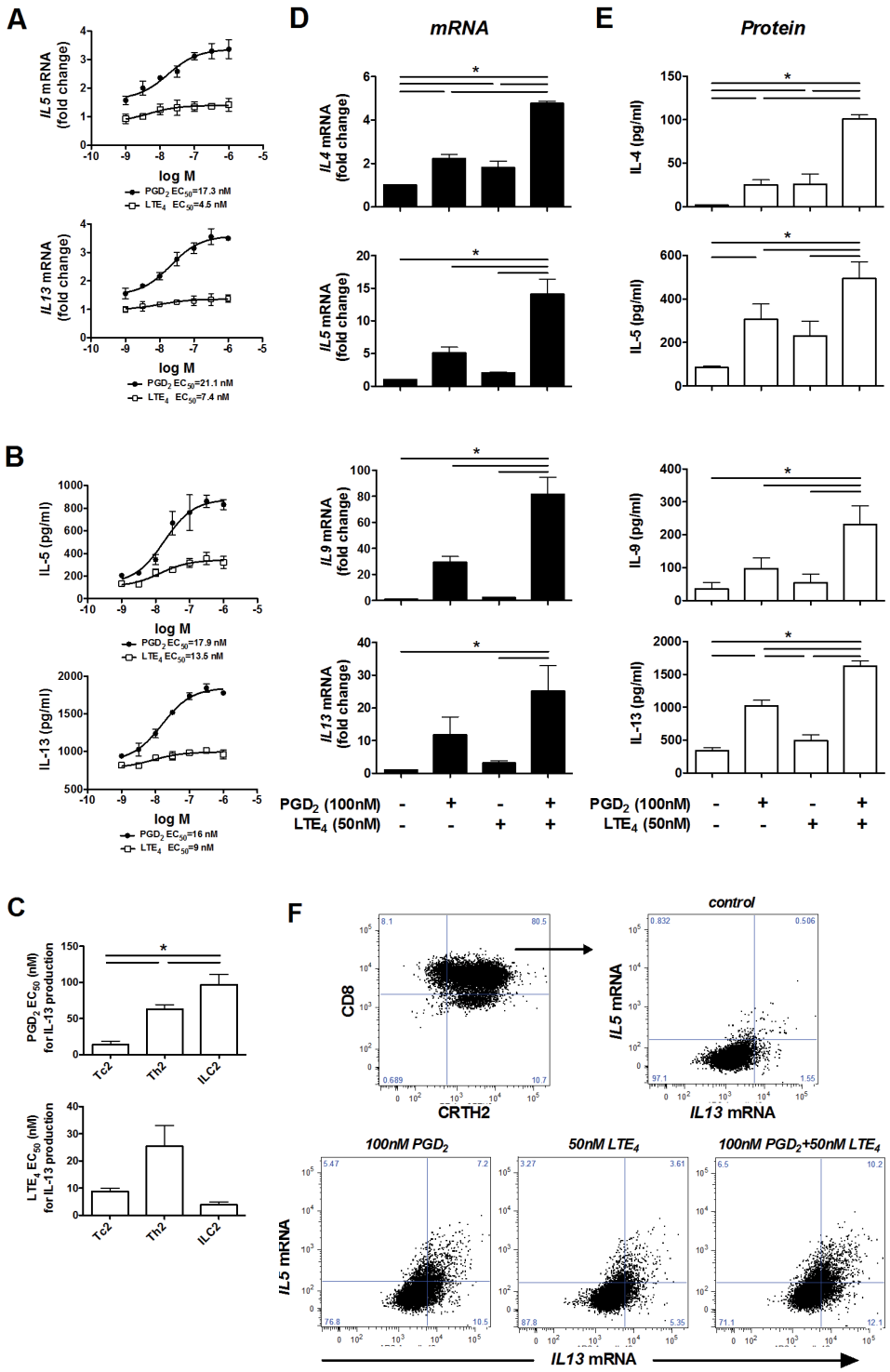
Figure 3: Tc2 cells migrate in response to PGD_2 and LTE_4.

(A) Cell migration in response to various concentrations of PGD_2 and LTE_4 in chemotaxis assays. **(B)** Cell migration in response to the combination of PGD_2 and LTE_4 in the absence or presence of TM30089 and montelukast. **p*<0.0001, (n=3).

We then further examined type-2 cytokine production by Tc2 cells in response to the lipids alone (100 nM for PGD_2 and 50 nM for LTE_4) or combination (**Figures 4D-E**). Both PGD_2 and LTE_4 increased type 2 cytokine (IL-4/5/9/13) production. As previously noted (**Figures 4A-B**), the effect of LTE_4 was weaker compared with that of PGD_2. However, the combination of these lipid mediators significantly enhanced the response, which was synergistic rather than additive. Using PrimeFlow assays to analyse the mRNA of IL-5 and IL-13 at individual cell level confirmed these data (**Figure 4F**). IL-5/13 positive cells were increased from 2.88% (untreated cells) to 23.17%, 12.23% or 28.8% after treatment with PGD_2, LTE_4 or their combination respectively (**Figure 4F**). Among these positive cells, a fraction expressed IL-5 dominantly, a fraction IL-13 dominantly, and only some produced IL-5 and 13 simultaneously, although almost all these cells (>90%) were capable of producing both cytokines following PMA/ionomycin stimulation (**Figure S7**).

Figure 4: PGD_2 and LTE_4 promote type 2 cytokine production in cultured Tc2 cells. →

(A) mRNA levels in Tc2 cells and **(B)** protein levels in the cell supernatants for IL-5 and IL-13 after treatment with various concentration of PGD2 and LTE4. **(C)** EC_{50} of PGD_2 or LTE_4 for IL-13 production in Tc2 cells compared with that in Th2 and ILC2 cells. **(D)** mRNA levels in Tc2 cells and **(E)** protein levels in the cell supernatants for type 2 cytokines after treatment with PGD_2 and LTE_4 alone or their combination. The mRNA levels in control samples were treated as 1-fold. **(F)** Increase of IL-5- and IL-13-mRNA positive Tc2 cells after treatment with PGD_2 and LTE_4 alone or their combination detected by using PrimeFlow RNA assay. **p*<0.05, (n=3 for **A**, **B**, **C** and **F**; n=6 for **D** and **E**).



Effect of PGD2 and LTE4 on the gene expression profile of Tc2 cells

To broadly explore the pathogenic features of Tc2 cells and their interaction with PGD_2 and LTE_4, we investigated the cellular transcriptional responses to the lipids added either alone or in combination using microarrays. Three experimental replicates were prepared for each of the 4 groups (control, LTE_4, PGD_2, and their combination). The data showed broad transcriptional changes after treatment. The mRNA levels of 1104, 3360, and 4593 genes were significantly modulated (P<0.05) (including upregulation and downregulation) by LTE_4, PGD_2, or their combination, respectively (**Figure S8A**). The effect of PGD_2 was much broader and stronger than that of LTE_4, and the effect of the combination treatment was mainly contributed by PGD_2 (**Figure S8B**).

To focus on the gene regulation potentially relevant to T cell-mediated inflammation, we next studied the genes encoding cytokines, chemokines, their receptors, and cluster of differentiation (CD) molecules (**Figure 5A**; **Table S1**). A total of ~90 of these genes were significantly modulated, most of them upregulated, and the most obvious effects were on cytokines (*IL3*, *IL5*, *IL8*, *IL13*, *IL22*, *CSF2*, *TNF* and *XCL1*) (**Table S1**). Although a few of these were induced by LTE_4 alone (*e.g.*, *GDF11*, *TNFSF13*, *CCL7*, *IL6R*, *CD14*, *CD46*, *CD99* and *CD99L2*), most were driven by PGD_2 alone or by the combination. Some transcriptional changes were regulated only by the combination treatment (*e.g.*, *IL21*, *IL22*, *LASS1*, *CCL3*, *CCL4*, *IL1RL1*, *CD1E* and *CCL21*). The microarray data were largely confirmed by PCRarray on human common cytokines although some significant effects (*IL9* and *CSF1*) were detected only in PCRarray (**Figure 5B**).

To further verify these finding, we conducted q-PCR (**Figure 5C**) and Luminex (**Figure 5D**) assays on selected cytokines. At the mRNA level, most of genes (except *CSF1*) showed synergistic effects of PGD_2 and LTE_4. At the protein level, the effects of LTE_4 were marginal in some cytokines (IL-3, IL-21 and IL-22), while the stimulatory effects of PGD_2 were obvious in all the genes, and particularly significant in IL-8, IL-22, GM-CSF, CSF1, TNFα and CCL4. Combination treatment either additively (CSF1) or synergistically (all other genes) enhanced cytokine production. We also noticed that IL-2 enhanced the effect of the lipids on some cytokines (IL-5/8/13 and GM-CSF) production, particularly in PGD_2 stimulation (**Figure S9**). The stimulatory effects of PGD_2 and LTE_4 on cytokine production by Tc2 cells were inhibited by TM30089 and montelukast respectively (**Figure 5E**).

Cytotoxic proteins are signature proteins of $CD8^+$ cells. We therefore examined the effects of PGD_2 and LTE_4 on the production of perforin and granzymes (GZMA, GZMB and GZMK) in Tc2 cells (**Figure S10**). The cells expressed these without stimulation, and there was no significant effect of the lipids, although the activation of the cells by PMA (25 ng/ml) and ionomycin (750 ng/ml) down-regulated their expression.

Effect of endogenous PGD2 and LTE4 on the activation of Tc2 cells

To investigate the mechanism of Tc2 activation under more physiological conditions, we evaluated the impact of endogenously synthesized eicosanoids on Tc2 function. Human

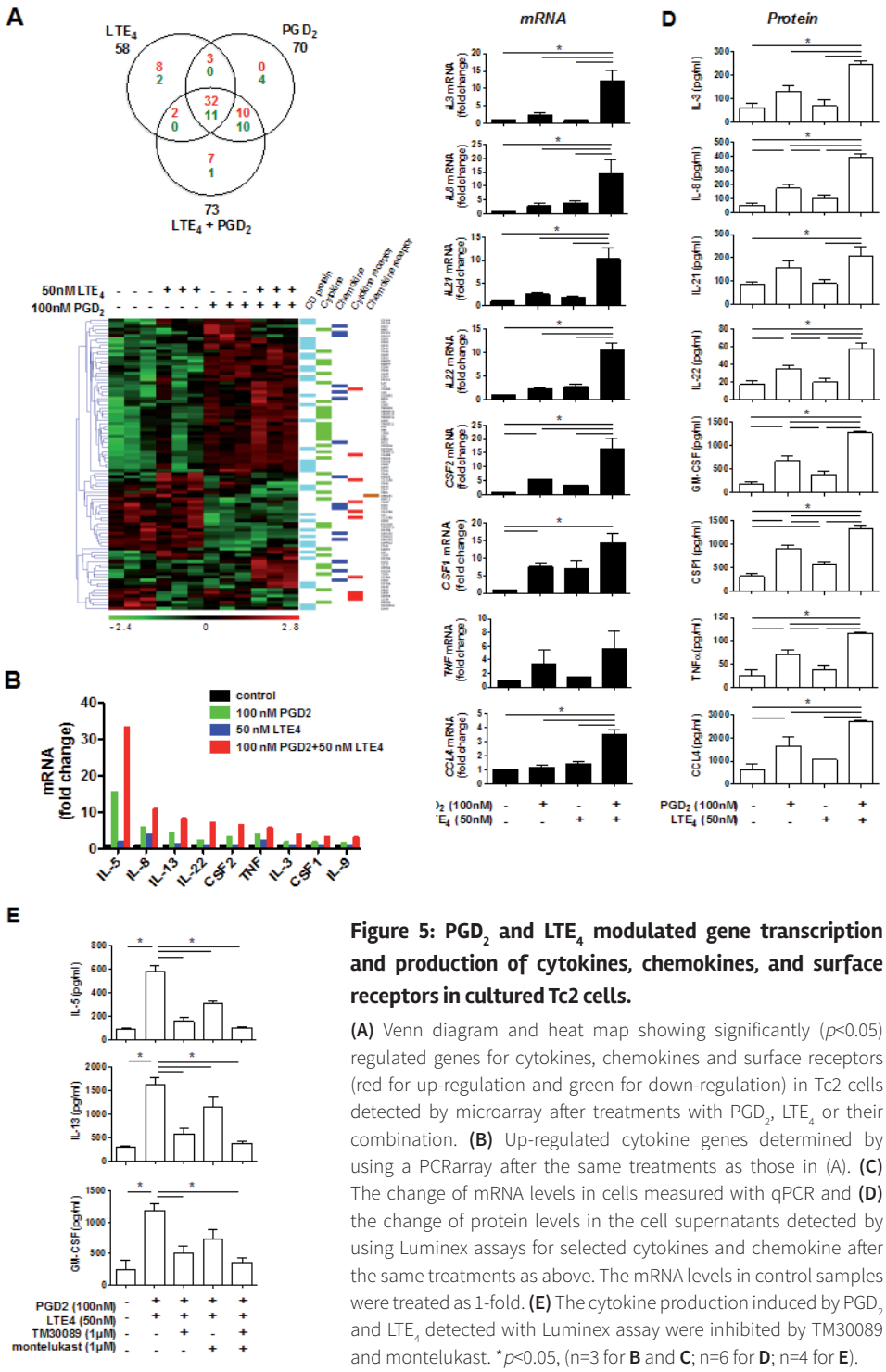


Figure 5: PGD_2 and LTE_4 modulated gene transcription and production of cytokines, chemokines, and surface receptors in cultured Tc2 cells.

(A) Venn diagram and heat map showing significantly (p<0.05) regulated genes for cytokines, chemokines and surface receptors (red for up-regulation and green for down-regulation) in Tc2 cells detected by microarray after treatments with PGD_2, LTE_4 or their combination. **(B)** Up-regulated cytokine genes determined by using a PCRarray after the same treatments as those in (A). **(C)** The change of mRNA levels in cells measured with qPCR and **(D)** the change of protein levels in the cell supernatants detected by using Luminex assays for selected cytokines and chemokine after the same treatments as above. The mRNA levels in control samples were treated as 1-fold. **(E)** The cytokine production induced by PGD_2 and LTE_4 detected with Luminex assay were inhibited by TM30089 and montelukast. *p<0.05, (n=3 for **B** and **C**; n=6 for **D**; n=4 for **E**).

mast cells were treated with IgE followed by crosslinking using an anti-IgE antibody (**Figure 6A**). Only low levels of PGD_2 (~0.1 ng/ml) and LTE_4 (~6 ng/ml) were detected in the supernatant from the mast cells before stimulation (supernatant UM). The supernatant from IgE/anti-IgE activated mast cells (supernatant SM) contained high levels of PGD_2 (~11.5 ng/ml) and LTE_4 (~86.8 ng/ml), which were similar to the concentrations detected in sputum from patients with asthma (**Figures 2C-D**). Using these supernatants to treat Tc2 cells illustrated that the supernatant SM induced Tc2 cell migration (**Figure 6B**) and marked cytokine (IL-5 and IL-13) production (**Figure 6C**). Both of these effects were reduced by TM30089 and montelukast and completely inhibited by the combination of the two compounds.

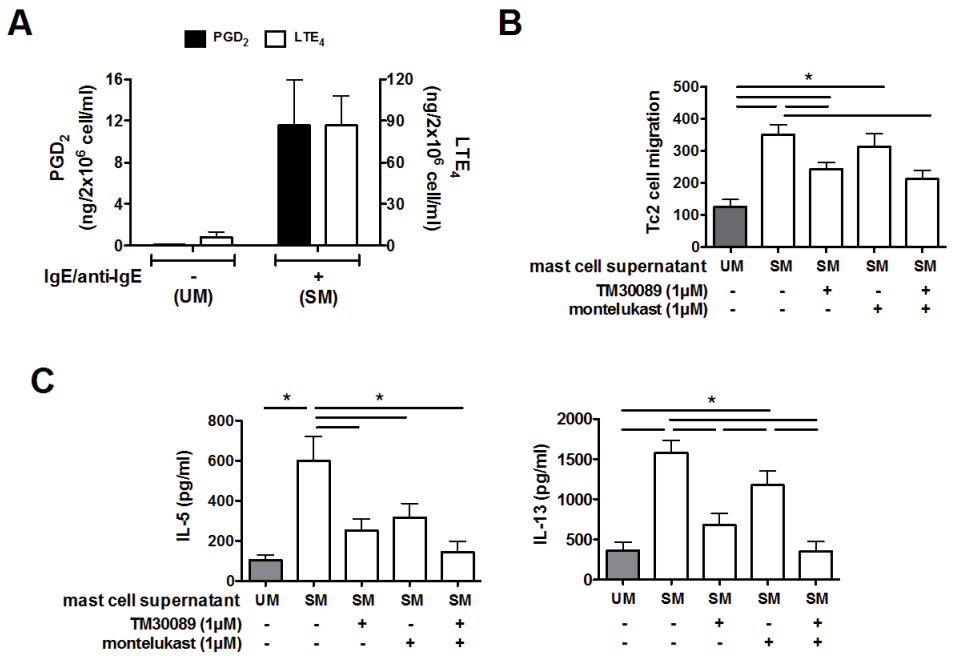


Figure 6: Tc2 cell activation in response to mast cell supernatant containing endogenous PGD_2 and LTE_4 is mediated by receptors sensitive to TM30089 and montelukast respectively.

(A) Levels of PGD_2 (black bars) and LTE_4 (white bars) were increased in supernatants from mast cells stimulated with IgE and anti-IgE antibodies (supernatant SM) compared with supernatants from cells without stimulation (supernatant UM). **(B)** More Tc2 cells migration to supernatant SM (white bars) than to supernatant UM (gray bar) in a chemotaxis assay was reduced by TM30089 and montelukast. **(C)** IL-5 and IL-13 productions in Tc2 cells were significantly increased in response to supernatant SM (white bars) compared with that to the supernatant UM (grey bars), and were inhibited by TM30089 and montelukast. * $p<0.05$, (n=3 for **A** and **B**; n=4 for **C**).

