The Role of CRTH2^{+} CD8^{+} T cells in Severe Asthma



Ryan David Hoyle

Magdalen College, University of Oxford

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Supervisors: Professor Luzheng Xue, Professor Paul Klenerman, Professor Ian Pavord, and Dr Timothy Hinks

Abstract

Type 2 inflammation of the airways is present in most patients with asthma, and some patients with chronic obstructive pulmonary disease (COPD). The molecular drivers of the type 2 immune response include the cytokines IL-4, IL-5, and IL-13, produced by activated type 2 T helper (Th2) and type 2 cytotoxic T (Tc2) cells. Expression of the prostaglandin D_2 (PGD_2) receptor chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) has been associated with type 2 T cells, and $CRTH2^+$ T cells in the blood and lung have been linked to the severity and endotype of asthma. In a new study of 127 participants, blood $CRTH2^+$ T cell counts were not associated with asthma severity or endotype, and instead appeared to be driven by other factors such as smoking history and biological sex. Airway $CRTH2^+$ T cells were detectable in the sputum, and airway $CRTH2^+$ Th cells correlated with sputum eosinophils. The sensitivity and specificity of CRTH2 as a marker of type 2 T cells was assessed *ex vivo*, showing that CRTH2 is a more sensitive marker of Tc2 (~80 %) than Th2 (~50 %) cells. $CRTH2^+$ $CD8^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) and expanded without addition of polarising cytokines, retaining a stable type 2 identity. Cultured $CRTH2^+$ $CD8^+$ T cells could be stimulated by multiple pathways: through the T-cell receptor, by PGD_2-CRTH2 interactions, or by alarmin cytokines. The transcriptomic response of $CRTH2^+$ $CD8^+$ T cells was specific to each stimulation. The gene expression patterns of activated $CRTH2^+$ $CD8^+$ T cells highlighted their capacity to contribute to type 2 inflammation, but also revealed novel links to genes and biological processes associated with asthma. These data support future study of $CRTH2^+$ $CD8^+$ T cells in the human airways.

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Covid-19 Impact Statement

The University of Oxford has released guidance to graduate students regarding the effects of the coronavirus pandemic on our studies. We have been advised to either apply for an extension, or to submit a thesis on time containing details of how our work has been disrupted. I was advised by my supervisors and the departmental director of graduate studies to choose the second of these options.

Broadly speaking, I have been fortunate in my experience of the Covid-19 pandemic so far, in that I have not suffered major personal loss or illness. However, there was some disruption to my work due to the general University closures. Our laboratory was shut (and patient research visits ceased) between March 13th and August 7th, 2020. I used this closure time to analyse clinical data I had collected up to that point, which became Chapter 2 of this thesis. Access to the labs has been slightly restricted for the remaining time (i.e., until August 2021) in line with University and national guidance.

More disruptive than the closure of lab space was the limitations on availability and use of human tissue samples, especially of human airways. Fortunately, we had some historical cell cultures available which I was able to use to generate the data in Chapter 4. Due to safety concerns, I did not get clearance to work with human blood until the end September 2020, which facilitated generation of the data presented in Chapter 3.

Much of my DPhil project plan that was approved during the Transfer of Status exam at the end of 2018 revolved around the study of sputum. I aimed to generate the first sputum single cell atlas, and was awarded some funding from the MRC to pursue this. However, we encountered protracted problems with sampling which I failed to solve before the outbreak of SARS-CoV-2 in March 2020. Since the induction procedure produces a lot of aerosols, it has not been safe to resume sampling at any point since. The MRC kindly approved my application to adapt this project to look at lung tissue resection margins instead of sputum. However, the raw data was not generated until June 2021. This was too late to begin analysis and interpretation before my return to clinical training, so the data are not included in this thesis.

Abbreviations

Abbreviation	Long format
ACQ	Asthma Control Questionnaire
AM	alveolar macrophage
ANOVA	analysis of variance
AP-1	activator protein 1
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BP	biological processes (domain of GO database)
cAMP	cyclic adenosine monophosphate
CCL	C-C chemokine ligand (e.g., CCL17, CCL18 etc.)
CCR	C-C chemokine receptor (e.g., CCR3, CCR7 etc.)
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
CRSwNP	chronic rhinosinusitis with nasal polyps
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
DAG	diacylglycerol
DAMP	damage-associated molecular pattern
DC	dendritic cell
DEG	differentially expressed gene
DMSO	dimethyl sulphoxide
DNT2	type 2 double negative T
DP1	prostaglandin D2 receptor 1
DP2	prostaglandin D2 receptor 2 (CRTH2)
DREAM	Dose Ranging Efficacy And safety with Mepolizumab in severe asthma
ECM	extracellular matrix
EGR	early growth response
ERS	European Respiratory Society
FACS	fluorescence-activated cell sorting
FC	fold change
FCS	foetal calf serum
FeNO	fractional exhaled nitric oxide
FEV1	forced expiratory volume in 1 second
FMO	fluorescence minus one
FVC	forced vital capacity
GINA	Global Initiative for Asthma
GO	Gene Ontology
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPCR	G protein-coupled receptor
GSEA	gene set enrichment analysis
H-PGDS	haematopoietic prostaglandin D synthase
HV	healthy volunteer
ICS	inhaled corticosteroids OR intracellular cytokine staining (context-dependent)
IFNγ	interferon gamma

Ig	immunoglobulin (e.g., IgA, IgE etc.
IL	interleukin (e.g., IL-4, IL-5, IL-9 etc.)
ILC	innate lymphoid cell
ILC2	type 2 innate lymphoid cell
ILR	interleukin receptor (e.g., IL6R, IL4R, etc.)
IP3	inositol triphosphate
KEGG	Kyoto Encyclopaedia of Genes and Genomes
M2	alternatively-activated macrophage
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMA	mild-moderate asthma (or non-severe asthma)
MRC	Medical Research Council
mTORC	mammalian target of rapamycin complex (e.g., mTORC1 or mTORC2)
NES	normalised enrichment score
NFAT	nuclear factor of activated T-cells
NMU	neuromedin U
NPV	negative predictor value
OAS	Oxford Airways Study
OCS	oral corticosteroids
ORA	Oxford Research Archive
OUH	Oxford University Hospitals
padj	adjusted p-value
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PGD_2	prostaglandin D_2
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PPV	positive predictor value
PRR	pattern recognition receptor
PYH	pack-year history
Q-Q	quantile-quantile
RAST	radioallergosorbent test
REC	research ethics committee
REM	rapid expansion medium
RIN	RNA integrity number
RSV	respiratory syncytial virus
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
scRNA-seq	single cell RNA sequencing
SEA	severe eosinophilic asthma
SFK	Src family tyrosine kinase
SNA	severe non-eosinophilic asthma

STA	severe asthma treated with eosinophil-depleting therapy
T2	type 2
T2C	type 2 cytokine
Tc	cytotoxic T
Tc1	type 1 cytotoxic T
Tc2	type 2 cytotoxic T
TCR	T-cell receptor
Tfh	T follicular helper
Th	T helper
Th1	type 1 T helper
Th17	type 17 T helper
Th2	type 2 T helper
Th2A	proallergic Th2
TNF	tumour necrosis factor
TP	thromboxane receptor
Tpath2	pathogenic Th2
Treg	regulatory T
Trm	tissue-resident memory T
TSLP	thymic stromal lymphopoietin
US	unstimulated

Word Count

Abstract, 289; main text, 49 639 (ex. bibliography, appendices, diagrams and tables).

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Chapter 1

General Introduction

1.1 Asthma and COPD

1.1.1 Current definitions

The current definition of asthma encompasses: four variable core symptoms of wheeze, breathlessness, chest tightness and cough; underlying chronic inflammation; and variable airflow limitation (Global Initiative for Asthma, 2021). Chronic obstructive pulmonary disease (COPD) is defined in part by persistent symptoms of breathlessness, cough, and sputum production. The other component of its definition is persistent (in contrast to variable) airflow limitation, usually due to lung damage from environmental exposure, especially cigarette smoke (Global Initiative for Chronic Obstructive Lung Disease, 2021).

Both asthma and COPD are obstructive (exhalation-limiting) lung diseases, involve complex aetiology, pathophysiology, and management (Table 1), and are incurable with current therapies. Both conditions are subject to exacerbations, defined as an acute worsening of symptoms and, in the case of asthma, acute and temporary reduction in lung function (Global Initiative for Asthma, 2021; Global Initiative for Chronic Obstructive Lung Disease, 2021), which can require hospitalisation or become life-threatening.

1.1.2 Historical account

1.1.2.1 Early history

Asthma is a chronic respiratory disease that has affected humans since prehistory. Physicians of antiquity identified a condition characterised by sudden periods of breathlessness (Marketos & Ballas, 1982), and Aretaeus of Cappadocia wrote a clinical description in 1st century CE that included asthma attacks (Aretaeus of Cappadocia, 1972; Marketos & Ballas, 1982). The first English text was Sir John Floyer's *A Treatise of the Asthma* (John Floyer, 1698), in which he identified intermittent obstruction ('periodic

Table 1 Summary of current understanding of Asthma and COPD

Information collated from current reviews of asthma (Global Initiative for Asthma, 2021; Stephen T. Holgate et al., 2015; Papi et al., 2018) and COPD (P. J. Barnes et al., 2015; Ferrera et al., 2021; Global Initiative for Chronic Obstructive Lung Disease, 2021; Rabe & Watz, 2017). Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; AERD, aspirin-exacerbated respiratory disease; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; GOLD, Global Initiative for Chronic Obstructive Lung Disease; ICS, inhaled corticosteroids; mMRC, modified Medical Research Council scale; NSAID, non-steroidal anti-inflammatory drug; SABA, short-acting beta agonist; SGRQ, St George's Respiratory Questionnaire.

	Asthma	**COPD**
Symptoms	- Wheeze, shortness of breath, cough, and/or chest tightness - Fatigue/insomnia	
	- Trigger avoidance - Often worse at night/morning - Symptoms highly variable	- Chronic sputum production - Symptoms are chronic and progressive
Natural history	- Onset at any age - Can regress, progress, or neither - Periodicity of disease	- Onset in mid to late life - Progressive worsening of disease - Constancy of disease
Common Classifications	- Symptom severity and treatment requirements (e.g., GINA steps 1-5, difficult-to-treat, severe, etc.) - Controlled vs uncontrolled - Allergic vs non-allergic - By trigger (e.g., occupational, aspirin AERD, aspergillus ABPA)	- By FEV1 (GOLD stages 1-4) - Degree of breathlessness (mMRC grades 1-4) - symptoms and exacerbation risk (GOLD grades A-D)
	- Self-reported questionnaires e.g., SGRQ, with variable cut-off - Inflammatory endotype (e.g., T2-high) - Algorithmic clustering	
Lung function	- Variable airflow limitation (measured as FEV1, FEV1/FVC) - Airway hyperresponsiveness	- Progressive decline in FEV1 - Fixed postbronchodilator FEV1/ FVC < 0.7
Diagnosis	- Clinical history (e.g., symptoms, comorbidities, family history) - Spirometry - Chest sounds	
	- Reversibility test - Exhaled nitric oxide (FeNO) - Provocation challenge - Allergy tests/challenge	- Exercise test - Radiography - Lung volume test
Common Comorbidities	- Allergies and atopy (atopic dermatitis, rhinitis, nasal polyps) - Obesity - NSAID intolerance	- Smoking-related diseases (e.g., hypertension, diabetes, heart disease, lung cancer) - skeletal muscle dysfunction - osteoporosis - cognitive impairment
	- Recurrent respiratory infections (especially COPD) - Gastroesophageal Reflux Disease (GERD) - Lung disease (obstructive sleep apnoea, bronchiectasis) - Mental illness (e.g., anxiety and depression)	

Exacerbation Triggers	- Respiratory infections - Exposure to inhaled particles (e.g., allergens, irritants)	
	- Exercise - Cold air/weather changes - Diet/food - Emotion and stress - Drugs e.g., aspirin, β-blockers	- Heart problems (e.g., arrhythmia)
Gross pathology	- Airway wall thickening - Gas trapping	
	- Mucous hypersecretion and plugging - Increased smooth muscle mass & contractility - Airway damage and remodelling	- Emphysema (tissue breakdown) - Bronchiolitis (blockage of small airways due to infection) - Small airway disease (e.g., fibrosis, narrowing) - Hyperinflation
Cellular and molecular pathology	- T2-mediated airway eosinophilia - T1 or T17-mediated airway neutrophilia - Chronic airway inflammation	
	- IgE cross linkage and activation of mast cells	- Systemic inflammation - Increased airway macrophages - High oxidative stress - High protease burden
Risk factors	- Genetics - Exposure to particles (e.g., cigarettes, indoor smoke, pollution) - Impaired lung development (e.g., low birth weight) - Socioeconomic and/or psychological problems	
	- Presence of comorbidities - Exposure to allergens	- Age - Childhood respiratory disease - Asthma - Alpha-1 antitrypsin deficiency
Treatment options (exacerbation)	- Supplemental oxygen - Short acting bronchodilators (e.g., SABA) - Short course of oral corticosteroids - Non-invasive ventilation or invasive ventilation (if life-threatening)	
	- Epinephrine (if anaphylaxis) - Magnesium sulphate	- Antibiotics
Treatment options (maintenance)	- Education (e.g., inhaler training, adherence) - Risk and exposure reduction (especially smoking cessation) - General lifestyle changes (e.g., diet and exercise) - Self-management (e.g., action plans) - Management of comorbidities - Vaccination against common respiratory pathogens - Correction of deficiencies (e.g., Vitamin D) - Combined long-acting bronchodilator and inhaled corticosteroid - Add-on azithromycin - Add-on monoclonal antibodies (severe, T2-high or allergic)	
	- ICS alone (essential therapy – prevents exacerbation/death) - Weight reduction - Oral corticosteroids - Leukotriene receptor antagonists - Allergen immunotherapy - Surgery (e.g., bronchial thermoplasty)	- Long acting bronchodilator alone - Pulmonary rehabilitation - Mucolytics - Phosphodiesterase 4 inhibitors - Bronchoscopic intervention (e.g., endobronchial valves) - Long term oxygen therapy - Surgery (lung volume reduction) - Palliative care