The role of Tc2 in eosinophilia

Eosinophil enrichment (eosinophilia), intrinsic to severe eosinophilic asthma, plays a critical role in lung inflammation [8]. Cytokines produced by activated Tc2 cells included several that could potentially interact with eosinophils (**Figures 4-5**). To investigate the potential role of Tc2 cells in eosinophilia, we examined the ability of Tc2 products from Tc2 conditioned media to elicit relevant changes in human eosinophil behaviour (**Figure 7**). Since PGD_2, LTE_4, PMA and ionomycin are also strong stimulators to eosinophils, to avoid cross-stimulation, Tc2 cells were stimulated with anti-CD3 and anti-CD28 antibodies (**Figure 7A**). This treatment promoted secretion of IL-5 and GM-CSF from resting levels ~600 and ~1300 pg/ml respectively in the supernatant from unstimulated Tc2 cells (supernatant UT) to ~1290 and ~2900 pg/ml respectively in the supernatant from stimulated cells (supernatant 3/28).

First, we evaluated the effect of IL-5 derived from Tc2 on eosinophil recruitment using an eosinophil shape change assay, a biomarker of eosinophil migration (**Figure 7B**) [36]. Recombinant human IL-5 (rhIL-5) induced eosinophil shape changes in fresh blood in a dose-dependent manner (**Figure S11A**). This effect could be inhibited with a neutralizing antibody against IL-5. The Tc2 supernatant 3/28 containing a high concentration of IL-5 had a stronger capacity to induce eosinophil shape change *ex vivo* compared with supernatant UT (**Figure 7B**). The eosinophil shape change was significantly, but not completely, inhibited by IL-5 neutralizing antibody suggesting involvement of IL-5 and other eosinophil-active factors.

Next, we examined the influence of GM-CSF released by Tc2 cells on eosinophil survival by measuring its ability to rescue cells from serum starvation-induced apoptosis (**Figure 7C**). The increase of Annexin V-positive (apoptotic) eosinophils after serum withdrawal was inhibited by rhGM-CSF in a dose-dependent manner, which was reversed by a neutralizing antibody against GM-CSF (**Figure S11B**). Similar protection against apoptosis was observed when the supernatant from activated Tc2 cells (3/28) was substituted for rhGM-CSF (**Figure 7C**). The protective capacity of supernatant 3/28 was reduced by the GM-CSF neutralizing antibody.

Several lines of evidence suggest that eotaxins play a central role in eosinophil recruitment to the lung tissue of patients with asthma [37]. Bronchial epithelial cells are an important source of eotaxins in lung, and the expression of eotaxins in these cells is regulated by type-2 cytokines IL-4 and IL-13 [38]. We, therefore, investigated the potential role of IL-4 and IL-13 derived from Tc2 cells on eotaxin production in A549, a human alveolar epithelial cell line. As expected, the generation of eotaxins, particularly eotaxin-2 (CCL24) and eotaxin-3 (CCL26), by A549 cells was induced by rhIL-4 and rhIL-13 (**Figure S12A**), and was reversed by neutralizing antibodies against IL-4 or IL-13 (**Figure S12B**). Using the supernatant of Tc2 culture (UT) to replace rhIL-4/13 also up-regulated eotaxin production except eotaxin-1 (CCL11) in A549 (**Figure 7E**). The supernatants from Tc2 cells activated by anti-CD3/CD28 antibodies (3/28) or PGD_2 combined with LTE_4 (P/L) contain increased concentrations of IL-4 and IL-13 compared with the supernatant UT (**Figure 7D**), and showed even higher capacities to induce eotaxin production (**Figure 7E**). The eotaxin induction by Tc2 media was significantly inhibited by anti-IL-4 and IL-13 neutralizing antibodies.

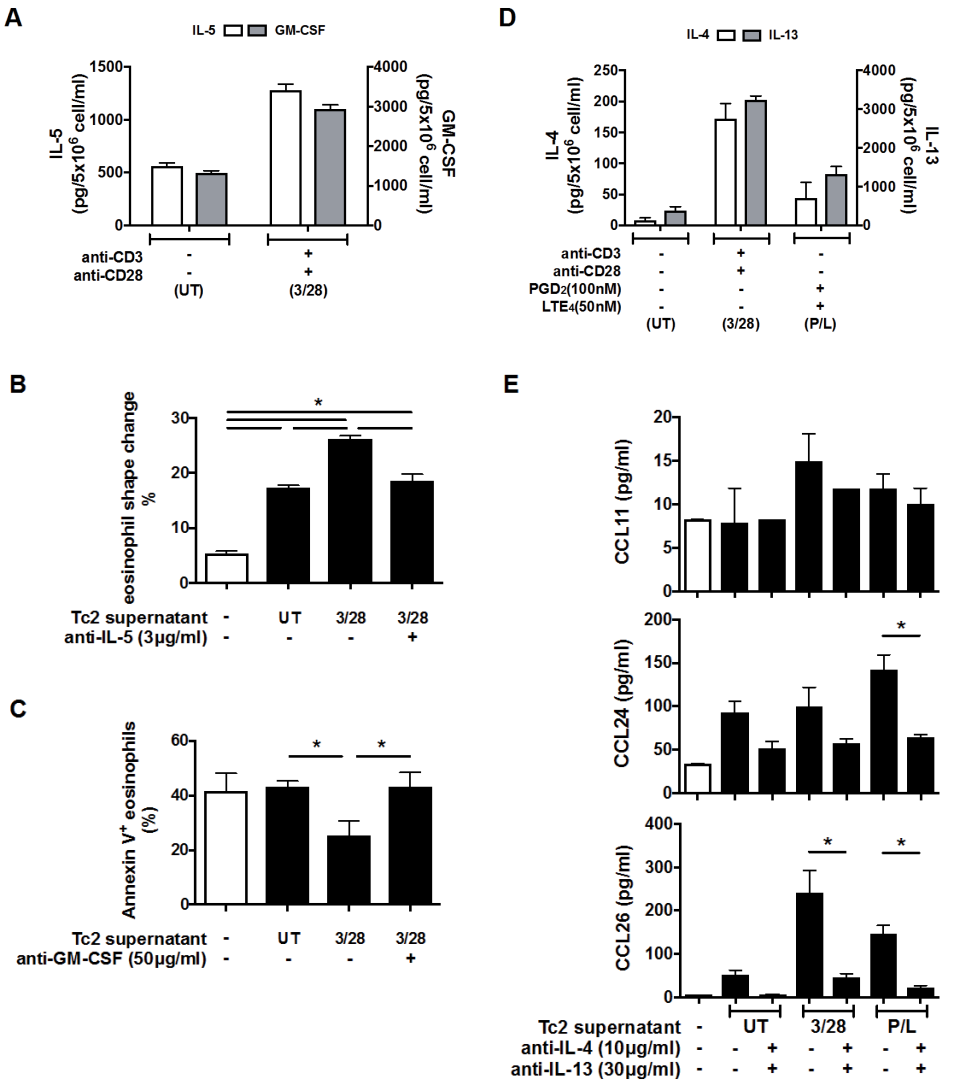


Figure 7: Cytokines released by human Tc2 cells promote eosinophil shape change and survival, and induce eosinophil chemokine production from airway epithelial cells.

(A) Concentrations of IL-5 (white bars) and GM-CSF (gray bars) were increased in the supernatants from Tc2 cells treated with anti-CD3 and CD28 antibodies (supernatant 3/28) compared with the supernatants from the cells without treatment (supernatant UT). **(B)** Supernatant 3/28 induced more eosinophil shape change in fresh blood than supernatant UT, which was reversed by adding anti-IL-5 antibody. **(C)** Supernatant 3/28 reduced annexin V positive eosinophils induced by serum withdrawal in the culture, which was reversed by adding anti-GM-CSF antibody. **(D)** Concentrations of IL-4 (white bars) and IL-13 (grey bars) were increased in the supernatants of Tc2 cells treated with anti-CD3 and CD28 antibodies (supernatant 3/28) or 100 nM PGD2 and 50 nM LTE_4 (supernatant P/L) compared with untreated cells (supernatant UT). **(E)** Tc2 supernatants, particularly from treated cells, induced CCL11/24/26 (eotaxin-1/2/3) productions in A549 cells, which were inhibited significantly by neutralizing antibodies against IL-4 and IL-13. **p*<0.05, (n=3 for A; n=4 for **B**, **C**, **D** and **E**).

Discussion

Tc2 cells are type-2 cytokine secreting $CD8^+$ T lymphocytes that highly express CRTH2. In this study, we found that Tc2 cells were enriched in patients with severe asthma and persistent eosinophilia despite treatment with corticosteroids. Importantly, a similar conclusion was achieved in two independent asthma cohort studies with different strategies - Tc2 cells were enriched in both peripheral blood and lung samples and correlated with disease severity. The lipid mediators PGD_2 and LTE_4 were also increased in the lungs in eosinophilic asthma. The interaction of Tc2 cells with these lipid mediators induced strong activation of Tc2 cells, leading to cell migration and pro-inflammatory protein production. In turn, the cytokines produced by activated Tc2 cells also promoted eosinophil recruitment and survival directly or indirectly, suggesting a mechanistic interplay between these two cell types. All these data provide compelling evidence for a role for Tc2 cells in the pathogenesis of eosinophilic asthma.

$CD4^+$ Th2 lymphocytes have long been believed to be the major driver in asthma pathogenesis, particularly to allergic asthma, as Th2 cytokines (such as IL-4, IL-5 and IL-13) play key roles in causing many features of the disease. Elevated levels of blood Th2 cells have been observed in severe asthma, although they are not associated with eosinophilia [39]. In a separate analysis of the Southampton cohort of asthma patients, we have shown blood and tissue $CD4^+$ T-cells to be elevated only in mild, but not severe asthmatics [33], even though a proportion of severe asthmatics had evidence of atopy and had high concentrations of type-2 cytokines in their airways. This suggested a potential different cell source for type-2 cytokines. Following the discovery of ILC2s, this novel group of type-2 cytokine producing lymphocytes has recently been considered as important new players mediating both innate and adaptive responses in asthma and allergy. In mouse asthma models, ILC2 can induce airway hyperreactivity in the absence of Th2 cells [40], and contribute to airway eosinophilic inflammation [21]. It has been reported that circulating blood ILC2 are increased in asthma patients [22]. In the asthma cohorts in the current study, we found that, although Th2 and ILC2 cells were slightly increased in asthmatic blood samples, these increases were not significant. Although we did not find raised ILC2 counts in blood, we cannot exclude the possibility that their numbers are increased in the lungs. In contrast, in the current study Tc2 cells were significantly increased in numbers and this enrichment was associated with the severity of the disease, the presence of nasal polyps and bronchodilator reversibility. Tc2 enrichment was also positively correlated with the increase of type-2 cytokine expression in the lung. In contrast, a Th2 increase was detected in mild asthma, and the frequencies of Th2 cells were negatively correlated with the expression of type-2 cytokine in the lung. Increase of Tc2 cells was also reported in atopic asthma and other non-atopic eosinophilic patients [41,42]. It was also noted in the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) project study that high submucosal $CD8^+$ cells in bronchial biopsies were associated with high blood, sputum and submucosal eosinophils, and high expression of type-2 cytokines [43]. The activation of circulating $CD8^+$ but not $CD4^+$ T-cells detected with microarray is associated with severe asthma [44]. Furthermore, in a mouse

model, prevention of Tc2 cell differentiation by vitamin D3 reduced asthma development [45]. Therefore, our findings strongly support the hypothesis that Tc2, a previously unappreciated lymphocyte subset, could be a key cell type contributing to type-2 immunity in asthma, particularly in the severe eosinophilic form of asthma.

Airway eosinophilia has long been associated with exacerbations of asthma [46] and has been suggested to play a role in airway remodelling [47]. Eosinophils are granulocytic leukocytes characterized by the ability to store and release multiple specific basic proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN) and a number of proinflammatory cytokines, chemokines and growth factors including IL-2/3/4/5/10/12/13/16/25, CCL5/11/17/22, TNF and TGFα/β [48]. These proteins can mediate eosinophil effector functions in the host defence against pathogens (*e.g.*, helminths). However, in asthma, these granule proteins are cytotoxic and disrupt the protective pulmonary epithelial barrier allowing further inflammatory responses to occur [48,49]. The relevance of eosinophilic inflammation in asthma is perhaps best illustrated by response to therapies. Sputum eosinophilia in asthma is associated with a good response to corticosteroid therapy [50]. Other potential clinical strategies such as anti-IgE, anti-IL5 and CRTH2 antagonism aimed to normalize sputum eosinophils reduced exacerbation frequency and severity in clinical trials in asthma [12,13,28,29,46]. Therefore, enrichment of eosinophils in the asthmatic lung is closely linked to the pathogenesis of eosinophilic asthma. Our *in vitro* mechanistic data have indicated that the activation of Tc2 cells can contribute directly or indirectly to airway eosinophilia. The IL-5 and GM-CSF secreted by activated Tc2 cells are sufficient to promote eosinophil migration and survival. Other Tc2 cytokines including IL-4 and IL-13 can stimulate airway epithelial cells strongly to produce eotaxins, potent chemokines to eosinophils via CCR3. Eotaxins function as orchestrators with IL-5 to promote eosinophilia in the inflamed lung [51]. In mice with allergic airway sensitization, $CD8^+$ T-cells were more critical than $CD4^+$ T-cells in mediating respiratory syncytial virus induced lung eosinophilia and airway hyperresponsiveness [52]. In a mouse model with virus-specific Tc2 cells, activation of Tc2 cells by viral infection induced lung eosinophilia [53]. All this evidence suggests an important role of Tc2 cells in eosinophil-mediated lung inflammation.

Unlike Th2 cells, *in vivo*-differentiated Tc2 cells retain some of their ability to produce the cytotoxins perforin, granzymes and granulysin when exposed to infected/dysfunctional somatic cells [54]. Through the action of these cytotoxic proteins, they trigger apoptosis in targeted cells, raising the possibility that the death of target cells in Tc2 reactions may potentiate the inflammatory response. In-vitro cultured Tc2 cells used in our study exhibited their capacity to release cytotoxins. However, no effect of PGD_2 and LTE_4 on the production of cytotoxins in Tc2 was detected.

Both PGD_2 and cysLTs are products of the oxidative metabolism of arachidonic acid derived mainly from activated mast cells in a IgE-dependent mechanism, although other types of inflammatory cells such as eosinophils, basophils, macrophages, dendritic and Th2 cells may also contribute to their production in some circumstances. These lipid mediators have

been detected in high concentrations at sites of allergic inflammation and play important roles in promoting airway inflammation and deterioration in lung function [55]. In our asthma cohort, PGD_2 was increased in the airways from both eosinophilic and non-eosinophilic severe asthma, while LTE_4 was significantly enriched only in severe eosinophilic asthma, as noted by other investigators [56]. Since most patients with severe eosinophilic asthma are non-atopic with low levels of IgE, the key cellular source of these lipids is still unclear. LTE_4 is a stable metabolic product of cysLTs and the dominant form detected in biological fluids [57]. Monitoring LTE_4 levels in the urine, sputum and exhaled air is an index of activity of the cysLT synthesis pathway [58]. LTE_4 has lower affinity than LTC_4 and LTD_4 for both $CysLT_1$ and $CysLT_2$ receptors [59,60]. However, our previous studies have demonstrated that LTE_4 showed higher efficacy than LTD_4 and LTC_4 in human Th2 and ILC2s, and the activity of LTE_4 in these cells was completely inhibited by the $CysLT_1$ antagonist montelukast [30,31,35]. Combination of PGD_2 and cysLTs synergistically amplifies their pro-inflammatory effects in these cells. Human Tc2 cells highly express the receptors for PGD_2 (CRTH2) and cysLTs ($CysLT_1$). The level of CRTH2 is slightly increased in the Tc2 cells from eosinophilic asthma. Treatment with PGD_2 or LTE_4 induces strong activation of Tc2 cells. Of relevance, we showed the potency of PGD_2 on Tc2 was higher than that on Th2 and ILC2 cells, and the potency of LTE_4 in Tc2 was also higher than that in Th2 cells. The combination of PGD_2 and LTE_4 synergistically enhanced the responses in Tc2 cells. These effects were confirmed by using endogenous lipid mediators from activated human mast cells. These findings provide a potential mechanistic insight into the large clinical benefit of CRTH2 antagonism seen in severe eosinophilic asthma [28,29].

To define the impact of these mediators on Tc2 cell function we performed gene expression analysis. This suggested that their effects are not limited to induction of type-2 cytokines but rather include a broad range of different genes. They upregulated transcription of >2000 genes including genes for bioactive cytokines and chemokines for eosinophils. For example, IL-3, IL-5 and GM-CSF promote eosinophil differentiation and migration [61,62]. TNFα delays eosinophil apoptosis and contributes to eosinophilic inflammation of airway [63]. IL-25 plays important role in eosinophilic airway inflammation in a mouse model [64] and transgenic overexpression of IL-25 resulted in eosinophilia [65]. CCL3, CCL4 and CCL7 are chemokines for eosinophils targeting CCR1 and CCR3 and CCR5 [66,67]. The data from our *in vitro* studies indicate the critical roles of these lipids in Tc2-mediated eosinophilic activities. One relevant finding was the impact of PGD_2, LTE_4 and their combination on expression of IL-5 by Tc2 cells. The EC_{50} for PGD_2 induced IL-5 production was 17 nM, compared to 118 nM for ILC2 populations [27] and 63 nM for Th2 cells [25]. This suggests that Tc2 cells stimulated in this way generate a strong and early IL-5 response, a finding that could be relevant to the clear clinical benefit of anti-IL-5 in severe eosinophilic asthma [13,68].