asthma') caused by bronchoconstriction, and triggers including common cold, exercise, and air quality (Sakula, 1984).

Progress was made by 19th century physician Henry Salter, publishing a lifetime of observations titled *On Asthma: its Pathology and Treatment* (Salter, 1860). Salter refined the term 'asthma' to refer only to periodic disease, established nervous bronchospasm as an underlying pathophysiology, and attempted probably the first sputum cytology (Figure 1A). A chapter on asthma was included in Sir William Osler's famed *The Principles and Practice of Medicine* (Osler, 1892) where inflamed mucous membrane is listed with bronchospasm as the central pathophysiology of asthma. Osler also mentioned recently discovered mucinous Curschmann spirals and proteinous Charcot-Leyden crystals in the sputum (Sakula, 1986).

Early reports of a disease likely to be COPD date back to the 17th century with clinical references to 'swollen' (hyperinflated) lungs and later to chronic cough and sputum production (Petty, 2006). These features were unified in Laënnec's 1837 description of 'emphysema' from dissecting hyperinflated lungs of his patients (Laënnec, 1827). Laennec

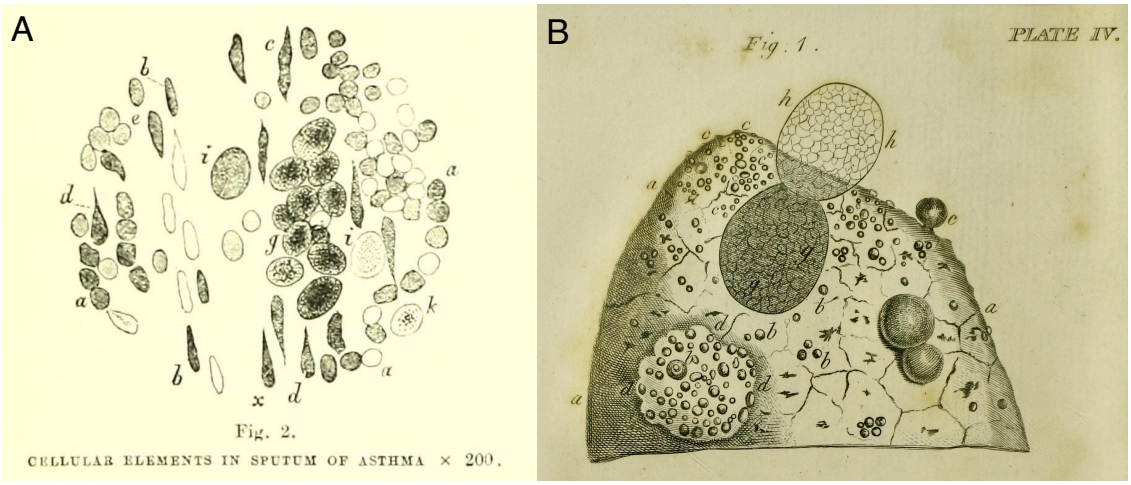


Figure 1 Early depictions of asthma and COPD

(A) Hand-drawn sputum cytology from Henry Hyde Salter's '*On Asthma'* (Salter, 1860). Salter noted the presence of cellular granules. (B) Diagram of lung tissue dissected from a patient with emphysema (COPD) (Laënnec, 1827), with particular reference to enlarged terminal air spaces. Both images are in the public domain and not subject to copyright.

describes the formation of air pockets (now known as focal lung pneumatoses) by distended and/or damaged lung structure (Figure 1B). Importantly, he distinguished the condition from periodic asthma, noting the constancy (rather than periodicity) of symptoms of dyspnoea, cough, and sputum expectoration.

1.1.2.2 Lung function tests

Advances in technology and medicine since the industrial revolution were paralleled by improved understanding of asthma and COPD. The first spirometer was reported in 1846 to aid in calculation of insurance premiums (Hutchinson, 1846). Pulmonary function testing developed further around the time of the second world war (T. D. Wu et al., 2018). With these tests, asthma and COPD were identified as obstructive (exhalation flow-limiting) lung diseases. This gave rise to the measurements of forced expiratory volume in one second (FEV1) and FEV1/FVC, where forced vital capacity (FVC) is the maximal exhaled volume of air. These concepts remain part of the diagnostic guidelines for asthma (Global Initiative for Asthma, 2021, p. 22) and COPD (Global Initiative for Chronic Obstructive Lung Disease, 2021, p. 24) and are commonly used as clinical trial outcome measurements.

1.1.2.3 Smoke inhalation

A longitudinal study through the 1970s showed that cigarette smoking irreversibly accelerates the natural decline in FEV1 in susceptible individuals, resulting in COPD (Fletcher & Peto, 1977). Further large scale studies showed that smoking cessation slows this decline and thus prevents COPD (Nicholas R. Anthonisen et al., 1994; Petty, 2006). Smoke inhalation induces widespread epigenetic, immune, and microbial changes in the lower airways (Ferrera et al., 2021), but it is not clear why only some individuals develop COPD (Petty, 2006). More recently, it has become accepted that other FEV1 trajectories

can end in COPD, such as low FEV1 during development (Lange et al., 2015). Although cigarette smoking is still the major risk factor for COPD (Global Initiative for Chronic Obstructive Lung Disease, 2021), other sources of smoke contribute, particularly indoor cooking. Air pollution also contributes to decline in lung function (Brunekreef & Holgate, 2002) and accounts for approximately a quarter of COPD mortality (Cohen et al., 2017). In asthma, the role of air pollution is less clear (Guarnieri & Balmes, 2014) but it is likely to play a role in sensitisation and exacerbation in some contexts. There is consensus that smoking increases risk of asthma, and worsens existing disease (Polosa & Thomson, 2013).