In conclusion, we have shown in clinical cohorts that type-2 cytokine-producing $CD8^+$ Tc2 cells are significantly enriched in in both peripheral blood and lung tissues in patients with eosinophilic asthma, particularly in severe disease. The lipid mediators PGD_2 and LTE_4 are also increased in the lung from the same patients. These lipids are strong chemokines and stimulators of Tc2 cells, and function synergistically in the recruitment and activation of Tc2

cells. Besides type-2 cytokines, Tc2 cells activated by the lipids produce a range of other proinflammatory cytokines and chemokines, which are sufficient to contribute directly or indirectly to eosinophilia in lung. Therefore, Tc2 cells are a potentially important target for therapeutic interventions to control severe eosinophilic asthma.

Materials and methods

Patients and clinical samples

For the Oxford cohort, patients meeting the latest ATS/ERS definition of severe asthma (*8*) with an induced sputum eosinophil count of >3% (eosinophilic, 24 subjects) or <3% (non-eosinophilic, 12 subjects), and 15 healthy control subjects were recruited from Churchill Hospital, Oxford (**Table 1**). The ethics was approved by Leicestershire, Nottinghamshire Rutland Ethics Committee, UK (08/H0406/189). For the Southampton cohort, 22 healthy non-atopic participants, 10 with eosinophilic asthma (sputum eosinophil count >3%), and 42 non-eosinophilic asthma (**Table 1**) were enrolled from NIHR Southampton Respiratory Biomedical Research Unit and outpatient clinics at University Hospital Southampton [33]. The study was approved by the Southampton and South West Hampshire Research Ethics Committee B.

Peripheral blood was collected and directly used for flow cytometry. Sputum was induced with nebulized saline solution (3-5%) after pre-treatment with salbutamol. Selected sputum plugs were broken down with 0.2% DTT, filtered and separated for cells for flow cytometry analysis and microarray, and supernatants for ELISA analysis. Bronchial biopsies and bronchoalveolar lavage (BAL) fluid were collected under bronchoscopy [33]. The biopsies were dispersed with collagenase for 1 h to obtain bronchial mucosal cells, and BAL fluids were treated with 0.1% DTT and filtered for single cells, for flow cytometric analysis. For microarray, the cells from sputum were immediately FACS sorted for $CD3^+$ T-cells using a FACS Aria IITM cell sorter (BD Biosciences).

Tc2 cell preparation, culture and treatment

Human $CD8^+CRTH2^+$ Tc2 cells were isolated from human CD Leucocyte Cones (National Blood Service, Oxford, UK). Briefly, PBMC were prepared by Ficoll-Hypaque gradient, followed by CD8 cell isolation using MACS $CD8^+$ T-cell isolation kit. After a week's culture in AIM V medium 50 U/ml rhIL-2, and 100 ng/ml rhIL-4, Tc2 cells were isolated from the $CD8^+$ cell culture by positive selection using anti-human CD294 MicroBeads. The harvested cells were further amplified in X-VIVO 15 medium containing 10% human serum and 50 U/ml rhIL-2 before use.

For further gene or protein analysis, Tc2 cells were treated with PGD_2, LTE_4 or their combination in the presence or absence of other compounds at indicated concentrations for 4 h. The cell supernatants were collected for ELISA or Luminex assays, and the cell pallets were used for qPCR, RNAarray or microarray studies.

For preparation of Tc2 conditioned supernatants, the Tc2 cells were treated with immobilized anti-CD3 and anti-CD28 antibodies or PGD_2 (100 nM) and LTE_4 (50 nM) in RPMI for 4 h, and then the supernatants were harvested and stored as Tc2 conditioned media for the treatment of fresh blood, eosinophils or A549 cells.

Human mast cell culture and activation

Human mast cells were cultured and treated as described previously [35]. Briefly, the cells were cultured from $CD34^+$ progenitor cells isolated from human cord blood (National Blood Service, Oxford) in a medium containing rhSCF (100 ng/ml) and rhIL-6 (50 ng/ml) for >12 weeks. The cells were stimulated with human myeloma IgE (5 mg/ml) followed by goat anti-human IgE antibody (1 µg/ml) for 1 h. The supernatants of the cells were collected and measured for PGD_2 and LTE_4 with ELISA, or used as mast cell conditioned media for the treatment of Tc2 cells.

PrimeFlow RNA assay

The levels of transcription for IL-5 and IL-13 in individual Tc2 cells in whole blood or Tc2 cultures were analysed with a PrimeFlow RNA Assay kit (eBioscience, Waltham, US) according to the manufacturer's instructions. Briefly, fresh blood or purified Tc2 cells were treated with conditions indicated for 4 h or without treatment, and then were stained with the antibodies (**Table S2**) and viability dye followed by fixation and permeabilisation. Then the cells were hybridised with RNA probes for IL-5 and IL-13. The signals were amplified and labelled with fluorescent probes. The results were analysed with a BD LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, US).

Microarrays

For $CD3^+$ cells sorted from sputa, RNAs were isolated using an Absolutely RNA Nanoprep Kit (Agilent, Santa Clara, US), and microarrays were performed using Affymetrix HT HG-U133+ PM GeneChips by Janssen Research & Development (Springhouse, Pennsylvania). For cultured Tc2 and $CRTH2^-CD8^+$ T-cells, RNAs were extracted with an RNeasy Mini kit (Qiagen, Venlo, Netherlands), and microarrays were conducted using an Illumina HumanHT-12v4 Expression Beadchip at the Transcriptomics Core Facility, The Jenner Institute, University of Oxford. Pre-processing data analysis was performed using R language (www.R-project.org) and Bioconductor packages (www.bioconductor.org/). Genes significant at a $p<0.05$ were selected by using Limma Bioconductor package [69]. Heat maps and gene hierarchal clustering were generated by using *tmev* microarray software suite.

ELISA

The levels of IL-4, IL-5, IL-13, and GM-CSF in the Tc2 supernatants were assayed with ELISA kits, and the concentrations of PGD_2 and LTE_4 in the mast cell supernatants were measured with a PGD_2–MOX enzyme immunoassay kit and LTE_4 enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, US) respectively according to the manufacturer's instructions. The results were measured in an EnVision Multilable Reader (PerkinElmer, Waltham, US).

Luminex assays

Multiple cytokine concentrations in the supernatants of Tc2 cultures or multiple eotaxin concentrations in the supernatants of A549 cells after various treatments as indicated in the results were measured using a Luminex Screening Assay kit (Bio-techne) as per the manufacturer's instructions. Results were obtained with a Bio-Plex 200 System (Bio-Rad, Hercules, US).

PCRarray

The levels of mRNA for cytokines in the RNA samples from the Tc2 cells after treatments were assessed with PCRarray by using an RT² Profiler PCRarray Human Common Cytokines PCR Array kit (Qiagen) in a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland).

Quantitative RT–PCR (qPCR)

qPCR was conducted as described previously [35]. Primers and probes (Roche) used are listed in **Table S3** in the Supplementary materials.

Flow cytometry analysis

For blood samples, fresh blood was labelled using antibodies (**Table S2**) followed by red blood cell lysis with a BD FACS Lysing Solution and washing. For cells from blood, sputum, BAL or bronchial biopsies, the cells were stained with multiple antibody cocktail and live/dead dye (**Table S2**). For intracellular IL-13 staining, the cells were rested overnight in AIM-V medium and stimulated with 25 ng/ml PMA and 500 ng/ml ionomycin in the presence of 2 μM monensin for 5 h. After surface marker staining, the cells were fixed with 1% formaldehyde and then treated with a Permeabilisation Buffer followed by incubation with anti-IL-13 antibody. The samples were analysed with a BD LSRFortessa flow cytometer at Oxford or a BD FACS Aria cell sorter at Southampton.

Chemotaxis assays

Tc2 cells were resuspended with RPMI 1640 media; 25 mL of cell suspension and 29-mL test compounds as indicated in the results or mast cell supernatants were applied to the upper and lower chambers, respectively, in a 5-μm pore sized 96-well ChemoTx plate (Neuro Probe). After incubation (37°C for 60 minutes), the migrated cells in the lower chambers

were collected and mixed with a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Fitchburg, US) and quantified by using an EnVision Multilable Reader.

Immunohistochemistry

Paraffin-embedded sections of bronchial biopsies were prepared by Oxford Centre for Histopathology Research. After deparaffin and rehydation, the sections were boiled in a Target Retrieval Solution (Dako, Glostrup, Denmark), followed by incubation with peroxidase blocking reagent (Bio-Rad, Veenendaal, The Netherlands) to block endogenous peroxidase activity and with normal horse serum to block non-specific-binding. The sections were then labelled with primary (goat anti-human CD3 and rabbit anti-human CD8) and secondary (peroxidase polymer-conjugated anti-rabbit followed by Cy5-tyramide) antibodies. After treatment with peroxidase blocking reagent again, the sections were further incubated with another secondary antibody (peroxidase polymer-conjugated anti-goat followed by Fluorescein-tyramide) and then 5 µg/ml DAPI solution. Images were acquired on an Olympus FV1200 inverted confocal microscope, and processed with ImageJ.

Eosinophil shape change assay

Fresh human blood was incubated with an equal volume of Tc2 conditioned media in the presence or absence of anti-IL-5 neutralizing antibody or other reagents as indicated in the results for 1 h. The samples were fixed with a Cytofix Fixation Buffer (BD Biosciences) followed by red blood cell lysis by using a RBC Lysis Solution (Gentra Systems, Minneapolis, US) and washings. Eosinophils were gated from granulocytes according to their autofluorescence during the flow cytometric analysis in a BD LSRFortessa flow cytometer. The eosinophil shape change was determined by the position shifting of the cells in forward scatter.

Eosinophil apoptosis assay

Eosinophils were prepared from human peripheral blood. Erythrocyte/granulocyte pellet was collected after Ficoll-Hypaque gradient, and then incubated with 3% dextran saline solution for sedimentation. Granulocytes were harvested from the supernatant of the sedimentation and further purified by lysis of the remaining erythrocytes with 0.6 M KCl hypotonic water, and followed by labelling with anti-CD16 microbeads. Unlabelled eosinophils were negatively selected by passing through a magnetic column and then resuspended in a RPMI 1640 medium. More than 80% purity of eosinophils was obtained for the assay.

After treatments with an equal volume of diluted Tc2 conditioned media in the presence or absence of anti-GM-CSF neutralizing antibody or other reagents as indicated in the results for 12 h, the eosinophils were labelled with Annexin V and then analysed with a BD LSRFortessa flow cytometer.

Eotaxin production assay

A549 cells (ATCC) were incubated with rhIL-4 and rhIL-13 in RPMI 1640 (5% FCS) or Tc2 conditioned media in the presence or absence of anti-IL-4 and IL-13 neutralizing antibodies as indicated in the results for 16 h. The supernatants of the cells were harvested for ELISA and luminex assays.

Statistics

Data for clinical samples in **Figures 1**, **2**, **S2** and **S3** were presented as median with interquartile range (IQR) and other data were presented as mean with SEM. Data were analysed using one-way ANOVA followed by the Newman–Keuls test or Student's t test. Groups ranked according to disease severity were tested for linear trend using Jonckheere-Terpstra test. Values of $p<0.05$ were considered statistically significant.

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Supplementary Material

Materials and methods

Reagents

PGD_2, and LTE_4 were purchased from Enzo Life Science; TM30089 was supplied by ChemieTek; rhIL-5, rhGM-CSF, anti-CD3, anti-CD28 antibodies, human $CD8^+$ T-cell isolation kit and anti-human CD294 or CD16 MicroBeads were from Miltenyi Biotec Ltd; BD FACS Lysing Solution was supplied by BD Biosciences; X-VIVO 15 medium were purchased from Lonza; AIM V medium was from Invitrogen; Ficoll-Paque™ Plus was supplied from GE Healthcare; RNeasy Mini kit and Omniscript reverse transcription kit were supplied from Qiagen; Real time quantitative PCR (qPCR) Master Mix and probes were from Roche; Primers were synthesized by Eurofins MWG Operon; rhSCF, rhIL-6 and anti-IL-4/5/13 neutralizing antibodies were purchased from Bio-techne; goat anti-human CD3 was from Santa Cruz; rabbit anti-human CD8 was from Abcam; peroxidase polymer-conjugated anti-rabbit and peroxidase polymer-conjugated anti-goat antibodies were from Vector Laboratories; Fluorescein-tyramide and Cy5-tyramide were from PerkinElmer; human myeloma IgE was from Calbiochem; Anti-human GM-CSF antibody, Annexin V-APC and Human GM-CSF ELISA kit were obtained from BioLegend; Permeabilisation Buffer, human IL-4, IL-5 and IL-13 ELISA kit were from eBioscience; and rhIL-2, rhIL-4 and rhGM-CSF were from PeproTech and other chemicals were from Sigma-Aldrich.

Flow cytometric analysis for isolated Tc2 cells

For isolated Tc2 cells, the cells were stained with antibodies for surface markers (**Table S2**) and live/dead dye, and then the cells were fixed with 1% formaldehyde. For further cytotoxic molecule studies, the cells were treated with a Permeabilisation Buffer followed by incubation with antibodies against cytotoxic effector molecules diluted in the Permeabilisation Buffer. The samples were analysed with a BD LSRFortessa flow cytometer.

Analysis of correlation

The values of R_s were calculated by using Spearman's rank correlation.

Figures

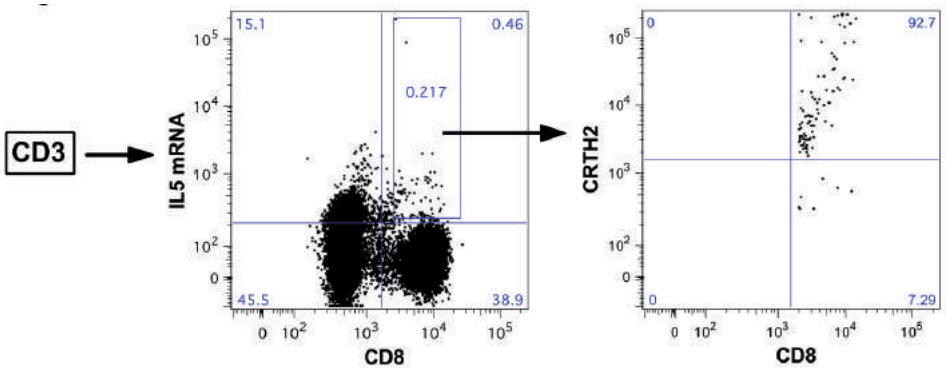

Figure S1: IL–5 producing CD8⁺ T lymphocytes are CRTH2 positive.

IL-5-producing CD3⁺CD8⁺ T-cells in human peripheral blood ex-vivo from a healthy subject are detected by using PrimeFlow RNA assay. More than 90% of IL-5⁺ cells express CRTH2 on the cell surface. (n=3).

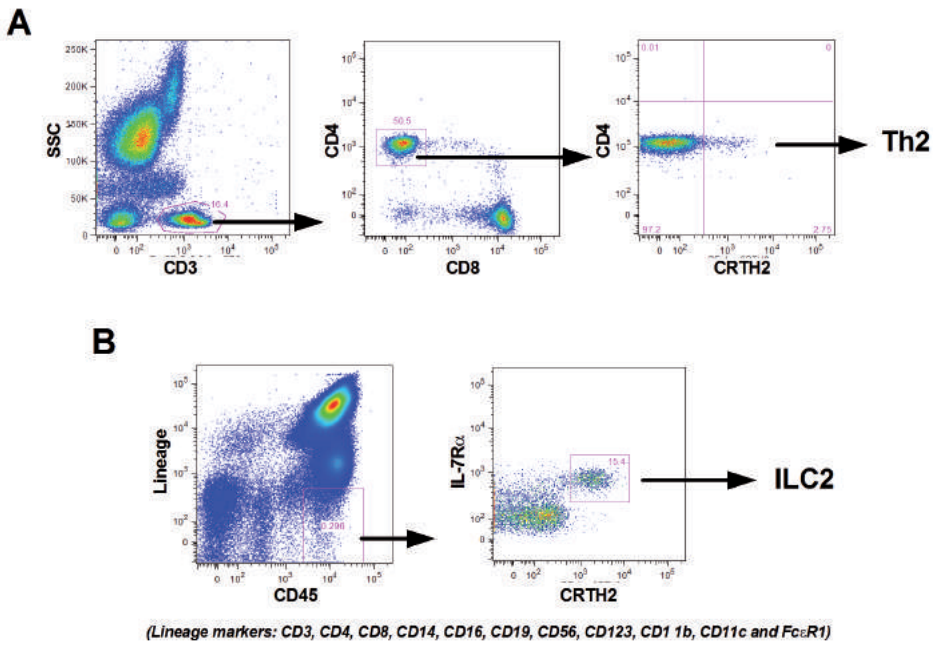

Figure S2: Gating strategies for Th2 and ILC2 cells in peripheral blood from the Oxford cohort.

Fresh bloods were stained with a mixture of antibodies against immune cell surface markers and analysed with flow cytometry. **(A)** Th2 cells were gated as CD3⁺CD4⁺CD8⁻CRTH2⁺ cells. **(B)** ILC2s were gated as lineage marker negative (CD3, CD4, CD8, CD14, CD16, CD19, CD56, CD123, CD11b, CD11c and FcεRI), CD45^{high}, IL-7Rα (CD127)⁺ and CRTH2⁺.

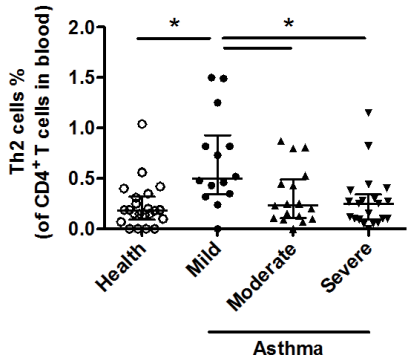

Figure S3: Blood Th2 cells are increased in mild asthma.