1.1.2.4 Bronchodilators

Bronchospasm theory explained the relief offered to asthma patients by bronchodilators, which expand the airways by relaxation of surrounding smooth muscle (Cazzola et al., 2012). Natural relaxants were described by ancient Egyptians (S. W. Stein & Thiel, 2017), but modern drugs are specifically formulated beta-agonists, antimuscarinics or xanthines (Cazzola et al., 2012). However, bronchodilators offer symptomatic relief and do not address underlying disease. Overreliance on bronchodilators in the 20th century was eventually linked to increased loss of asthma control (Sears et al., 1990) and death (Spitzer et al., 1992). Today, the most effective general use inhalers for asthma (Beasley et al., 2019; L. A. Lee et al., 2021) and COPD (Lipson et al., 2018; Rabe et al., 2020) contain slow acting bronchodilators in combination with an anti-inflammatory corticosteroid. However, poor inhaler technique and adherence are a barrier to asthma control (Pavord et al., 2018).

1.1.2.5 Allergy and IgE

What causes bronchospasm? The first theory came with the development of the concept of allergy in the late 19th century, soon linked to asthma (Igea, 2013). Francis Rackemann used skin prick testing to classify asthma as *extrinsic* (younger onset and allergic) or *intrinsic* (older

onset and not allergic) (Rackemann, 1947). A causative transferable agent of allergic reaction, 'reagin', was identified as immunoglobulin (Ig) E in the 1960s (Stanworth, 1993). It was shown that cross linking of IgE bound to mast cells underlies anaphylaxis, including smooth muscle contraction (Blank et al., 2013). Much later it was confirmed that mast cells are enriched in the asthmatic airway (Christopher E. Brightling, Bradding, et al., 2002). In parallel, histamine was identified as a key mediator of anaphylaxis in the early 20th century and was linked to mast cells in the 1950s (Emanuel, 1999). From these early discoveries, a network of interacting cells and molecules was described representing the current model of allergic inflammation (Gould & Sutton, 2008). Elements of this network are therapeutic targets for allergic asthma. For example, omalizumab targets the IgE fragment crystallizable (Fc) region and is an adjunct therapy for severe allergic asthma (Global Initiative for Asthma, 2021). Mast-cell derived tryptase is being targeted, with promising results in a mouse model of IgE-mediated anaphylaxis (Maun et al., 2019).

1.1.2.6 Eosinophils and IL-5

After the discovery of the eosinophil in the 19th century by Paul Ehrlich and others (Barry Kay, 2017), high counts were reported in the blood and sputum of some patients with asthma (Herrick, 1911; Sakula, 1986) and later COPD (Hargreave et al., 1996). Rackemann observed eosinophilia in both extrinsic and intrinsic asthma, suggesting independence of allergic and eosinophilic inflammation (Rackemann, 1918). Later studies linked eosinophil activity in the airway with remodelling and disease severity (Jean Bousquet et al., 1990). Importantly, corticosteroids were shown to be effective in management of eosinophilic asthma (Morrow Brown, 1958) and later COPD (Christopher E. Brightling et al., 2000), whereas early trials failed because the importance of eosinophil counts was not known. Inhaled steroids also perform better when scaled by airway eosinophils rather than disease

severity (Green et al., 2002; Siva et al., 2007). The mechanism of action of glucocorticoids is incompletely understood (R. S. Hardy et al., 2020), but T cell and eosinophil apoptosis are both increased.

The eosinophil-specific cytokine interleukin (IL) 5 was identified in the 1980s (Lopez et al., 1986; Sanderson, 1992) and connected to eosinophilic asthma (Foster et al., 1996; Hamid et al., 1991). Monoclonal antibodies targeting the IL-5 pathway struggled in trials because, echoing failed steroid trials, the patients were not selected for eosinophilia (Pavord et al., 2018). However, a renewed focus on eosinophilic patients showed that anti-IL-5 reduces exacerbations of eosinophilic asthma (Farne et al., 2017) and COPD (Donovan et al., 2020). Antibodies targeting other aspects of eosinophil biology such as Charcot-Leyden crystals, are in development (Persson et al., 2019).

1.1.2.7 Th2 cells and type 2 cytokines

The predominant theory explaining both allergic (Kubo, 2017) and eosinophilic (Jacobsen et al., 2021) inflammation is aberrant production of type 2 (T2) cytokines by type 2 T helper (Th2) cells. The subclassification of T helper (Th) cells into type 1 T helper (Th1) cells and Th2 cells began in the 1980s (T R Mosmann et al., 1986). Over the next decade, Th2 cells (Douglas S. Robinson et al., 1992) and their products (Foster et al., 1996; Grunig et al., 1998; Wills-Karp et al., 1998; Z. Zhu et al., 1999) were linked to asthma in some of the field's most widely cited papers. The Th2 cell forms the central node of the contemporary understanding of type 2 inflammation (Wynn, 2015). Many aspects of the network have been targeted with novel therapeutics at various stages of development, and with varying degrees of success, in asthma and COPD (P. J. Barnes, 2018; Chupp et al., 2020). For example, the anti-IL-4-receptor drug dupilumab reduces asthma exacerbations

and increases lung function, with benefits generally linked to the extent of type 2 inflammation (Castro et al., 2018).

1.1.2.8 Beyond Th2: the complexity of asthma and COPD

The heterogeneity of asthma was appreciated by Henry Hyde Salter (Salter, 1860), and Francis Rackemann, who acknowledged that his extrinsic/intrinsic classification system was too simple (Rackemann, 1958). Type 2 inflammation may be present in only half of patients with asthma (Fahy, 2015), and roughly a third of those with COPD (George & Brightling, 2016). It is now understood that multiple inflammatory processes (e.g., type 1 or type 17 inflammation) can coexist in both diseases, incorporating a broad range of immune and structural cells (P. J. Barnes, 2016; Hammad & Lambrecht, 2021). Furthermore, large scale genome-wide association studies (GWAS) have identified hundreds of contributing genetic loci (El-Husseini et al., 2020; Sakornsakolpat et al., 2019). Focus has therefore turned to defining the heterogeneity of these diseases in the form of *endotypes* (Anderson, 2008), with the end goal of pursuing *treatable traits* in individual patients (Pavord et al., 2018). Broad patient clustering was achieved using basic clinical measurements (Haldar et al., 2008). Since then, it has become possible to make enormously detailed airway measurements by single-cell sequencing (Travaglini et al., 2020; Vieira Braga et al., 2019). However, the inconvenience and cost of this technology has so far limited its use to very small numbers of patients. In the coming decades we can expect much larger studies to describe airway heterogeneity at the cellular and molecular level.