The frequencies of IL-Thirteen secreting $CD3^+CD4^+$ T cells in peripheral blood determined by flow cytometry with intracellular cytokine staining were increased in patients with mild asthma but not in moderate or severe asthma from the Southampton cohort.

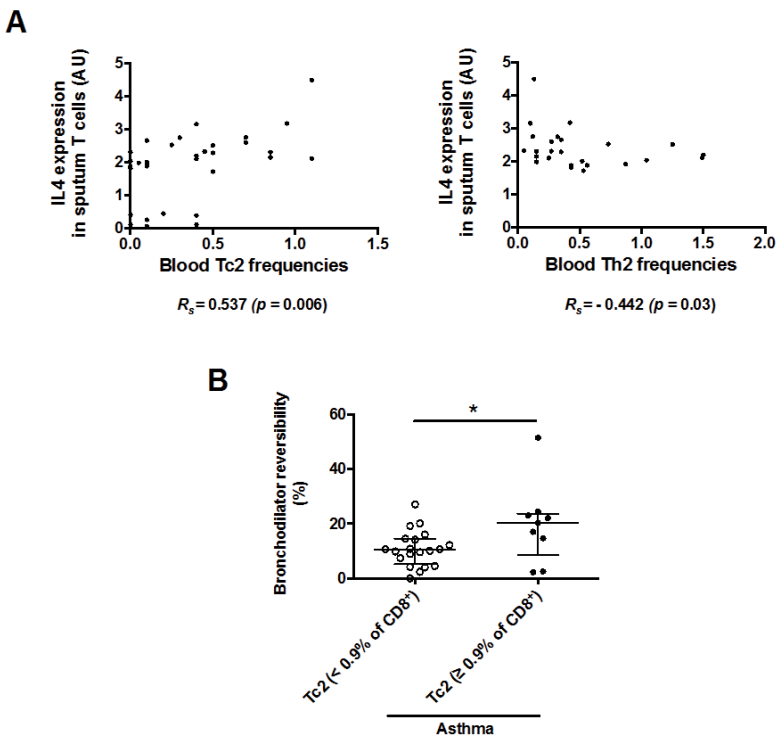

Figure S4: Frequencies of Tc2 but not Th2 are correlated with lung inflammation.

(A) Relative expression of IL-4 in T-cells measured by microarray in FACS sorted $CD3^+$ cells from induced sputum from the Southampton cohort, is correlated positively with peripheral blood $CD3^+CD8^+IL\text{-}13^+$ (Tc2) frequencies, but negatively with peripheral blood $CD3^+CD4^+IL\text{-}13^+$ (Th2) frequencies. **(B)** High Tc2 frequencies (≥ 0.9%, equivalent to the top decile of Tc2 frequencies in healthy controls) in bronchial biopsies are associated with high salbutamol bronchodilator reversibility in asthmatic subjects. R_s = Spearman's correlation coefficient.

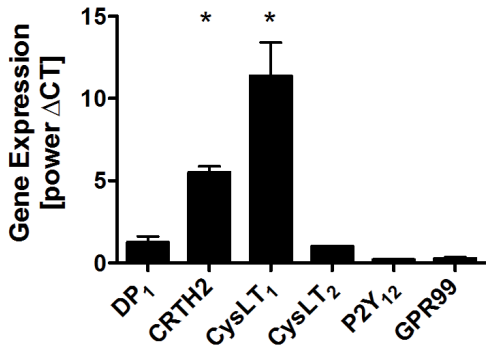

Figure S5: CRTH2 and CysLT$_1$ are highly expressed in Tc2 cells.

The levels of mRNA for the receptors for PGD$_2$ (DP$_1$ and CRTH2) and for cysteinyl leukotrienes (CysLT$_1$, CysLT$_2$, P2Y1$_2$ and GPR99) in Tc2 cells were compared by using qPCR. All the genes were normalized with GAPDH, and CysLT$_2$ was treated as 1. The levels of CRTH$_2$ and CysLT$_1$ are significantly higher than other receptors. *p<0.05, (n=8).

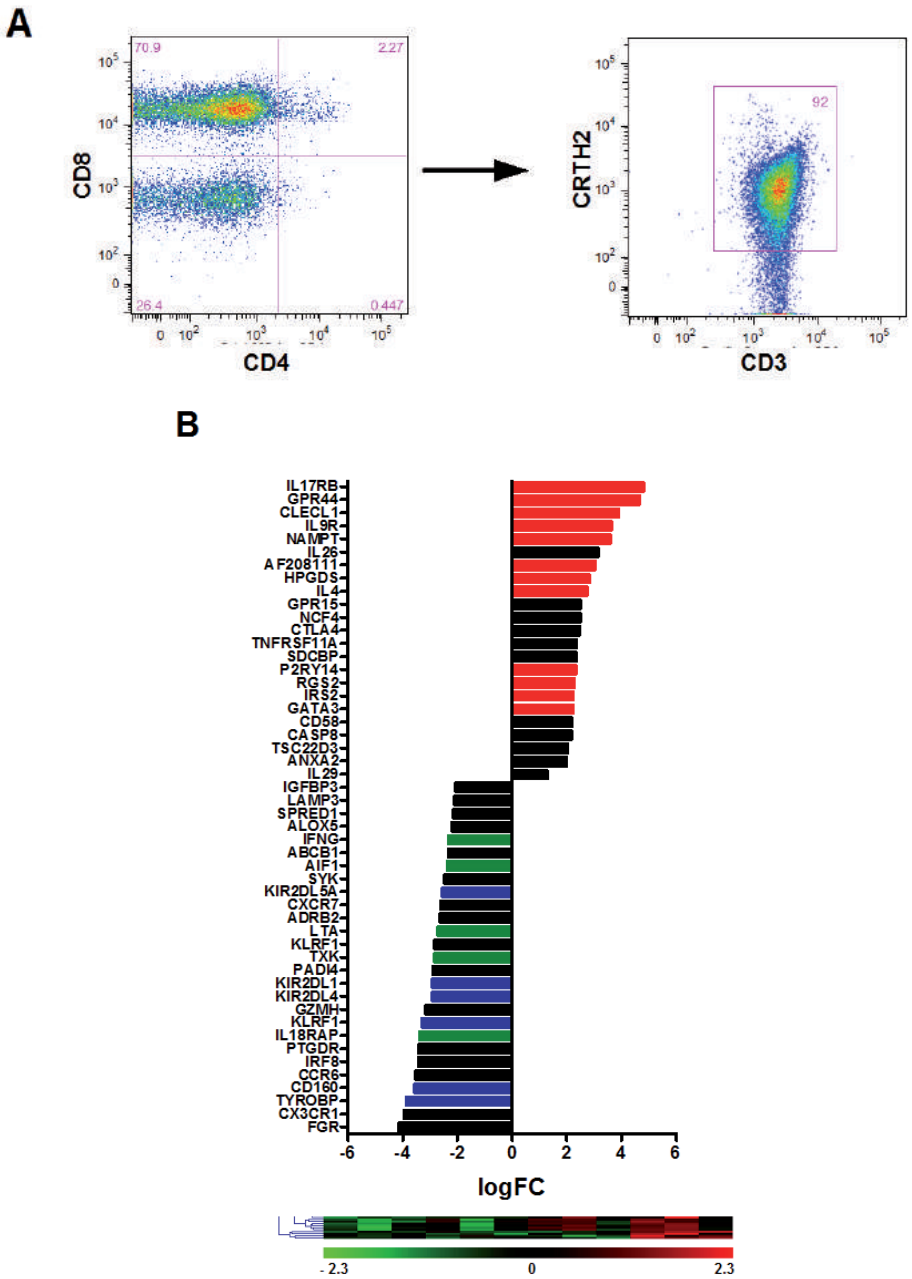

Figure S6: Tc2 cell culture shows high type–2 immunity related gene signature.

(A) Cultured Tc2 cells were >70% CD8 positive and >90% CRTH2 positive. **(B)** Significant (p<0.05) differences in gene transcriptions between Tc2 cells ($CD8^+CRTH2^+$) and other $CD8^+$ cells ($CD8^+CRTH2^-$) was detected by using microarray. Tc2 cells express higher type-2 immunity related genes (red) but lower type-1 (green) and killer cell related genes (blue) than other $CD8^+$ cells. p<0.05, (n=3 for B).

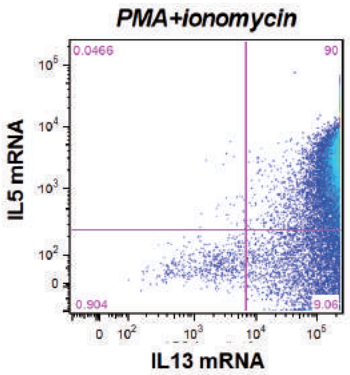

Figure S7: All Tc2 cells are capable of producing both IL–5 and IL–13.

More than 90% of cultured Tc2 cells showed IL5 and IL13 double positive after stimulation with 25 ng/ml phorbol myristate acetate (PMA) and 750 ng/ml ionomycin examined for 4 h by using PrimeFlow RNA assay. (n=2).

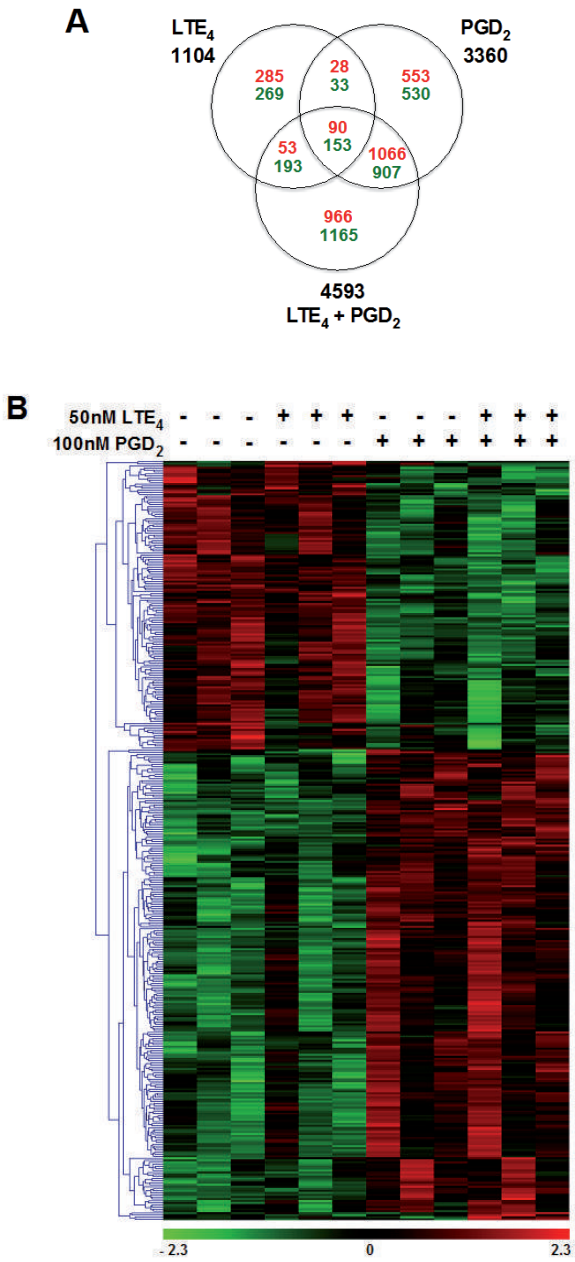

Figure S8: Large numbers of gene transcripts in Tc2 cells are modulated by PGD_2 and LTE_4.

(A) Venn diagram and **(B)** heat map representing total numbers of genes significantly regulated (p<0.05), including up-regulations (red) and down-regulations (green), by 50 nM LTE_4, 100 nM PGD_2 or their combination for 4 h in Tc2 cells detected with microarray. (n=3).

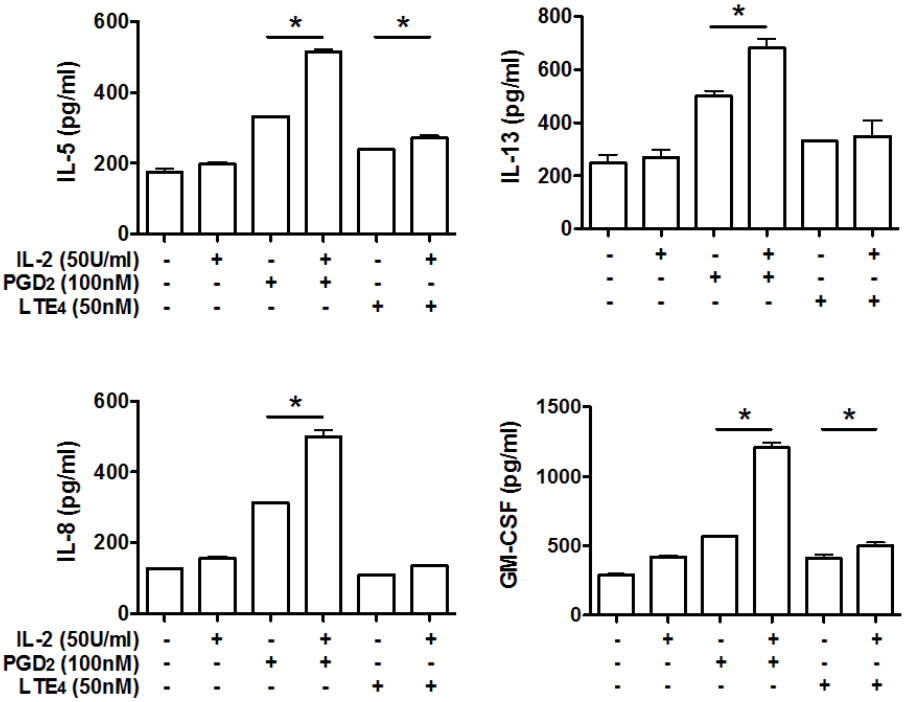

Figure S9: IL–2 enhances cytokine production in Tc2 cells in response PGD_2 and LTE_4.

Release of IL-5, IL-8, IL-13 and GM-CSF by Tc2 cells in response to 100 nM PGD_2 or 50 nM LTE_4 detected with Luminex, particularly to PGD_2, were increased in the presence of 50 U/ml IL-2. * p<0.05, (n=3).

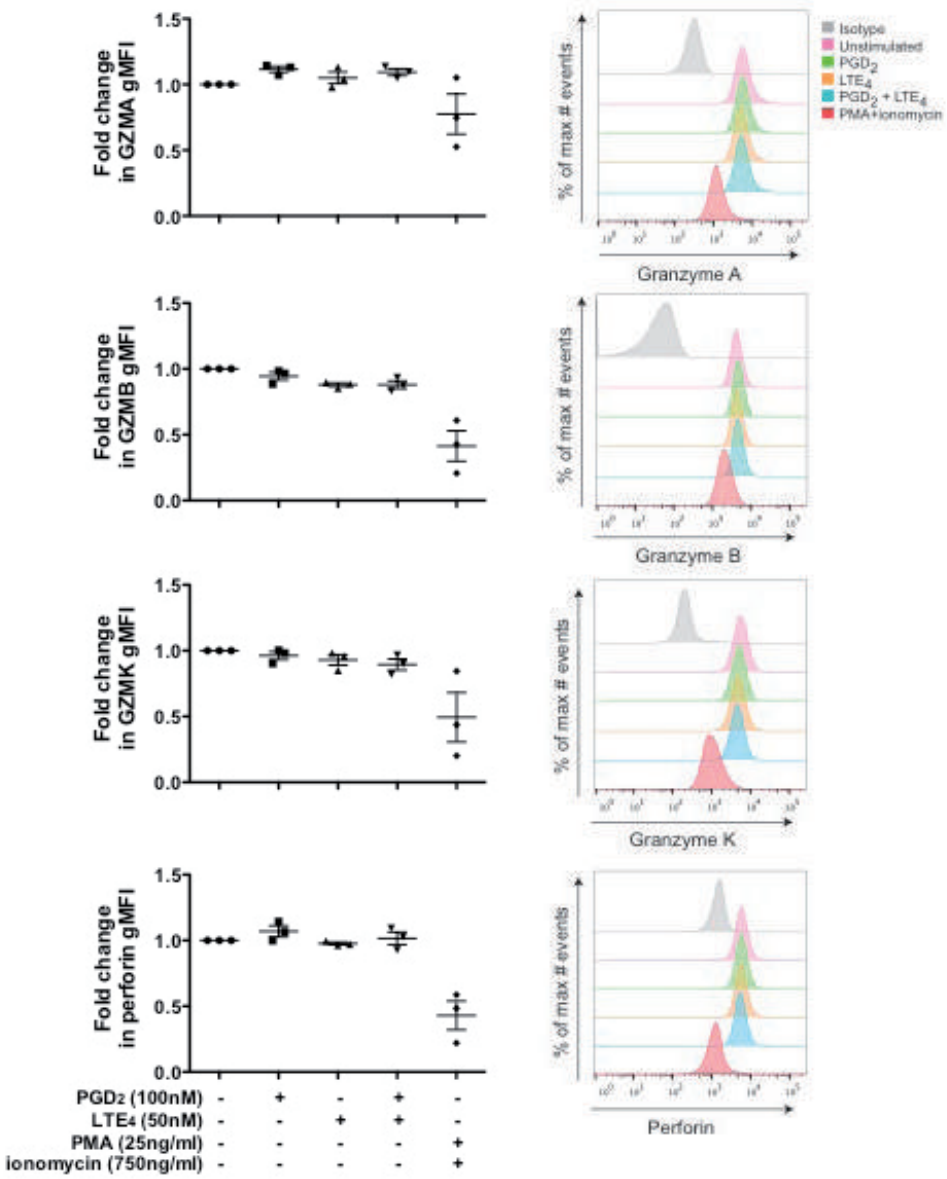

Figure S10: Production of cytotoxins in Tc2 cells are not affected by PGD_2 and LTE_4.