The importance of the airway microbiome is also increasingly appreciated (Barcik et al., 2020; Sze et al., 2012), supported by the beneficial effects of the macrolide antibiotic azithromycin in both asthma (Gibson et al., 2017) and COPD (Albert et al., 2011). Indeed, the beneficial effects of antibiotics for COPD have been known since the 1980s (N. R.

Anthonisen, 1987). An elegant study of genetically similar Amish and Hutterite children showed that the interaction between microbiome and innate immunity affects allergic sensitisation and asthma development (M. M. Stein et al., 2016). Furthermore, both asthma and the microbiome are connected to metabolic changes including obesity (U. Peters et al., 2018). Application of high-throughput single cell sequencing to prokaryotes is more challenging than to eukaryotes (Imdahl et al., 2020), so a complete picture of microbial changes in asthma and COPD remains distant. Viral infections are also closely linked to exacerbations of disease (Guo-Parke et al., 2020; Hammad & Lambrecht, 2021).

Finally, researchers are focussing on the initial development of asthma and COPD, and the effects of lung age. A landmark study in the 1990s identified children with persistent wheezing during infancy and childhood at greater risk of developing asthma (Martinez et al., 1995). Similarly in COPD, the effect of childhood factors such as early severe respiratory infection are at least as predictive of COPD development as cigarette smoking (Svanes et al., 2010). So, there appears to be consensus that disease trajectories can begin very early in life. However, disease can also develop in later adulthood, especially in the context of COPD. Indeed, there are significant overlaps between the physiology of normal lung aging and of COPD (Schneider et al., 2021). Late-onset asthma is also common and it is agreed to be biologically distinct from early-onset, although exactly in what ways has not been fully explored (R. M. Dunn et al., 2018). It is likely that development of molecular lung atlases will also begin to address these developmental questions. The long term goal will be prevention of disease, which is perhaps a more realistic goal than cure.

1.1.3 Epidemiology and clinical need

Around 545 million people suffer from chronic respiratory disease, the vast majority of them suffering from asthma or COPD (Soriano et al., 2020). Prevalence of asthma is

greater in more developed countries, and has increased for several decades but may now be plateauing (Dharmage et al., 2019). However, most asthma mortality happens in less developed countries (Papi et al., 2018). Studies of migrant populations show that the environment of developed urban areas inherently promotes asthma (Cabieses et al., 2014), so prevalence is likely to continue to increase with development. COPD is responsible for more deaths and greater morbidity than asthma both in the UK and globally (Figure 2). There are conflicting claims in the literature about whether the global burden of COPD is getting better (Soriano et al., 2020) or worse (Global Initiative for Chronic Obstructive Lung Disease, 2021).

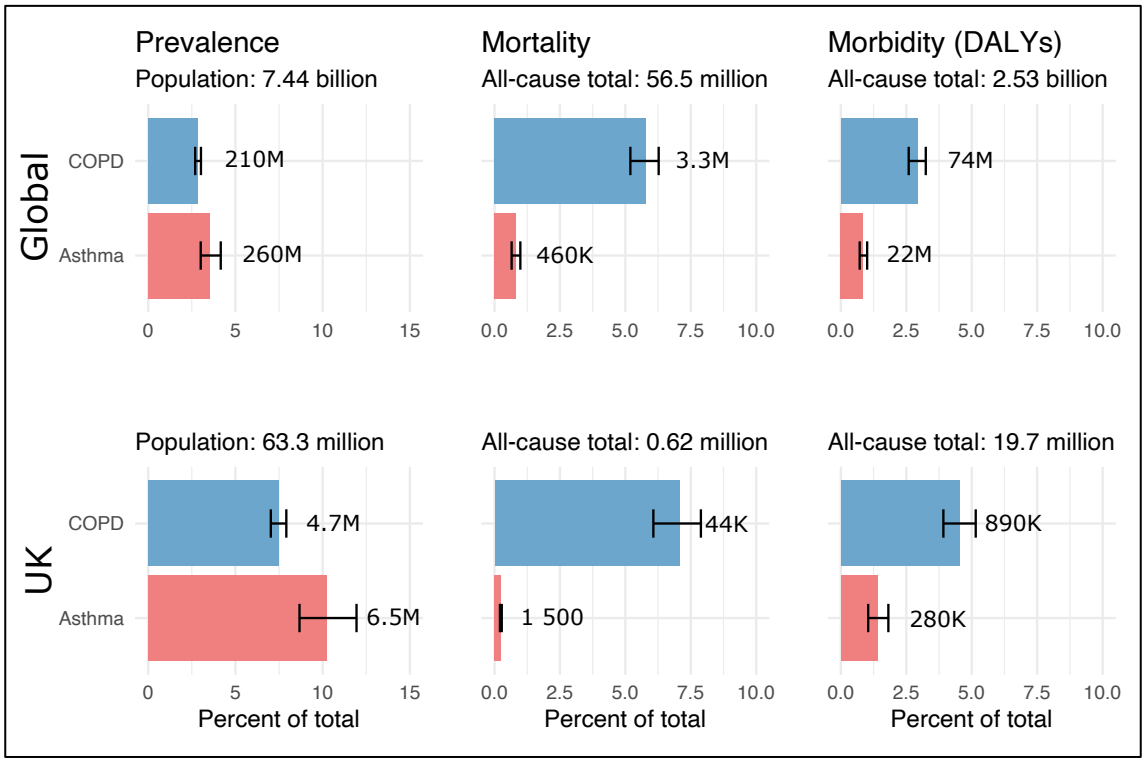


Figure 2 Current epidemiological data for asthma and COPD.

Data downloaded from the Global Burden of Disease databases (Global Burden of Disease Collaborative Network., 2020). All data are approximate and rounded to two significant figures. Figure generated in RStudio (RStudio Team, 2020), an environment for working with R (R Core Team, 2020) using package ggplot2 (Wickham, 2016), and arranged in Inkscape (Harrington, 2004). Error bars represent a 95% confidence interval. Abbreviations: DALY, Disability-adjusted life year; UK, United Kingdom.

Currently, between one-third and one-half of patients with asthma remain uncontrolled on conventional therapies (Buhl et al., 2020). Around 3 % of patients exhibit persistent T2 inflammation even with good adherence to steroids (Pavord et al., 2018), and even highly specific biological therapies do not work for all T2-high patients (Hammad & Lambrecht, 2021). The situation is worse in COPD where most patients are resistant to steroids (P. J. Barnes, 2013). Neither disease can be cured with current treatments. Clinical need exists for optimising delivery of existing therapies, and for the identification of new ones.

1.2 Type 2 Immunity and the respiratory system

1.2.1 Defence of the respiratory system

The respiratory system (Waugh & Grant, 2018) can be divided into the upper and lower respiratory tracts. The upper respiratory tract consists of the nose and nasal cavity, larynx and pharynx. The lower respiratory tract includes the trachea and the lungs. The airways form a branching tree structure classically divided into the conducting and respiratory zones (Figure 3). The primary function of the respiratory system is respiration, meaning the movement of oxygen into the body and removal of excess carbon dioxide. This process is facilitated by breathing, in which the chest expands and air flows into the lungs (inspiration), followed by the reverse process (expiration). In addition to oxygen, atmospheric air may contain a wide variety of gases and particles, some of which can be infectious or damaging to tissue. The function of the immune system is to protect healthy tissue from disease and damage. Therefore, the surfaces of the respiratory system are immunologically active sites.

The airway has multiple defence mechanisms against potential threats (reviewed in Naish & Court, 2019). Firstly, the largest foreign particles are filtered by nasal hair. Secondly, the

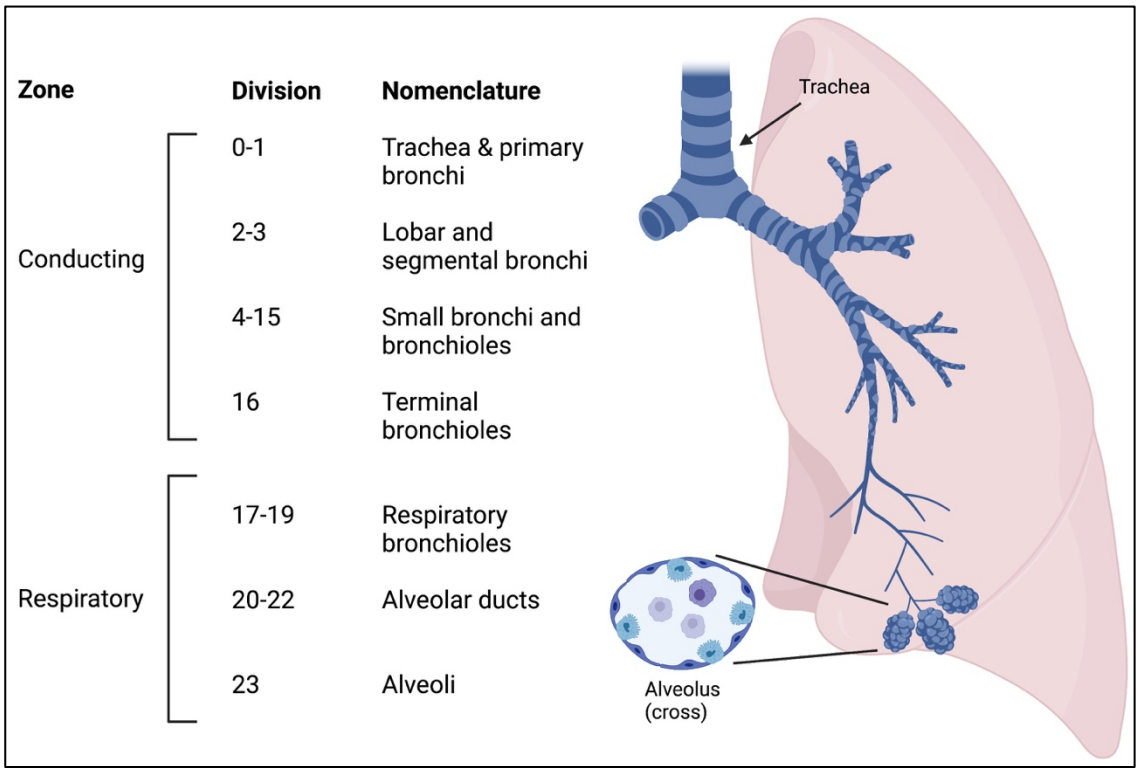


Figure 3 Divisions of the lower respiratory tract

Information sourced from a medical textbook (Naish & Court, 2019). Created in Biorender.com.

airway is lined with a layer of sticky mucous that traps small particles. Beneath the mucous is a layer of ciliated epithelium: the cilia beat upwards to produce a *mucociliary escalator* – trapped particles are expelled or swallowed. In addition to ciliated epithelium, there are a broad range of innate cells involved in airways defence (Table 2). The purpose of this complex system is to manage the host's interaction with foreign organisms and particles in an anatomically delicate site (Hartl et al., 2018), only mounting an inflammatory response when necessary. Inappropriate or excessive activation of immune responses at the lung can compromise its fundamental function of gas exchange, and this is thought to underlie pathologies such as asthma (Hammad & Lambrecht, 2021) and viral pneumonia (Lucas et al., 2020). Indeed, for some cells, such as eosinophils and mast cells, it has been difficult to parse their normal physiological function from pathological roles.

Table 2 Innate cells contributing to airways defence

Cell type	Cell subtype	Distribution	Defensive functions	Reference
Epithelial	Goblet	Conducting airways	Mucous production	(Rogers, 2003)
	Columnar epithelial	Throughout, except alveoli	- Production of complement proteins - Mucociliary escalator - Immunological sensor and regulator	(Kulkarni et al., 2018) (Hewitt & Lloyd, 2021)
	Club (Clara)	Bronchioles	Detoxification enzymes	(Blundell, 2006)
	Neuroendocrine (PNEC)	Throughout, clustered	Chemosensory/mechanosensory	(Noguchi et al., 2021)
	Type II pneumocyte	Alveoli	Production of collectins that bind pathogens	(Kuroki et al., 2007)
Macrophage	Alveolar (AM)	Airway lumen, especially alveoli	- Phagocytosis - Organise immune response and repair	(Hussell & Bell, 2014).
	Interstitial (IM)	Lung interstitium	Immunoregulatory roles	(Liegeois et al., 2018)
Muscle	Airway smooth muscle	Throughout	Various immunomodulatory properties	(Damera et al., 2009)
Nervous	Sensory and autonomic nerve endings	Lung interstitium, close to the airway surface	- Reflex arc for coughing/sneezing - Neuroimmune crosstalk - Chemosensory and mechanosensory	(Blake et al., 2019)
Granulocyte	Neutrophil	Pooled in lung vasculature	- 'First responder' - Direct killing of microbes	(Hartl et al., 2018).
	Eosinophil	Throughout	Debated, often pathological	(Jacobsen et al., 2021)
	Basophil	Rare	Anti-inflammatory regulation of AMs	(M. Cohen et al., 2018)
	Mast cell	Throughout	- Microbial sensing and killing - Tissue repair and remodelling - Initiation of inflammation and recruitment	(Virk et al., 2016) (Erjefält, 2014)
Lymphocyte	Natural Killer (NK)	Throughout	Killing of infected host cells	(Ardain et al., 2020)
	Innate Lymphoid Cell (ILC)		Surveillance and cytokine production	
	Mucosal-associated Invariant T (MAIT)		Detect and respond to microbial metabolites	(Provine & Klenerman, 2020)
Megakaryocyte		Rare	Innate regulatory role	(Pariser et al., 2021)

An extensive network of alveolar and intraepithelial dendritic cells (DCs) link innate and adaptive respiratory immunity (Lambrecht & Hammad, 2012). The primary function of DCs is to sample the airways and present antigen along with co-stimulation or co-inhibition, depending on integrated input signals. The lungs have extensive lymphatic drainage to a network of lymph nodes (Drake et al., 2018) and tertiary bronchus-associated lymphoid tissue (BALT) (Marin et al., 2019). Together, these lymphatic tissues facilitate priming of the adaptive T and B cell responses against aero-antigens.