The expression levels of GZMA, GZMB, GZMK and perforin in Tc2 cells determined with flow cytometry were not changed by treatment with 100 nM PGD_2, 50 nM LTE_4 or their combination for 8 h. The reduction of the cytotoxin levels in Tc2 cells by 25 ng/ml PMA/750 ng/ml ionomycin was used as positive controls. Histogram shows a representative experiment for these assays (n=3).

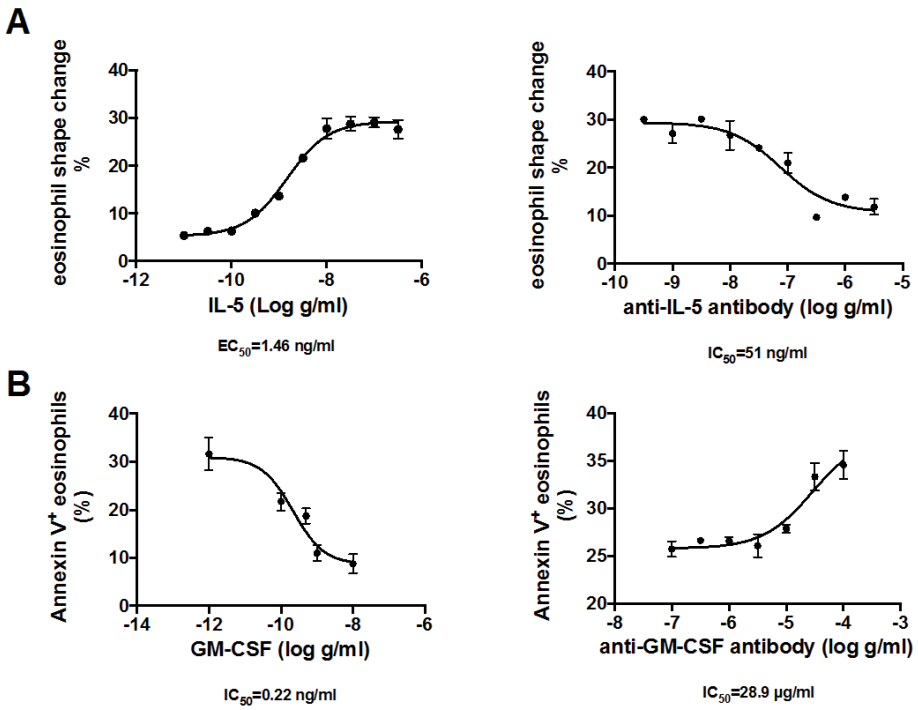

Figure S11: Effects of IL-5 and GM-CSF on eosinophil shape-change and apoptosis are inhibited by the neutralizing antibodies against these cytokines.

(A) Fresh blood was incubated with various concentrations of rhIL-5 (left) or with 10 ng/ml rhIL-5 in the presence of various concentrations of anti-IL-5 neutralizing antibody (right) for 1 h, and then the shape change of eosinophils in the blood was measured using flow cytometry. The percentage shape change was increased with the concentration of IL-5, which was reversed by anti-IL-5 antibody in a dose-dependent manner. **(B)** Eosinophil apoptosis was induced by serum deprivation in the presence of various concentrations of rhGM-CSF (left) or 1 ng/ml GM-CSF and various concentrations of anti-GM-CSF neutralizing antibody (right), and then cell apoptosis was determined with Annexin V staining by flow cytometry. Annexin V$^+$ eosinophils were reduced with increasing concentrations of GM-CSF, which was inhibited by anti-GM-CSF antibody in a dose-dependent manner. (n=10 for A left; n=2 for **A** right; n=3 for **B**).

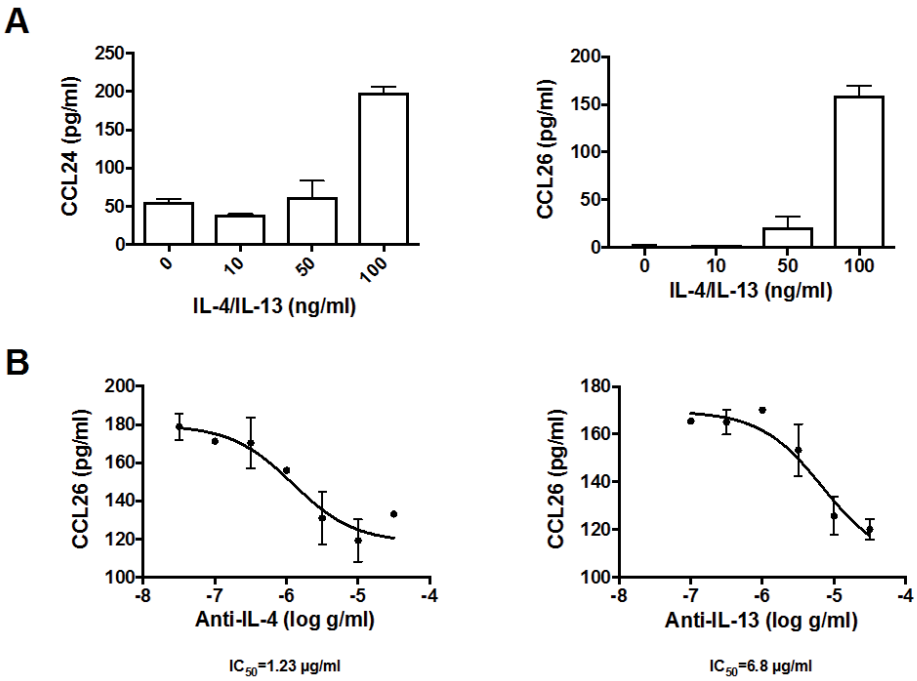

Figure S12: Effects of IL–4 and IL–13 on eotaxin 3 production in A549 cells were inhibited by neutralizing antibodies against these cytokines.

(A) A549 cells were incubated with or without increased concentrations of rhIL-4 and rhIL-13 overnight. Concentrations of CCL24 and CCL26 in cell culture supernatants of the cell culture were measured by luminex assay. CCL26 increased with increasing concentrations of IL-4 and IL-13. (**B** and **C**) A549 cells were treated with 100 ng/ml rhIL-4 and 100 ng/ml rhIL-13 in the presence of various concentrations of anti-IL-4 **(B)** or anti-IL-13 **(C)** neutralizing antibodies. The production of CCL26 induced by the cytokines was partially inhibited by the antibodies in a dose-dependent manner. (n=3 for **A**; n=2 for **B** and **C**).

Table S1: List of cytokine, chemokine, their receptor and CD molecule genes regulated by PGD_2, LTE_4 or their combination in Tc2 cells detected by microarray*

Up-regulation				Down-regulation			
	Sample treatment				Sample treatment		
Gene	PGD_2	LTE_4	PGD_2 + LTE_4	Gene	PGD_2	LTE_4	PGD_2 + LTE_4
IL3	+	+	+++**	BMP8B	-	-	-
IL5	+	+	++	CYFIP2	-	-	-
IL8	+	+	+++	FLT3LG	-		-
IL10	+	+	+	GDF11	-		-
IL13	+		++	LASS1	-		
IL16	+	+	+	TNFSF13	-		-
IL21			+	TSLP	-	-	-
IL22			++	CCL2		-	
IL24	+	+		CCL21			-
IL25	+	+	+	IL3RA	-		
IL26	+	+	+	IL6R	-		-
BMP1	+		+	IL7R	-	-	-
CSF2	+	+	++	IL11RA	-		
GDF11		+		CCK1	-	-	-
TNF	+	+	++	CNTFR	-	-	-
TNFSF8	+	+	+	LEPR	-	-	-
TNFSF11	+	+	+	CMKLR1	-		-
TNFSF13		+		CD2	-	-	-
TNFSF14	+	+	+	CD14	-		-
LASS1			+	CD40	-	-	-
LIF	+	+	+	CD46	-		-
LTA	+	+	+	CD58		-	
NAMPT	+		+	CD99	-		-
XCL1	+	+	++	CD79A	-		
FASLG	+	+	++	CD79B	-	-	-
CCL2	+		+	CD99L2	-		-

Table S1 continues on next page.

Table S1: *Continued*

Up-regulation				Down-regulation			
	Sample treatment				Sample treatment		
Gene	PGD_2	LTE_4	PGD_2 + LTE_4	Gene	PGD_2	LTE_4	PGD_2 + LTE_4
CCL3			+	CD300LG	-	-	-
CCL4			+	GPR44	-		-
CCL7		+					
CCL21	+	+					
CCL22	+	+	+				
CCL25	+		+				
CD2BP2	+	+	+				
CMTM4	+		+				
PF4V1	+		+				
PPBP	+	+	+				
IL1RL1			+				
IL2RB	+	+	+				
IL2RG	+	+	+				
IL3RA		+	+				
IL6R		+					
IL11RA	+	+					
CD1E			+				
CD7	+	+	+				
CD14		+					
CD28	+	+	+				
CD40LG	+	+	+				
CD44	+		+				
CD46		+					
CD53	+		+				
CD55	+	+	+				
CD58	+		+				
CD59	+	+	+				

Table S1 continues on next page.

Table S1: *Continued*

Up-regulation				Down-regulation			
	Sample treatment				Sample treatment		
Gene	PGD_2	LTE_4	$PGD_2 + LTE_4$	Gene	PGD_2	LTE_4	$PGD_2 + LTE_4$
CD69	+	+	+				
CD79A		+	+				
CD82	+	+	+				
CD99		+					
CD99L2		+					
CD109	+	+	+				
CD151	+	+	+				
CD164	+	+	+				
CD226	+	+	+				

* The concentrations of PGD_2 and LTE_4 were 100 nM and 50 nM respectively.
** ++ indicates fold change ≥3; +++ indicates fold change ≥6.

Table S2: Antibodies used for flow cytometry and PrimeFlow RNA assays

Antigen	Clone	Supplier	Used for
CD3	SK7	BioLegend	Flow cytometry
CD3	UCTH1	eBioscience	Flow cytometry, PrimeFlow
CD4	L200	BD Biosciences	Flow cytometry
CD4	OKT4	BioLegend	Flow cytometry, PrimeFlow
CD8	SK1	BioLegend	Flow cytometry, PrimeFlow
CRTH2	BM16	Miltenyi Biotec	Flow cytometry, PrimeFlow
$CysLT_1$	polyclonal	Novus Biologicals	Flow cytometry
Granzyme A	356412	R&D Systems	Flow cytometry
Granzyme B	GB11	BD Biosciences	Flow cytometry
Granzyme K	24C3	Immunotools	Flow cytometry
IL-13	11711	R&D Systems	Flow cytometry
perforin	B-D48	BioLegend	Flow cytometry

Table S3: Primers and probes used for q–PCR

Gene	Primer	Probe No.
CSF1	5'-GCAAGAACTGCAACAACAGC-3' 5'-ATCAGGCTTGGTCACCACAT-3'	19
CSF2	5'-TCTCAGAAATGTTTGACCTCCA-3' 5'-GCCCTTGAGCTTGGTGAG-3'	1
CYSLT1	5'-ACTCCAGTGCCAGAAAGAGG-3' 5'-GCGGAAGTCATCAATAGTGTCA-3'	29
CYSLT2	5'-CTAGAGTCCTGTGGGCTGAAA-3' 5'-GTAGGATCCAATGTGCTTTGC-3'	48
DP1	5'-CCTGGAGGAGCTGGATCA-3' 5'-GCTCCATAGTAAGCGCGATAAA-3'	18
GAPDH	5'-AGCCACATCGCTCAGACAC-3' 5'-GCCCAATACGACCAAATCC-3'	60
GPR44	5'-CCTGTGCTCCCTCTGTGC-3' 5'-TCTGGAGACGGCTCATCTG-3'	43
GPR99	5'-CAACCTGATTTTGACTGCAACT 5'-GGATAATCGTGGTATAGCAAAGTG	16
IL3	5'-TTGCCTTTGCTGGACTTCA-3' 5'-CTGTTGAATGCCTCCAGGTT-3'	60
IL4	5'-CACCGAGTTGACCGTAACAG-3' 5'-GCCCTGCAGAAGGTTTCC-3'	16
IL5	5'-GGTTTGTTGCAGCCAAAGAT-3' 5'-TCTTGGCCCTCATTCTCACT-3'	25
IL8	5'-AGACAGCAGAGCACACAAGC-3' 5'-ATGGTTCCTTCCGGTGGT-3'	72
IL9	5'-CTTCCTCATCAACAAGATGCAG-3' 5'-AGAGACAACTGGTCACATTAGCAC-3'	59
IL13	5'-AGCCCTCAGGGAGCTCAT-3' 5'-CTCCATACCATGCTGCCATT-3'	17
IL21	5'-AGGAAACCACCTTCCACAAA-3' 5'-GAATCACATGAAGGGCATGTT-3'	7
IL22	5'-CAACAGGCTAAGCACATGTCA-3' 5'-ACTGTGTCCTTCAGCTTTTGC-3'	6
P2Y12	5'-TTTGCCTAACATGATTCTGACC-3' 5'-GGAAAGAGCATTTCTTCACATTCT-3'	27
TNF	5'-CAGCCTCTTCTCCTTCCTGAT-3' 5'-GCCAGAGGGCTGATTAGAGA-3'	29

GENERAL DISCUSSION

Asthma is a heterogeneous disease that is highly prevalent in the western world. Around 4% of this large asthma population suffers from severe asthma which is an invalidating disease [1,2]. Today only a small group of these difficult-to-treat patients with specific characteristics receive biologicals that reduce exacerbations and have marginal side effects. Identifying the right patient for the right treatment has been proven challenging but essential (Chapter 1.2) [3,4]. Therefore, the research in this thesis focuses on improving diagnostic value of blood tests and sputum sample-tests in asthma patients. Three research directions were explored to improve the diagnostic value of blood and sputum cells for asthma phenotyping.

- The value of eosinophil and neutrophil activation in vivo in blood and sputum
- Non-linear principal component analysis to study a combination of clinical parameters and measures of activation of blood cells
- Improvement of flow cytometry data analysis to evaluate the added value of a multivariate approach for disease diagnosis in general and for asthma in particular.

Activation state of eosinophils and neutrophils in peripheral blood

Peripheral blood eosinophils need to become (pre)activated to arrest on the endothelium and extravasate into the airways [5,6]. Eosinophilic asthma is characterized by airway eosinophilia and hence peripheral blood eosinophils need to be (pre)activated to migrate. We hypothesized that the (pre-) activation state of peripheral blood eosinophils was different in this specific phenotype compared to eosinophils from neutrophilic asthma and controls (Chapter 2.1).

There was already evidence to support the activation state of eosinophils is changed in asthma. Johansson and colleagues suggested eosinophil priming and activation is deficient in peripheral blood during active eosinophilic inflammation in the airways [7]. Luijk *et al*. found active FcγRII (CD32), the main IgG receptors on blood eosinophils, was upregulated after segmental lung challenge [8,9]. Both findings seem contradictive because there is deficiency in priming and activation in one study and activation after lung challenge in the other. However, in our studies we found very similar patterns. The activation state of granulocytes was tested by *in vitro* stimulation with N-Formylmethionine-leucyl-phenylalanine (fMLF). fMLF is a strong activation and chemotactic factor for polymorphonuclear leukocytes [10]. In the Utrecht cohort and Oxford test cohort (Chapter 2.1) we observed that peripheral blood eosinophils did not respond to the fMLF stimulation in patients with a clear airway eosinophilia which is similar to the findings of Johanson *et al*. [11]. In eosinophilic asthma the activation markers Mac1 (CD11b) and to a lesser extent active FcγRII (CD32 recognized by the antibodies A17 and A27 [12]) were less upregulated after stimulation with fMLF compared to the other inflammatory phenotypes diagnosed by sputum induction. Eosinophils isolated from patients with mild asthma and neutrophilic or paucigranulocytic airway inflammation were hyper-responsive for fMLF compared to controls and patients with eosinophilic asthma.

This is in line with the findings of Luijk *et al.* describing granulocyte responsiveness in a group of mild-asthma patients [8]. The main findings (**Figure 1**) were (i) that patients with airway eosinophilia have refractory peripheral blood eosinophils and (ii) different states of activation of granulocytes correlate with different states of inflammation in asthma.

The presence of refractory eosinophils in peripheral blood indicative for systemic inflammation in asthma patients is in line with refractory neutrophils in peripheral blood after inflammatory complications following femur fracture and more severe COPD patients [13,14]. The same or very similar methods were used to measure the activation state of neutrophils in these latter studies. Hietbrink *et al.* assessed neutrophil responsiveness by *in vitro* stimulation with fMLF in trauma patients that were admitted to the ICU with a femur fracture [13,15]. Some of these patients developed late (>3 days) inflammatory complications,

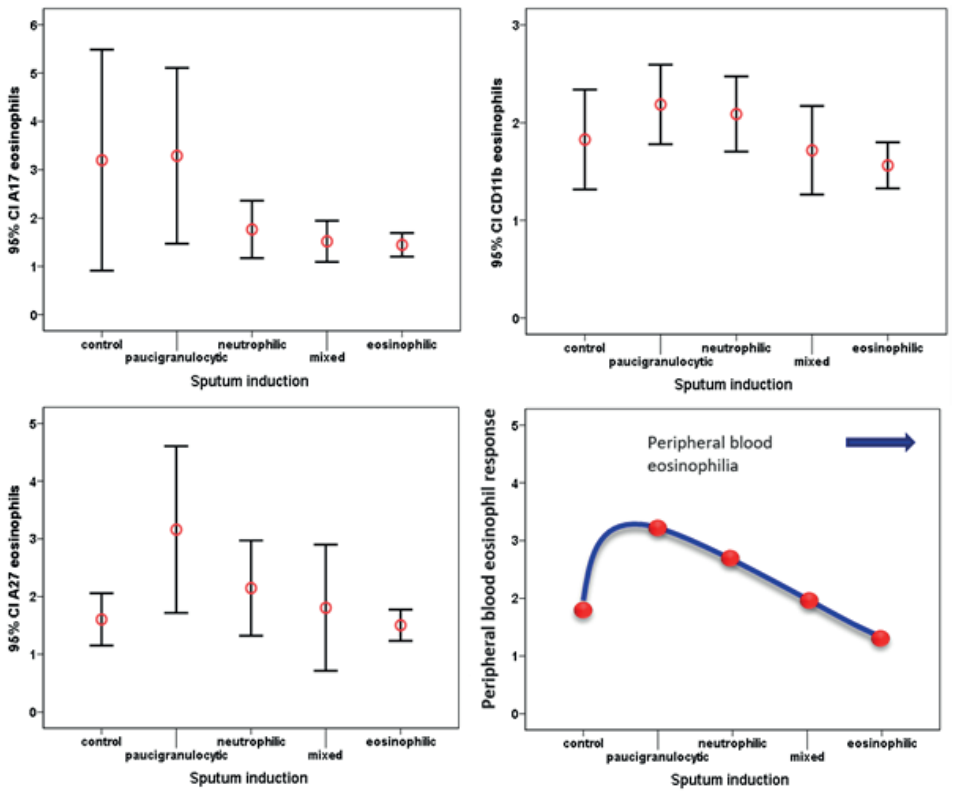

Figure 1 A–D: A–C: responsiveness of blood granulocytes for the innate stimulus fMLF.