The importance of B cell responses at the lung is indicated by the susceptibility to respiratory infection suffered by patients with primary antibody deficiencies (Cinetto et al., 2018). Mucosal IgA and IgG are particularly important for prevention and clearance of viral infection (Chiu & Openshaw, 2015). Likewise, the importance of functional T cell responses is shown by the frequent and diverse respiratory infections that can present with human immunodeficiency virus (HIV) induced acquired immunodeficiency syndrome (AIDS) (Benito et al., 2012). In addition to lymphoid-derived adaptive cells, permanently tissue-resident memory T (Trm) (Snyder & Farber, 2019) and tissue-resident memory B (Brm) (Allie et al., 2019) cells have been identified and their functional contribution to immunity has been implied in animal models.

Recent advances in single-cell transcriptomics have significantly extended our understanding of respiratory immunology. There are now estimated to be 58 transcriptomically distinct cell populations across the airways, far more than I have discussed here (Travaglini et al., 2020), including many subtypes of stromal and endothelial cells. All of these are likely to directly or indirectly be involved in the defence of the lung, and many are partially defined by immune response genes. Study of the airway microbiome has also benefitted greatly from high throughput sequencing, and there is increasing focus

on the role of commensal microbes in the development and maintenance of an immunologically healthy respiratory system (Man et al., 2017).

1.2.2 The airway type 2 immune response

Type 2 immunity describes a coordinated arm of the immune system classically defined by the action of effector type 2 cytokines IL-4, IL-5, and IL-13 (reviewed in Gause et al., 2020). Known physiological functions of type 2 immunity are defence against helminth infection, tissue repair and wound healing, and general tissue homeostasis. However, type 2 immunity is perhaps better known for its pathological role in allergic inflammation and fibrosis. Although there are general principles of the type 2 response, the process varies between tissues and individuals, with specific consequences for the lungs (Akdis et al., 2020).

The innate immune system detects pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) through pattern recognition receptors (PRRs), and these signals are integrated to decide on an appropriate immune response (K. Murphy & Weaver, 2017). Specific combinations of PAMPs and DAMPs can induce a type 2 immune response through a wide range of poorly understood mechanisms (Gause et al., 2020). Type 2-inducing PAMPs include omega-1 secreted by the eggs of *Schistosoma masoni* (Everts et al., 2009), and type 2-inducing DAMPs include adenosine triphosphate (ATP), chitinase-like-proteins, and nucleic acids. Sources of tissue damage that might induce a type 2 response include helminths and other infections, allergens, pollutants and toxic or irritating chemicals (Figure 4). Furthermore, physical structures such as alum, silica, and Charcot-Leyden crystals also induce type 2 immunity through multiple signalling pathways. The lung is at particularly high risk of damage by environmental particles given its constant exposure to large volumes of air.

Sentinel cells (e.g., epithelium, neurons) that have been stimulated by type 2-inducing triggers respond by releasing specific alarm signals. The most well established example of these is the release of alarmin cytokines (IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)) by the respiratory epithelium, although these proteins can be produced and sensed by many other cell types (Gause et al., 2020). Alarmins together exert two key functions. First, they directly stimulate a range of innate cells involved in type 2 inflammation at the local tissue site: type 2 innate lymphoid cells (ILC2s), basophils, eosinophils, mast cells, and alveolar macrophages (AMs) (Hammad & Lambrecht, 2021), as well as local type 2 memory Th2 cells (Endo et al., 2015). Secondly, alarmins license DCs to migrate to the lymph nodes and prime the adaptive type 2 response (Rank et al., 2009; Soumelis et al., 2002). Alarmins also polarise airway macrophages towards the alternatively-activated (M2) state (Kurowska-Stolarska et al., 2009). Beyond alarmins, other mechanisms of type 2 induction are being uncovered: for example, the release of substance P by sensory neurons in response to allergen (Perner et al., 2020), and the release of neuromedin U (NMU) in response to helminths (Cardoso et al., 2017), or the release of calcitonin gene-related peptide (CGRP) by pulmonary neuroendocrine cells (PNECs) (Sui et al., 2018).

Once induced, type 2 inflammation is propagated by a suite of effector cells and molecules. Activated ILC2 produce type 2 cytokines IL-4, IL-5, IL-9, amphiregulin, and IL-13 (Vivier et al., 2018), with a range of downstream effects (Gause et al., 2020; Wynn, 2015). IL-4 promotes polarisation of T cells towards a type 2 phenotype: in the event of an adaptive response, Th2 and type 2 cytotoxic T (Tc2) cells join ILC2 at the tissue site and exhibit a similar cytokine profile. IL-5 stimulates eosinophil release from the bone marrow, and also activates mature eosinophils and prolongs their survival through the IL-5 receptor, and