Responsiveness is depicted as fold induction of respectively MoPhabA17, CD11b and MoPhabA27 with on the x-axis the different asthma phenotypes based on sputum induction. Only CD11b reaches a significant difference in the eosinophilic group compared to the other groups. However in the NLPCA analysis in Chapter 2.1 all these measures were essential to discriminate between patients by making use of 'multivariate advantage'.

D: overall model of Mac-I and FcγRII priming/activation and inflammatory subtypes.

which was associated to impaired responsiveness of neutrophils at the day of trauma. Lo Tam Loi *et al*. studied neutrophils activation states in COPD patients [14]. They applied the unique characteristics of a CD11b antibody clone, VIM12, to activate respiratory burst of neutrophils. This response was markedly decreased in COPD patients compared to controls. Among the COPD patients, the group with the lower half intensity of respiratory burst had a more severe disease state. Also the response to fMLF was lower in the more severe COPD group who had a higher symptom score, lower diffusion capacity and impaired exercise tolerance. This finding in neutrophils shows striking similarity to refractory eosinophils in eosinophilic asthma and is integrated in **Figure 2**. Notably, the pattern of low neutrophil responsiveness was not observed in asthma patients.

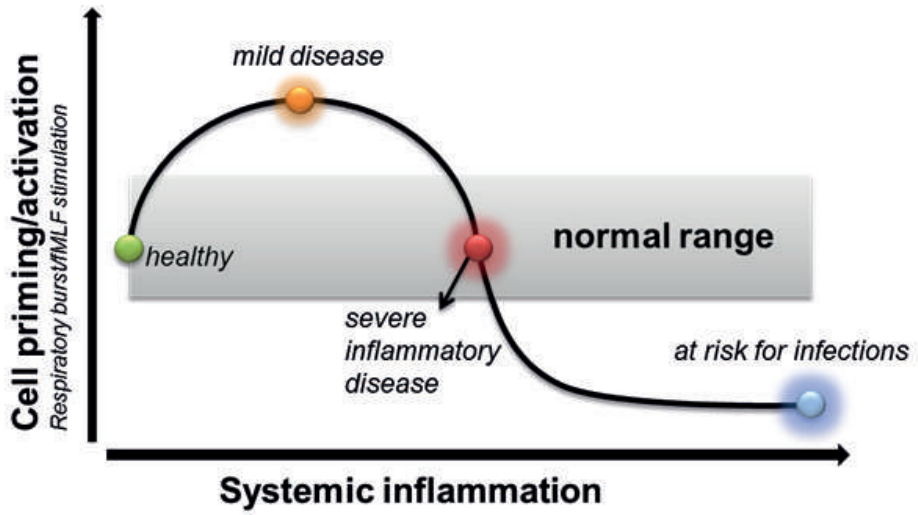

Figure 2: Integrated identification of Asthma, COPD and Trauma subtypes by analysis of the functional phenotype of blood eosinophils (fMLF) and neutrophils (fMLF and respiratory burst).

With the increase of systemic inflammation the priming and activation state changes from a low state (green) to a high state (orange) and in severe systemic inflammation from (red) to a dysfunctional refractory state (blue). Adapted from Chapter 6 of the thesis of Dr. Lo Tam Loi. https://dspace.library.uu.nl/handle/1874/298639 and from Chapter 4 of the thesis of Dr. Hietbrink et al. https://dspace.library.uu.nl/bitstream/1874/30517/1/hietbrink.pdf.

State of eosinophils in airway tissue

Recently, Mesnil *et al*. described phenotypically different eosinophils in airway tissue of healthy and diseased mice and airway tissue of asthma patients. They found that eosinophils appear to exist in two forms in the airways of mice: resident eosinophils (rEos) present in the parenchyma and inflammatory eosinophils (iEos) in the peribronchial tissue [16]. After house dust mite challenge in mice rEos remained unchanged and present in the parencyma and

at the same time iEos were recruited into the airway tissue. Mice lacking rEos showed an increase in Th2 cell responses to inhaled allergens (Mesnil *et al*. used ΔdblGATA, C.Cg-Gata1 tm6Sho/J mice purchased from The Jackson Laboratory). In non-asthmatic human lungs rEos (Siglec-8^{+}CD62L^{+}IL-3R^{lo}) *in tissue* could be differentiated from (Siglec-8^{+}CD62L^{lo}IL3R^{hi}) iEos *in sputa* of asthmatic patients. However, taking our results (in Chapter 2.2) into consideration, the activation profile of sputum cells is overall high. Therefore, comparing sputum cells to tissue cells does not seem to be a strong point to prove the existence of two cellular phenotypes. In this line of reasoning, low expression of CD62L (shedding in active state) and high expression of IL3R does not necessarily prove the iEos are actually different phenotypes of eosinophils in 'human asthma' as was stated by Mesnil and colleagues [16]. On the other hand the finding of subsets of eosinophils in the airways could be in line with findings of changes in activation state in peripheral blood eosinophils (Chapter 2.1) [7,8]. In severe eosinophilic asthma there is a high concentration of highly activated sputum eosinophils and a high concentration of refractory peripheral blood eosinophils, yet it is unclear what the activation state is of eosinophils in lung tissue.

One hypothesis could be that responsive eosinophils extravasate into the lung tissue under influence of signaling chemokines such as IL-5, which results in a remaining signal unresponsive group in peripheral blood (refractory eosinophils) and activated eosinophils in the tissue (inflammatory eosinophils). These iEos are likely to have shed CD62L during migration, have high expression of activation markers, and accumulate and degranulate around the bronchial wall [17,18]. Finally this eosinophil phenotype ends up degranulated and still highly activated in the airways and can eventually be found in sputum. Parenchymal rEos might respond to the degranulation and activation of iEos by becoming more activated and could even start to function as sources for IL-5 production thereby causing a positive feedback loop that recruits again influx of more iEOs. The feedback loop could explain the pathophysiology in persistent eosinophilic asthma and is suspected to be refractory to treatment with oral steroids [19].

One of the relatively novel treatment options for airway eosinophilia is anti-IL-5 therapy, which should affect eosinophil dynamics throughout the human body as IL-5 is important in eosinophil production, differentiation, proliferation, survival, chemotaxis and priming [20]. Mice deficient in IL-5 (IL-5$^{-/-}$ knock-out) do not show a response to helminth infection but do have normal eosinophil levels in peripheral blood with standard morphology [21]. Mice deficient in IL-5 have a functional difference in response to specific triggers compared to wild type mice. In IL-5$^{-/-}$ knock-out mice allergen challenge does not lead to eosinophilia, airway hyperresponsiveness and lung damage [22]. Those phenomena do occur in wild type mice. IL-5 plays in important role in airway remodeling as was shown in IL-5 knockout mice challenged by OVA which had significantly less peribronchial fibrosis and significantly lower peribronchial smooth muscle thickness compared with WT mice challenged with OVA [23]. In humans 20 weeks of anti-IL-5 treatment (Mepolizumab) lead to a median reduction of 100% of eosinophils in peripheral blood and only to an average of 52% reduction in bone marrow eosinophils, 55% reduction of eosinophil number in airway mucosa and a 79% reduction

of eosinophils in bronchial alveolar lavage (BAL) fluid [24]. This illustrates the sensitivity of peripheral blood for a blocking signal of IL-5 and at the same time it shows the insensivity of bone marrow, tissue and also the airways for relatively short term IL-5 blockade.

Activation state of eosinophils and neutrophils in the airways (sputum)

In Chapter 2.2 we studied the endobronchial compartment by measuring sputum eosinophils and neutrophils and more specifically the association of sputum eosinophilia with neutrophil activation in sputum. There was no association between sputum eosinophilia and neutrophil activation nor was there a difference in sputum eosinophil activation between eosinophilic and non-eosinophilic asthma. However, both sputum neutrophils and eosinophils were characterized by an activated and degranulated phenotype compared to peripheral blood cells; degranulation of all granule types were detected in sputum cells, combined with an increased expression of the activation markers (activated) Mac-1 (CD11b), Programmed Death-Ligand 1 (CD274) and a decreased expression of CD62L. CD69 expression was only increased on sputum eosinophils.

Interestingly, sputum granulocytes show an overall highly activated state and concurrently express an immune-suppressive marker (PDL-1). Blockade of the PD-1 receptor (CD274) was shown to restore $CD4^+$ cell functioning *in vitro*, although this was not specifically tested in neutrophils [25]. The expression of CD274 on sputum cells of asthma patients suggests an ability of these cells to modulate inflammation in asthma instead of merely causing tissue damage and contributing to the inflammatory cascade.

The state and function of eosinophils in peripheral blood, tissue and airways in eosinophilic asthma

The combination of refractory eosinophils in peripheral blood, the highly activated eosinophils in sputum and the data published by Mesnil *et al*. seem to fit a hypothesis of one cell type with seemingly opposing functions; rEos down-regulating a type-2 response and in their absence iEos causing eosinophilic inflammation. This paradigm of counter acting functions within one cell type fits with a hypothesis launched by Lee *et al*. named the LIAR hypothesis, stating eosinophils are regulators of **L**ocal **I**mmunity **A**nd/or **R**emodeling/**R**epair in both health and disease [26]. This perspective goes against the view that eosinophils are always destructive and damaging end-stage inflammatory effector cells. And it launches the idea of eosinophils having a role in healthy tissue homeostasis with a damaging role only in non-homeostatic situations. Therefore, in eosinophilic asthma it seems important to objectify the role of tissue eosinophils and ultimately to aim for restored healthy tissue homeostasis regulated by eosinophils.

Non–linear Principal Component Analysis on clinical and cellular data

The clinical and cellular data variables gathered in the Utrecht and Oxford asthma cohorts consist of multiple data levels: continuous and categorical. Dimension reduction was required to test whether a combination of multiple variables with different data levels has the potential to improve asthma phenotyping [27,28]. A widely used unsupervised method to reduce data dimensionality is Principal Component Analysis (PCA). However, PCA is only suitable for continuous data (linear) and, therefore, we choose to use non-linear principal component analysis (NLPCA) and thereby aim for multivariate advantage [29–31].

NLPCA brought two main findings: (i) The granulocyte response contributes to diagnostic accuracy (see Chapter 2.1) (ii) 6 clinical markers were important to discriminate between patients with eosinophilic and non-eosinophilia asthma: FeNO, eosinophil count, ACQ, nasal polyposis, aspirin sensitivity and medication use (**Figure 3, bottom panel**, NLPCA). At least as important was the demonstration of clinical markers that were excluded; among these markers were for example presence of allergy, BMI, age and smoking history. This is specifically interesting, because there is a lot of interest in the asthma research field for the importance of allergy, BMI, age and smoking in the pathogenesis of asthma. Our cohort consisted of patients visiting tertiary centers in The Netherlands or the UK with an overrepresentation of severe asthma patients. This precludes generalization for the disease as a whole, but it does provide direction for the better understating of the pathogenesis of severe asthma. In severe asthma clearly eosinophilic disease is important and of lesser importance is the presence of allergy, obesity and age (Chapter 2.1). This is in line with other unbiased cohort studies focusing on more severe asthma such as the Leicester cohort and goes against specific hallmarks of the disease in general [32].

The question arises: Does the selection of a multidimensional analysis method influence the interpretation or conclusions of these cohort studies? NLPCA unlike PCA is not a common method to be used for dimension reduction in clinical studies, but it is in an early stage of development for this type of analysis [30,31]. It is only recently that larger studies such as the Leicester cohort of Haldar and colleagues, the American SARP (Severe Asthma Research Programme) cohort, the ADEPT-cohort (Airway Disease Endotyping for Personalized Therapeutics) and U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome) consortium gathered high-dimensional and high-density data for which advanced statistical methods were required [32–36]. In these large cohorts data analyses were chosen based on the research question, nature of the data and preference of the authors. For these four cohorts clustering techniques were chosen such as k-means clustering, Ward's minimum-variance hierarchical clustering method or fuzzy partition-around-medoid clustering (FPAM). Disadvantages of these methods are supervision, for example k-means clustering and FPAM requires prior choice of the number of clusters. Another disadvantage is sensitivity for outliers, which is a weakness of Ward's method. Therefore, the preference of the authors for the different methods also included the degree of supervision or bias in the data [37]. Prosperi *et al.* described the challenges in variable and analysis selection in

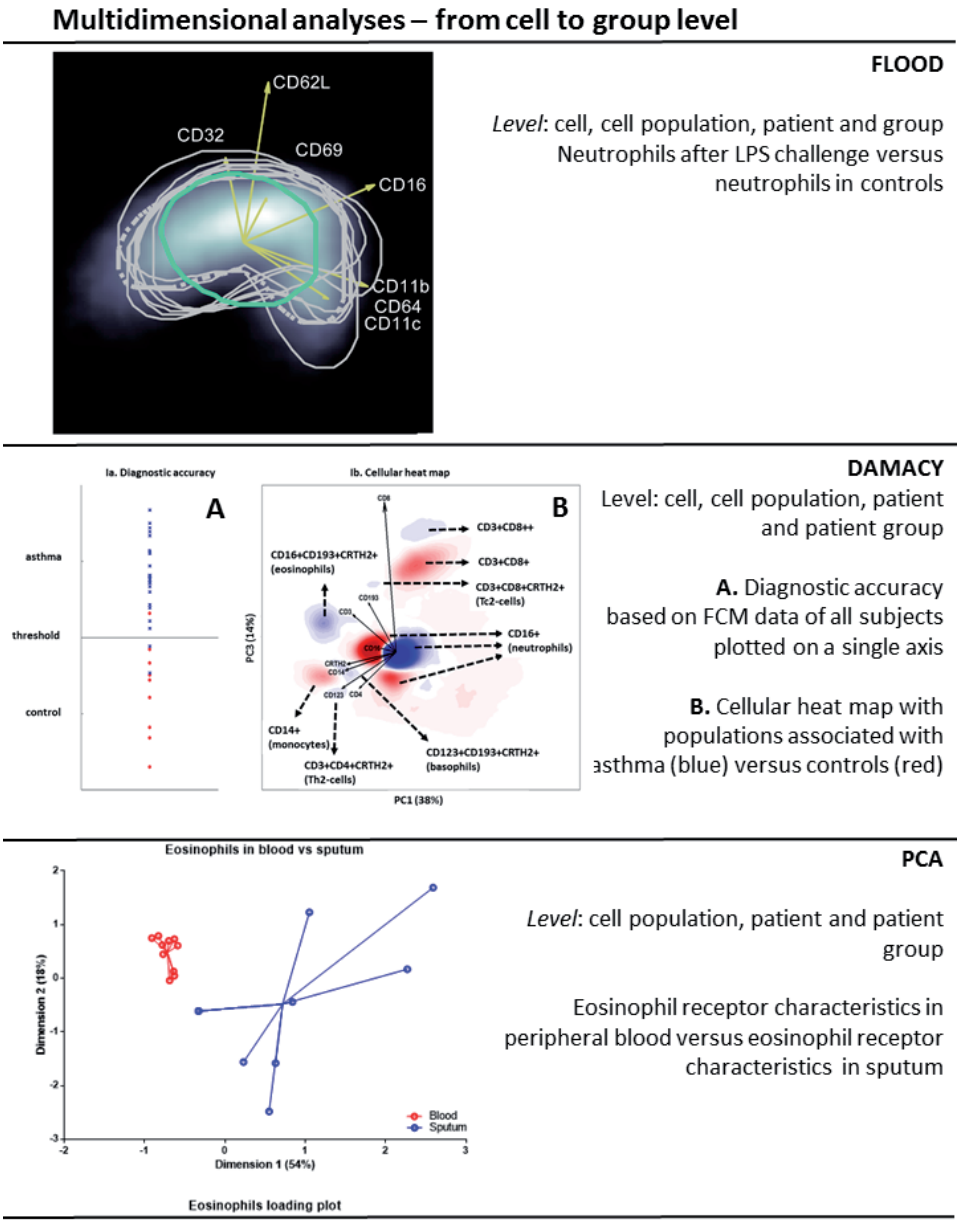

Figure 3: MFC and/or clinical data analysis methods on different hierarchical levels.

FLOOD: Output from the Response Model of FLOOD (Chapter 3.1) containing neutrophils in white dots with grey lines representing 80% CI of individual subjects after LPS challenge and a cyan line containing the 80%CI of neutrophils from healthy controls. **DAMACY:** Output from DAMACY with a diagnostic score **(A)**. In blue are asthma patients from the oxford cohort and in red healthy controls. The right hand cellular heat map **(B)** contains characteristic cells from asthma patients (blue) and cells from healthy controls (red). **PCA:** eosinophils in blood (red) and in sputum (blue), not visible here are the surface marker expressions underlying the differences of location in this graph.

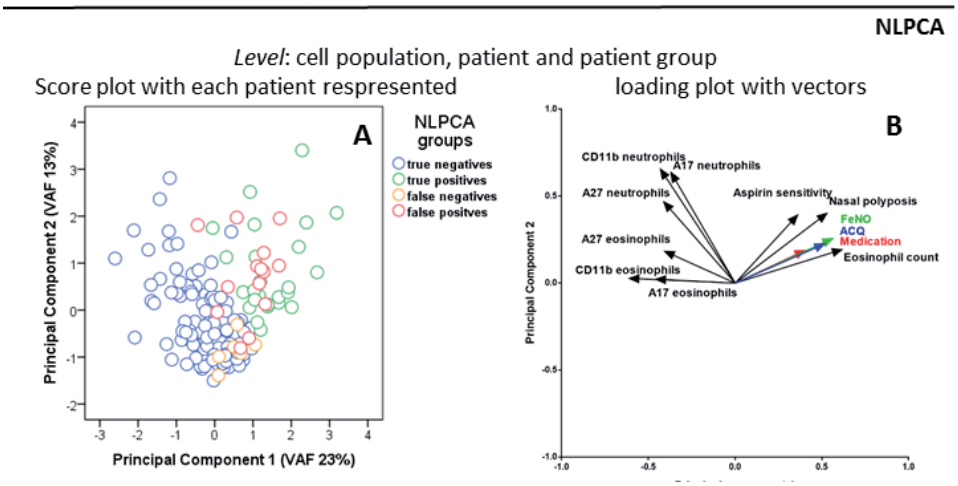

Figure 3: *Continued.*

NLPCA: (A) Score plot with four groups of patients (rounds). 1. Green rounds = the patient has sputum eosinophilia and is predicted to have it by the developed prediction model in Chapter 2.1. 2. Blue rounds = no sputum eosinophilia and was predicted so 3. Orange rounds = patients with sputum eosinophilia not identified by the prediction model. 4. Red rounds = patients without sputum eosinophilia that were identified to have sputum eosinophilia. **(B)** Loading plot with the 12 markers that remained after dimension reduction. The vectors represent each marker and can be used to determine the characteristics of each score (rounds in A).

multidimensional datasets with clinical data and advised to carefully select variables for inclusion and to carefully select the analysis method depending on the research question and nature of the data [38].

The method that was chosen for the analysis of clinical and laboratory data in Chapter 2.1, NLPCA, is unsupervised. It can integrate different types of variables: categorical and continuous. NLPCA reduces the dimensionality of data and results in this study in a score per patient (Chapter 2.1) and a certain number of axes (Principal Components) interpretable for analysis, preferably three or lower. In Chapter 2.1 these axes represent the data of 12 variables. The subsequent discriminant analysis was logically supervised and aimed to test the possibility of predicting sputum eosinophilia based on the original data coming from blood measurements, FeNO and clinical markers. Finally, an important advantage of NLPCA is the insightfulness of the output of the analysis (score and loading plot) instead of other visualization methods such as k-means clustering plots that can be very challenging in interpretation [39].

Studies taking the step from asthma phenotype to phenotype-based treatment decisions are limited [32]. Based on Chapter 2.1 it would be attractive to develop an intervention study using cell activation data and clinical parameters to diagnose eosinophilic asthma in patients

not on oral steroids. Such intervention or treatment itself could be any of the biologicals known to suppress eosinophilic inflammation. The Utrecht and Oxford cohort data could serve as a training set and would improve the diagnostic accuracy on forehand [40].

Multidimensional analysis of Multicolour Flow Cytometry data

Multicolor Flow Cytometry (MFC) has become an elementary technique to study receptor profiles on single cells. The technique has been rapidly improved and, by using multiple lasers with different wavelengths and innovation in antibody-bound fluorescent labels, an increasing amount of epitopes can be measured simultaneously on a single cell. However, with this increase of labels the analysis of flow cytometry data has become more complex. Immunologists are now confronted with multi-dimensional datasets while being used to analyse flow cytometry data two-dimensionally [41–43]. The amount of biplots required to fully interpret a flow cytometry experiment with 10 labels is ((10*10)-10)/2 = 45 biplots. Clearly MFC biplots with fluorescence intensity are very important for many research questions, yet a considerable amount of information is lost by two-dimensional analysis only (Chapter 3.1).

In this thesis several analysis methods for MFC data have been described and used to answer a variety of research questions. Roughly MFC data contains four hierarchical levels for interpretation (DAMACY asthma Chapter 3.3):

1. The multivariate (co)-expression of markers on single cells
2. Aggregation into cell populations with similar marker expression
3. Representation of cell populations in a specific individual/subject
4. The cellular representation in a specific (clinical) phenotype

The ideal flow cytometry analysis technique combines these four levels. However, it is very challenging to develop a method incorporating all levels and hence a variety of analyses exists with a focus on one or two levels. Logically, the choice of analysis depends on the research question. Within this thesis several methods were used to analyse MFC data and/or clinical data, all of these are displayed in **Table 1**. and in **Figure 3**.

FLOOD (**FL**ow cytometric **O**rthogonal **O**rientation for **D**iagnosis) is a PCA based method that reveals disease specific marker patterns (**Figure 3, top panel**, FLOOD). Benchmarks are used to highlight deviations of challenged from unchallenged individuals based on cell concentration and surface marker expression. We demonstrate its power in an *in vivo* study of the response of healthy humans to lipopolysaccharide (LPS) challenge and an *in vivo* study of the response of elite rowers to an intense anaerobic exercise stimulus (rowing).

In the LPS challenge (Chapter 3.1) in healthy human volunteers, FLOOD reveals a reproducible pattern of challenge specific markers on blood neutrophils. An automated gating approach identified two directions of the response (score plot) and the relevant vectors responsible for these directions (loading plot). In summary, the unsupervised analysis based on two directions identified two new neutrophil types, one with low L-selectin expression

Table 1: Multivariate MFC and/or clinical data analysis techniques

Technique	Short description	Research Questions	Applied to
FLOOD	**FL**ow cytometric **O**rthogonal **O**rientation for **D**iagnosis: visualizing cellular responses on an individual and group level.	What is the cellular response to a stimulus? What is the difference between cells at point A and point B in time?	• LPS challenge • Response to exercise
DAMACY	**D**iscriminant **A**nalysis of **M**ulti**A**spect **Cy**tometry: Discriminating between cell populations relevant for one group of patients versus the other. Creating a diagnostic score (accuracy).	• Is a surface marker profile typical of a disease state? • What surface marker profile is typical of a disease state? • Are cell populations present in one and not in the other group?	Oxford Asthma cohort
PCA	Principal component analysis	Are there patterns visible in the principal component space between scores (cells or patients), for example grouping? Which characteristics are important in these patterns (loadings)?	Asthma cohort Utrecht, sputum data
NLPCA	Non-linear principal component analysis	What is the distribution of patients with multidimensional data characteristics (clinical and cellular) in a reduced dimensional space (scores)? What are the important characteristics influencing this distribution(loadings)?	Utrecht Asthma cohort and Oxford Asthma cohort

and elevated expression of integrin chains CD11c, CD11b and activation marker CD69 corresponding to hypersegmented neutrophils and the other population that has higher L-selection levels and lower CD16, CD11b, CD11c and CD69 that is similar to neutrophils with banded nuclei described earlier by Pillay and colleagues [44].

In high-intensity anaerobic exercise (Chapter 3.2) FLOOD analysis was performed on MFC data of leukocytes and neutrophils of elite rowers. Although the amount of leukocytes increases significantly, the cells show less surface marker variation after the exercise stimulus. This is mostly due to the relatively high increase in neutrophils. The neutrophil count rises from ~3.5 million neutrophils/mL at time point t=pre to ~9 million neutrophils/mL 2h after exercise.

Subsequent FLOOD analysis on gated neutrophils shows a large population with neutrophils with similar characteristics as neutrophils present before exercise. This is caused by a known exercise-induced shift from the marginated pool to the freely circulating pool of neutrophils [45]. The marginated pool is supposed to consist of neutrophils present in the capillary bed of organs such as in the liver. The exercise-induced neutrophil shift was studied into most detail shortly after mild exercise and not after intense anaerobic exercise and is generally considered to consist of 'regular' mature neutrophils [46]. There are a few studies on exercise-induced neutrophilia that show results pointing into the direction of exercise induced mobilization of neutrophils with other characteristics compared to neutrophils present in homeostasis [47,48]. The origin of these neutrophils was not studied.

After intense anaerobic rowing exercise we found an increase of $CD16^{dim}$ and $CD64^{dim}$ neutrophils compared to neutrophils present before exercise. These $CD16^{dim}CD64^{dim}$ 'exercise-associated' neutrophils have an overall higher expression of activation markers CBRM1/5, CD11b, CD11c and CD62L compared to the pre-exercise neutrophils. In percentage of total neutrophils the 'exercise-associated' neutrophils increase from 8% to 20%. Corrected for an increase in total neutrophil count, this type of cell goes from $0.3x10^9$/L to $1.8x10^9$/L, which is a 6-fold increase.

The function of the exercise-associated neutrophils is unclear, yet they do show similarities in surface marker expression with banded neutrophils that also have a lower CD16 expression and high expression of CD62L. However, banded neutrophils express less CD11b, CD11c and CBRM1/5 compared to 'mature' cells [44]. Unpublished results of Leliefeld *et al.* (Koenderman group) indicate $CD16^{dim}CD62L^{bright}$ neutrophils mobilised after LPS challenge are superior in bacterial killing capacity compared to other neutrophil phenotypes. If exercise-associated neutrophils are in fact superior in bacterial killing capacity compared to 'mature' neutrophils requires additional future experiments.

One can only speculate about the origin of neutrophils mobilized after exercise. The vast majority of neutrophils has similar characteristics as neutrophils present before exercise and is likely to originate from organ vasculature such as present in the liver, spleen and lung. The exercise-induced neutrophils with a $CD16^{dim}CD64^{dim}$ profile and upregulated activation

markers could originate from another location such as the bone marrow which is more associated with younger neutrophils. Future studies should focus on the morphology and detailed receptor characteristics of exercise-associated neutrophils to establish their origin.

In summary both LPS challenge and anaerobic exercise induce neutrophilia in peripheral blood. FLOOD can identify and describe subpopulations of cells that were changed or mobilized as a result of a stimulus. It does so in a very detailed manner based on surface marker expression. In the future FLOOD based sorting strategies might improve cell selection for laboratory assays by the ability to pre-select stimulus associated cells based on multiple surface marker characteristics simultaneously.

DAMACY (**D**iscriminant **A**nalysis of **M**ultiAspect **CY**tometry) was used in Chapter 3.3 to analyse MFC data of asthma patients and test whether (i) cell profiles in peripheral blood would discriminate between asthma patients and controls, (ii) what cell populations were characteristic of severe eosinophilic asthma and (iii) what cell populations were typical of severe non-eosinophilic asthma (**Figure 3, second panel**, DAMACY). Eosinophilic asthma was defined based on the biomarkers blood eosinophilia (>0.27x10^9/L) and/or the presence of sputum eosinophilia (>3% of non-squamous cells). The main findings in this chapter are the typical pattern of blood eosinophils, basophils, Th2 cells and Tc2 cells in eosinophilic asthma and, in non-eosinophilic asthma, a pattern with a very homogeneous neutrophil population and the presence of cells that highly express the CD8 receptor. Whether these cells are in fact Tc1 cells is unclear, the presence of intracellular IFN-γ was not measured. The identification of Tc2 cells in eosinophilic asthma by DAMACY strengthens the finding in the last chapter of this thesis that describes a higher concentration of Tc2 cells in peripheral blood of severe eosinophilic asthma patients with asthma defined by the ERS/ATS guideline (Chapter 4) [2].

DAMACY also provided diagnostic scores which reached fair accuracy discerning asthma patients from healthy controls with 90% accuracy. Separating eosinophilic asthma from controls resulted in 71% accuracy, and non-eosinophilic asthma from controls in 80% accuracy. The explanation of a lower accuracy in eosinophilic asthma versus the overall asthma population is likely due to low numbers of patients included in the analysis. Future studies should aim for high numbers of patients to increase diagnostic accuracy and optimized flow cytometry panels to further improve inflammatory pattern recognition in peripheral blood of asthma patients.

Although multidimensional analysis techniques for MFC analysis are becoming more common, it is challenging to combine this technology with clinical datasets to unravel pathophysiology, diagnose disease, personalize medicine and monitor treatment [49]. There are no studies yet that incorporate MFC data to their full extent and capacity in clinical studies on asthma. In Chapter 3.3 (DAMACY) flow cytometry data were analysed in an unbiased way, but not yet combined with clinical data such as sex, age, FeNO, symptom expression etc. The combination of MFC data and clinical data is likely to lead to new discoveries as shown in a preliminary way in Chapter 2.1 where refractory eosinophils were found to associate with

known markers of eosinophilic asthma such as FeNO, nasal polyps and peripheral blood eosinophilia. A next step would be to integrate FLOOD and DAMACY with clinical data to further investigate asthma phenotypes and ultimately asthma endotypes 3.

Tc2 cells in severe eosinophilic asthma

In Chapter 4 a strong association was found between levels of Tc2 cells in peripheral blood and severe eosinophilic asthma defined by blood eosinophilia and/or sputum eosinophilia. Additionally, the lipid mediators Prostaglandin D2 (PGD_2) and cysteinyl Leukotriene E_4 (LTE_4) were found to be present in high concentration in the airways of these patients. The *in vitro* susceptibility of Tc2 cells to stimulation with PGD_2 and LTE_4 and the ability of Tc2 cells to secrete type-2 cytokines upon activation suggest an important role of these cells in severe eosinophilic asthma.

Targeting this cell could be a promising treatment approach for patients with severe eosinophilic asthma by blocking the PGD_2 receptor with $CRTH_2$-antagonists. The first clinical studies with CRTH2-antagonists showed varying results in general asthma populations [50–53]. Adequate pre-selection of patients has proven to be essential to reach clinical effect. Pre-selection of patients for treatment with CRTH2-antagonists could comprise of MFC measurements and subsequent DAMACY analysis to identify possible responders on forehand; DAMACY analysis found Tc2 cells to be characteristic of eosinophilic asthma defined by blood and/or sputum eosinophilia independent of the ERS/ATS classification that was used in Chapter 4.

In this same study type-2 innate lymphoid cells (ILC2s) were not found to be present in higher concentrations in peripheral blood nor in the airways of patients with severe eosinophilic asthma [54]. ILC2s are hypothesized to be essential producers of type-2 cytokines such as IL-4, IL-5 and IL-13 in non-allergic asthma [55]. The absence of these cells seems to indicate the role of this cell type is limited in severe eosinophilic asthma, although it is important to notice it is very challenging to study these cells in the airway tissue compartment due to low numbers present and technically demanding laboratory assays for cell identification.

A fundamental question that has not been addressed in both Chapter 3.3 and Chapter 4 is what the link is between cytotoxic T-cells and asthma. In the classic allergic cascade there is no place for these cells. A possible explanation for their presence in severe eosinophilic asthma was described by Coyle *et al*. who found virus-specific $CD8^+$ T-cells become potent IL-5 producers in the presence of TH2 cells [56]. They switch off IFNγ production and contribute to increased airway eosinophilia in mice. This study possibly links viral infections in asthma patients to airway eosinophilia. Whether this mechanism actually plays a role in human severe eosinophilic asthma has to be established.

Conclusion

In this thesis the activation state of blood eosinophils and neutrophils was discovered to contribute significantly to the diagnostic accuracy of a quick and low-invasive test for eosinophilia asthma. In patients with eosinophilic airway inflammation, blood eosinophils are more refractory to stimulation with fMLF.

A multi-dimensional reduction approach based on Non-linear PCA reduced dimensions of cellular and clinical parameters, making use of the multivariate advantage. Subsequent Discriminant Analysis of PCA scores of the Utrecht asthma cohort lead to a prediction model that was validated by the independently measured Oxford cohort of asthma patients. In contrast to blood, sputum eosinophils and neutrophils were found to be overall highly activated, independent of the asthma phenotype being eosinophilic or non-eosinophilic. Hence, sputum eosinophil and neutrophil activation do not have diagnostic value for asthma phenotypes.

In the subsequent two chapters, the MFC analysis method FLOOD was described. FLOOD identifies and describes subpopulations of cells that are changed or mobilized as a result of a stimulus. FLOOD showed its potential in the human LPS challenge study by clearly visualizing two subpopulations in an unsupervised fashion that were already known to be present but were not yet described in so much detail regarding specific receptor profile. FLOOD also identified exercise-associated neutrophils in elite rowers after intense anaerobic exercise. The exercise-associated neutrophils show similarities to banded cells appearing after LPS challenge. Additional functional assays (*e.g.* bacterial killing capacity) will be required to verify whether these neutrophils are in fact similar.

DAMACY, a second multivariate MFC analysis method, proved helpful in diagnosing asthma in general and eosinophilic asthma specifically. Moreover, it identified an important unknown subset of $CD3^+CD8^+CRTH2^+$ cells, known to be Tc2 cells. In the last chapter of this thesis Tc2 cells are found to be present in higher numbers in peripheral blood and airways of patients with severe eosinophilic asthma. *In vitro* assays show an activation response of Tc2 cells by PGD_2 and LTE_4, both lipid mediators that were measured in higher concentrations in the airways of patients with severe eosinophilic asthma. Therefore Tc2s might play an important role in severe eosinophilic asthma and could be a promising target of therapy.

In summary, this thesis shows that a combination of detailed cellular measurements and improved multi-dimensional and unbiased analysis methods of MFC data lead to the development of promising diagnostic steps to further phenotype asthma with the ultimate goal to improve asthma care.

Future directions

- To objectify the role of tissue eosinophils in health and disease.
- To aim for restored tissue homeostasis in eosinophilic asthma.
- To develop a prospective pharmaceutical intervention study using cell activation data and clinical parameters to select eosinophilic asthma in patients not on oral steroids. The Utrecht and Oxford cohort data can serve as a training set and would likely improve the patient pre-selection with the aim to reach maximum treatment effect. The pharmaceutical intervention could involve any compound known to suppress eosinophilic inflammation.
- To develop high-dimensional flow cytometry antibody panels relevant for eosinophilic asthma and use unbiased analysis such as DAMACY for cell population discovery, recognition of inflammatory patterns and monitoring treatment responses in asthma.
- To use FLOOD based sorting strategies that improve cell selection for laboratory assays by the ability to pre-select stimulus-associated cells based on multiple surface marker characteristics simultaneously.
- To target eosinophilic inflammation by CRTH2-antagonists in a preselected patient group that is characterized by high concentrations of eosinophils, basophils and Tc2-cells in peripheral blood which can be identified by MFC measurements and subsequent unbiased DAMACY analysis.

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Fenotypering van astma door multidimensionele analyse

Met de nadruk op kenmerken in perifeer bloed van patiënten met ernstig astma

Ernstig astma komt in Nederland bij 4% van de astmapatiënten voor en veroorzaakt een hoge ziektelast, meer dan de helft van de astma-gerelateerde zorgkosten en in zeldzame gevallen overlijden. Bij deze groep patiënten is er een grote behoefte aan biomarkers om het ontstekingstype vast te stellen, de therapierespons te voorspellen en de therapie te monitoren.

Dit proefschrift laat zien dat het mogelijk is om eosinofiel astma van andere astma fenotypes te onderscheiden door gebruik te maken van gedetailleerde celeigenschappen in het bloed van de patiënt in combinatie met klinische kenmerken zoals symptoomexpressie, FeNO (stikstofoxide in uitademingslucht) en aantallen eosinofielen.

Daarnaast toont het aan dat verbeterde analysemethodes van flowcytometriedata veelbelovend zijn om verschillende typen astma in het bloed te kunnen onderscheiden. FLOOD is een analysetechniek waarmee de cellulaire respons op een stimulus heel precies kan worden worden vastgesteld. DAMACY is een analysetechniek waarmee verschillende groepen van patiënten op basis van celeigenschappen van elkaar kunnen worden onderscheiden. Deze techniek laat ook zien welke celeigenschappen daarin de doorslag geven. De flowcytometrie-analysemethoden zijn overigens breed toepasbaar om onderzoeksvragen met betrekking tot celeigenschappen te beantwoorden en dus niet alleen geschikt voor astmapatiënten.

In het laatste hoofstuk wordt beschreven dat type-2 cytotoxische T-cellen een rol spelen bij ernstig eosinofiel astma. Deze cellen zijn niet eerder in verband gebracht met astma. Tc2 cellen zijn gemeten in hogere aantallen in bloed, weefsel en sputum van patiënten met ernstig astma en worden sterk geactiveerd door PGD_2 en LTE_4. PGD_2 en LTE_4 zijn lipide-mediatoren die in hoge concentraties voorkomen in de luchtwegen van patiënten met ernstig eosinofiel astma.

Samengevat laat het zien dat verdieping in de receptorexpressie van bloedcellen met behulp van multivariate data-analysemethoden en aanvullend fundamenteel immunologisch onderzoek toegevoegde waarde hebben voor (1) fenotypering van astmapatiënten en (2) het verkrijgen van inzicht in de mechanismen die een rol spelen bij de immuunrespons die zich voordoet bij verschillende astmapatiënten. Beide zijn nodig om behandelmethoden te ontwikkelen en de meest effectieve behandelmethoden op de juiste patiënt toe te passen.

Inleiding

Astma is een verzamelnaam voor verschillende ontstekingsvormen van de luchtwegen met uiteenlopende symptomen. De gemeenschappelijke overeenkomst van deze astma-subvormen is reversibele luchtwegobstructie en een zekere symptoomlast. Het komt wereldwijd bij ruim 300 miljoen mensen voor. In Nederland komen jaarlijks ~600.000 mensen bij de huisarts met astmatische klachten. De meeste van deze mensen zijn goed te behandelen met ontstekingsremmers (inhalatiesteroïden) en luchtwegverwijders (β2-sympaticomimetica). Een klein deel (4%) van deze 600.000 patiënten heeft ernstig astma. Deze kleine groep heeft een hoge ziektelast, komt veel bij verschillende artsen en moet regelmatig in het ziekenhuis worden opgenomen met een astma-aanval. Bovendien is dit deel verantwoordelijk voor meer dan de helft van de asthma-gerelateerde zorgkosten.

De definitie van ernstig astma bestaat uit het optreden van een hoge symptoomlast, met vaak een piepende ademhaling, benauwdheidsklachten, kortademigheid en hoestklachten. De symptoomlast is te meten met vragenlijsten, zoals de Asthma Control Questionnaire (ACQ). Tevens is het optreden van een astma-aanval onderdeel van de definitie, waarbij 2 of meer aanvallen per jaar als criterium voor de diagnose geldt. Een astma-aanval (of exacerbatie) is een plotselinge toename van symptomen gedurende langer dan 48 uur waarvoor een prednisonkuur wordt voorgeschreven door de huisarts of longarts. Er is sprake van een ernstige astma-aanval als een patiënt moet worden opgenomen in het ziekenhuis. Tot slot is persisterende luchtwegobstructie een criterium. Luchtwegobstructie wordt gedefinieerd door een verminderde hoeveelheid uitgeblazen lucht in 1 seconde (tot minder dan 80%) en een vermindering van de hoeveelheid uitgeblazen lucht ten opzichte van de maximaal uitgeademende lucht zonder tijdsbeperking.

De definitie van ernstig astma is een 'klinische definitie' waarbij er geen rekening gehouden is met de onderliggende onstekingsziekte. Om een onderliggende onsteking te meten wordt er gebruik gemaakt van biomarkers. Een biomarker is een objectief te meten, ziektegerelateerde indicator die bij voorkeur (1) specifiek is voor een aandoening, (2) geschikt is om de ziekte-activiteit over de tijd te meten en (3) patiëntvriendelijk te meten is. Voor astma zijn er diverse biomarkers die tot op zekere hoogte aan deze criteria voldoen. De drie belangrijkste biomarkers zijn (1) het percentage eosinofielen in sputum, (2) stikstofoxide in uitademingslucht (FeNO) en (3) het aantal eosinofielen in het bloed. Eosinofielen zijn witte bloedcellen die tot het type granulocyten behoren. Eosinofielen zijn bij een deel van de patiënten met ernstig astma in hoge mate aanwezig in bloed, sputum en in het longweefsel.

Studies uit het verleden hebben uitgewezen dat het starten van een prednisonkuur bij de aanwezigheid van meer dan 3% eosinofielen in het sputum leidt tot minder astma-aanvallen. Het meten van eosinofielen in sputum is echter beperkt gebleven tot een aantal specialistische klinieken.Dit heeft te maken de beschikbaarheid van expertise, tijd en middelen om de vereiste sputuminductietechniek te kunnen verrichten.

FeNO wordt beschouwd als een surrogaatmarker voor eosinofiele inflammatie in het sputum, maar is niet bewezen effectief als behandelindicator. De verrichte klinische studies lieten geen afname van longaanvallen zien bij gebruik van inhalatiesteroïden.

Tot slot is het aantal eosinofielen in bloed gebruikt als biomarker in studies met anti-IL-5 therapie bij patiënten met ernstig astma. IL-5 (Interleukine-5) is een cytokine dat een belangrijke rol speelt in de proliferatie, differentiatie en activatie van eosinofielen. Uit studies met anti-IL-5 is gebleken dat een hoger aantal eosinofielen van positief voorspellende waarde is voor het verlagen van longaanvallen en het verminderen van steroïdgebruik bij patiënten met astma. Uit deze studies blijkt echter ook dat sommige patiënten erg goed reageren en anderen niet.

Samengevat zijn er drie belangrijke biomarkers voor ernstig astma die in wisselende mate effectief zijn gebleken. Ondanks deze drie markers kan er bij een aanzienlijk deel van de patiënten met ernstig astma geen therapierespons voorspeld worden. Er is behoefte aan een (combinatie van) minimaal invasieve, specifieke biomarker(s) om therapierespons goed te voorspellen. Deze test moet toepasbaar zijn in de tweedelijnszorg (ziekenhuizen). Het grootste deel van dit proefschrift draagt in fundamentele zin bij aan de ontwikkeling van een dergelijke test.

Deel 1. Biomarkers en immunotherapie

In **Hoofdstuk 1.1** worden verschillende biomarkers voor het fenotyperen van astma beschreven. De huidige klinisch relevante biomarkers die worden verkregen uit sputum, bronchoalveolaire lavage, uitademingslucht, urine en bloed zijn niet specifiek genoeg, te tijdrovend en/of te invasief. De meest veelbelovende markers zijn te vinden in uitademingslucht en bloed. Beide technieken om markers te verkrijgen zijn minimaal invasief en de specificiteit van markers is aan het verbeteren. Het combineren van meerdere markers zou bovendien nog bij kunnen dragen aan een verbeterde fenotypering.

Hoofdstuk 1.2 is een overzicht van het gebruik van biomarkers om aan de juiste patienten de juiste immunotherapie te geven. Het is essentieel gebleken om biomarkers te gebruiken voor het selecteren van de juiste astmapatiënt voor een specifieke immunotherapie. Op dit moment zijn FeNO en periostinspiegels in serum van goede voorspellende waarde voor een therapierespons op anti-IgE en anti-IL4-α behandeling, terwijl het aantal bloedeosinofielen een effectieve voorspeller is voor de therapierespons op anti-IL-5. Op dit moment zijn er beperkte vooruitzichten voor patiënten met ernstig astma en een non-eosinofiel biomarkerprofiel. Neutrofiele ontsteking en Th17 gemedieerde processen zijn het onderwerp van huidige studies voor die groep patiënten.

Deel 2. Het fenotyperen van astma met behulp van de granulocytenrespons in bloed en sputum

Hoofdstuk 2.1 bevat de resultaten van de AIR-studie (Asthma Inflammatory Research). Deze studie had tot doel om een non-invasieve bloedtest te ontwikkelen om de gouden standaard voor astma-ontstekingstypen (sputumanalyse) te vervangen. De activatie-status van eosinofielen en neutrofielen is in de studie gebruikt om het bloedprofiel van patiënten in meer detail te beschrijven. De studie met 115 patiënten uit het UMC Utrecht leidde tot een multivariaat model bestaande uit klinische parameters zoals symptoomexpressie en de hoogte van FeNO in combinatie met cellulaire parameters zoals de respons van eosinofielen en neutrofielen op de stimulus fMLP (N-Formylmethionyl-leucyl-fenylalanine). De basis voor dit model werd gelegd door non-lineaire principale component analyse (NLPCA) te gebruiken, gevolgd door discriminantanalyse (DA).

Het multivariate model werd gevalideerd door 34 astmapatiënten uit Oxford te includeren en op dezelfde manier het bloedonderzoek te verrichten en klinische parameters te meten. De diagnostische test die op basis van het Utrecht-cohort ontwikkeld was, kon met 91% sensitiviteit en 92% specificiteit sputumeosinofilie vast te stellen in het Utrecht-cohort en met respectievelijk 77% en 71% sensitiveit en specificiteit in het Oxford-cohort. Het verschil in diagnostische kwaliteit tussen de twee cohorten is waarschijnlijk te wijten aan prednisongebruik in het Oxfort cohort.

Hoofdstuk 2.2 bevat eveneens data afkomstig van het cohort van de AIR-studie, maar was een sub-studie met als doel om te bestuderen of de activatiestatus van neutrofielen in het sputum gebruikt kan worden om klinische verschillen tussen patiënten te onderscheiden. Uit de resultaten blijkt dat neutrofielen in het sputum diffuus geactiveerd en gedegranuleerd zijn, onafhankelijk van de aanwezigheid van eosinofielen in het sputum of klinische kenmerken zoals de hoogte van FeNO en symptoomexpressie. Ook eosinofielen in het sputum waren diffuus geactiveerd en gedegranuleerd. Een opvallende nevenbevinding was de expressie van CD274 (PD-L1) op neutrofielen en in mindere mate op eosinofielen in sputum. Dit suggereert dat granulocyten in sputum een immuunregulatoire werking kunnen hebben.

Deel 3. Multidimensionele analyses van flowcytometriedata

Flowcytometrie is een analysemethode om cellen te meten in een vloeistofstroom door gebruik te maken van licht. Er kan ook gebruik gemaakt worden van fluorescent gelabelde markers om bijvoorbeeld receptoren op cellen te meten. In **Hoofdstuk 3.1** wordt een analysemethode beschreven, genaamd FLOOD, die het mogelijk maakt om complexe hoog-dimensionele flowcytometriedata te analyseren. De analysemethode FLOOD werd hier gebruikt om het verschil tussen receptor-eigenschappen op neutrofielen van gezonde individuen (controles) te onderscheiden van die van vrijwilligers die een stimulus hebben gekregen met deeltjes bacteriewand in de bloedbaan (LPS, lipopolysaccharide). LPS geeft een heftige fysiologische respons met hoge koorts, een versnelde hartslag en een

verlaagde bloeddruk. Dit hoofdstuk laat zien dat FLOOD in staat is om een zeer precieze beschrijving te geven van het verschil in receptorprofiel tussen neutrofielen van controles en LPS-gestimuleerde vrijwilligers. De methode kan ook gebruikt worden voor andere onderzoeksomstandigheden waarbij de receptorprofielen van de ene groep met de andere worden vergeleken of van het ene tijdspunt met het andere.

In **Hoofdstuk 3.2** wordt de neutrofielenrespons van roeiers op nationaal niveau op een inspanningsprikkel beschreven. De precieze verandering in receptorexpressie kon worden bepaald met FLOOD. Dit liet zien dat een anaerobe inspanningsprikkel zorgde voor een neutrofilie en dat er naast toename van het aantal neutrofielen ook neutrofielen met andere eigenschappen aanwezig waren dan bij een toestand van homeostase. Het bloed van de roeiers bevatte na de inspanning zesmaal meer neutrofielen (van 0,3 naar 1,8x10^6/mL cellen) met $CD16^{low}CD64^{low}$ expressie en over het geheel genomen meer CD11b, CD11c, CBRM1/5 en CD62L. De rol van deze neutrofielen is nog niet duidelijk en zal in de toekomst met functionele celtesten moeten worden vastgesteld.

In **Hoofdstuk 3.3** wordt DAMACY ingezet, een tweede flowcytometriedata-analysemethode, om verschillen tussen onstekingsvormen van astma te beschrijven. Een specifiek gekozen panel met fluorescent-gelabelde antistoffen werd gebruikt om bloedcellen van patiënten met astma te bestuderen en middels DAMACY te beschrijven. Hieruit bleek ernstig eosinofiel astma te worden gekenmerkt door basofielen, Th2 cellen en Tc2 cellen. Non-eosinofiel astma wordt gekenmerkt door een lagere receptorvariatie van CD16 op neutrofielen en de verhoogde aanwezigheid van $CD3^+CD8^{++}$ cellen ten opzichte van $CD3^+CD8^+$ cellen.

Deel 4. Type–2 cytotoxische T–cellen in patiënten met een ernstige vorm van eosinofiel astma

In **Hoofdstuk 4** wordt de aanwezigheid van type-2 cytotoxische T-cellen (Tc2) voor het eerst in verband gebracht met ernstig eosinofiel astma. In twee verschillende cohortstudies in Oxford en in Southampton werden deze cellen in hogere concentraties gevonden in bloed, sputum en weefsel van astmapatiënten met eosinofiel astma in vergelijking met andere astmapatiënten en gezonde controles. Daarnaast werden de lipide-mediatoren PGD_2 en LTE_4 in hoge concentraties in sputum gemeten, deze mediatoren kunnen Tc2 activeren en aantrekken. Tevens zorgen PGD_2 en LTE_4 voor de upregulatie van genen die geassocieerd zijn met type-2 ontstekingsreacties. Naast de reeds bekende Th2 (type-2 T-helpercellen) en ILC2 (lymphoide cellen van het aangeboren immuunsysteem) zijn ook Tc2 nu geassocieerd met ernstig eosinofiel-astma.

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Met veel plezier heb ik de afgelopen jaren onderzoek kunnen doen en veel mensen hebben in die tijd bijgedragen aan de totstandkoming van dit proefschrift. Graag grijp ik het best gelezen deel van dit proefschrift aan om iedereen te bedanken. Het gaat om patiënten, collega's, vrienden en familie uit alle windstreken, van Utrecht tot Nijmegen en van Coevorden tot Oxford.

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Curriculum vitae

Bart Hilvering was born on the 24th of October in Coevorden. After completing his secondary education at the Nieuwe Veste Gymnasium in Coevorden, he started studying Medicine at the Utrecht University in 2003.

During his studies he started a randomised controlled trial at the Meander Medical Center in Amersfoort to study intraperitoneal pain blockade during laparoscopic cholecystectomy. In 2010 he obtained his Medical degree and in 2011 he started his PhD on severe eosinophilic asthma at the Eijkman school of Infection & Immunity which is part of the University Medical Center Utrecht. The main focus of his PhD was the development of a blood test to phenotype severe asthma. The research was supervised by Prof. dr. Jan-Willem Lammers and Prof. dr. Leo Koenderman and co-promotor dr. René Schweizer.

In 2014 he was awarded with a long-term fellowship by the European Respiratory Society and went for a year to the Nuffield department of Medicine in Oxford where he worked as a clinical research fellow at the laboratory of Prof. Ian Pavord. Under co-supervision of the immunologist dr. Luzheng Xue, he studied the role of type-2 Innate Lymphoid Cells in peripheral blood, broncho-alveolar lavage and tissue of patients with severe asthma.

In 2015 Bart returned to the University Medical Center in Utrecht to start his training in Respiratory Medicine. Currently he works at the St. Antonius Hospital in Nieuwegein as a Registrar in Internal Medicine.

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