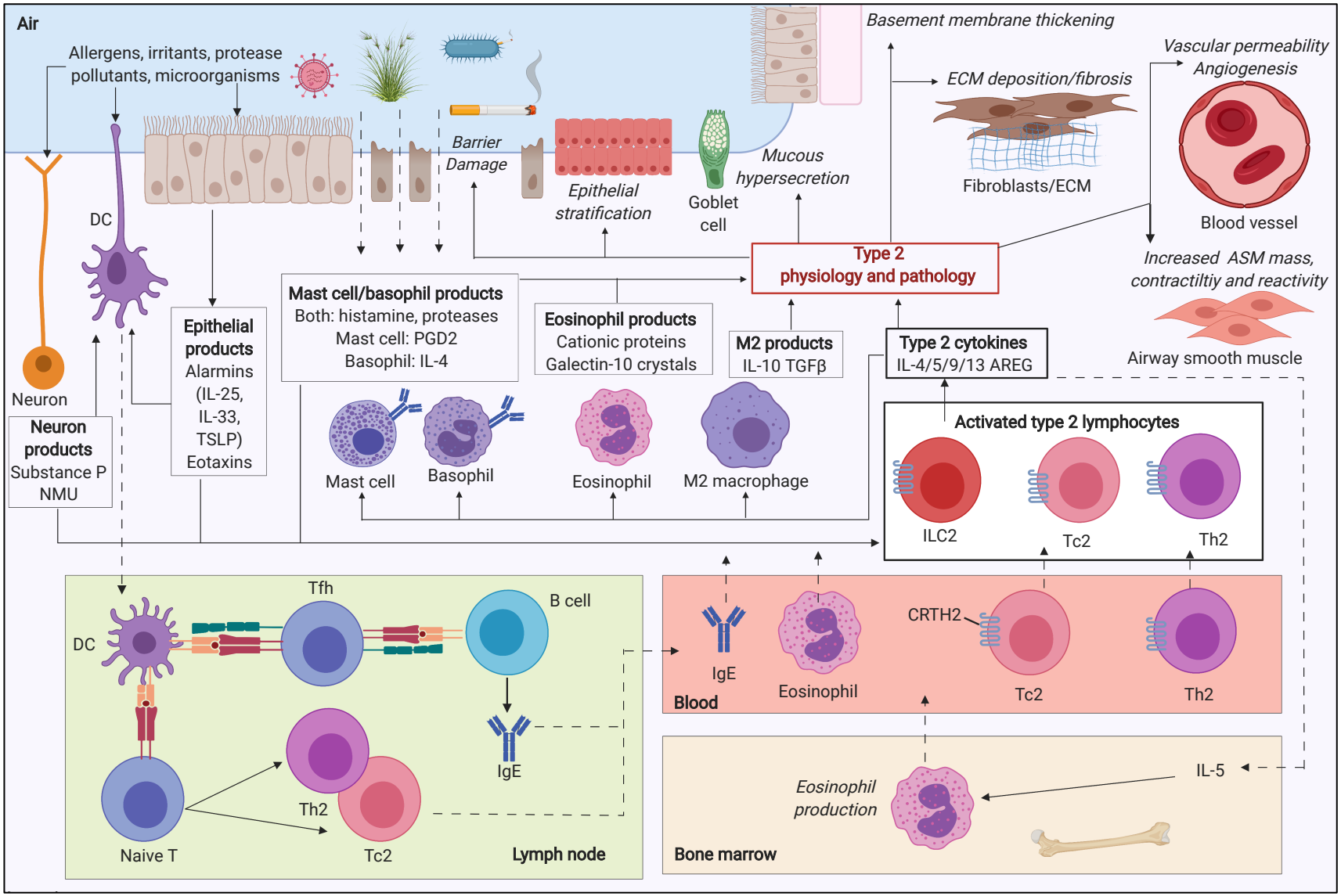

Air
Allergens, irritants, protease
pollutants, microorganisms
Barrier
Damage
Epithelial
stratification
Goblet
cell
Mucous
hypersecretion
Basement membrane thickening
ECM deposition/fibrosis
Fibroblasts/ECM
Vascular permeability
Angiogenesis
Blood vessel
Increased ASM mass,
contractiltiy and reactivity
Airway smooth muscle
DC
Neuron
Neuron
products
Substance P
NMU
Epithelial
products
Alarmins
(IL-25,
IL-33,
TSLP)
Eotaxins
Mast cell/basophil products
Both: histamine, proteases
Mast cell: PGD2
Basophil: IL-4
Eosinophil products
Cationic proteins
Galectin-10 crystals
M2 products
IL-10 TGFβ
Type 2
physiology and pathology
Type 2 cytokines
IL-4/5/9/13 AREG
Mast cell
Basophil
Eosinophil
M2 macrophage
Activated type 2 lymphocytes
ILC2
Tc2
Th2
Tfh
B cell
DC
IgE
Th2
Naive T
Tc2
Lymph node
IgE
Eosinophil
CRTH2
Tc2
Th2
Blood
Eosinophil
production
IL-5
Bone marrow

Figure 4 Type 2 immune responses in the airway

Information synthesised from recent reviews of type 2 immunity at the lung (Akdis et al., 2020; Chupp et al., 2020; M. C. Peters & Wenzel, 2020). Potential triggers of type 2 immunity include a range of airborne chemicals, biological compounds, pathogens and other particles. These are detected by sentinel cells such as airway epithelial cells and sensory neurons. Initial 'alarm signals' are released such as epithelial alarmin cytokines or neurotransmitters. These activate innate cells including ILC2 and local granulocyte populations. Dendritic cells sample the airway for antigen and after activation by alarm signals move to the lymph nodes where they polarise and activate type 2 T cells and T follicular helper (Tfh) cells. Tfh in turn induce class switching of B cells to produce IgE, which moves to the lung and binds to surface receptors on local mast cells and basophils. Type 2 T cells migrate to the circulation and home to the lung tissue where they release type 2 cytokines such as IL-4, IL-5, and IL-13. IL-5 plays a particularly important role in driving eosinophil production in the bone marrow and migration to the lung. The downstream physiological effects of type 2 cytokines include mucous hypersecretion and airway remodelling. Clinical sequelae of such changes are believed to underpin asthma. Arrows indicate positive regulation. Dotted arrows indicate movement or migration. Made in Biorender.com. Abbreviations: ASM, airway smooth muscle; AREG, amphiregulin; DC, dendritic cell; ECM, extracellular matrix; ILC2, type 2 innate lymphoid cell; NMU, neuromedin U; PGD_2, prostaglandin D_2; Tc2, type 2 cytotoxic T; Tfh, T follicular helper; TGF-β, transforming growth factor beta; Th2, type 2 T helper; TSLP, thymic stromal lymphopoietin.

collaborates with eotaxins through C-C chemokine receptor type 3 (CCR3) to recruit eosinophils to the lung. Eosinophils have a huge range of products and functions (Weller & Spencer, 2017) but contain mostly cationic proteins released on degranulation (Jacobsen et al., 2021), and Galectin-10 which forms Charcot-Leyden crystals. IL-4 and IL-13 promote class-switching of B cells towards IgE production. IgE binds to FcεR on the surface of tissue mast cells and crosslinks on encountering allergen, causing their degranulation. The main products of activated mast cells are histamine, proteases (e.g., tryptase and chymase), cytokines, and lipid mediators, especially prostaglandin D_2 (PGD_2) and leukotrienes (Komi et al., 2020). Basophils have similar effector functions to mast cells but can also produce IL-4 and can act as an antigen presenting cell (APC) (Douglas et al., 2021). IL-4 and IL-13 polarise macrophages towards an M2 phenotype (Shapouri-Moghaddam et al., 2018). M2 macrophages can form multiple sub-phenotypes and release mediators such as IL-10 and TGF-β, which contribute to type 2 inflammatory processes, and chemokines C-C chemokine ligand 17 (CCL17) and CCL18, associated with recruitment of type 2 lymphocytes.

Type 2 cytokines collaborate with the products of activated type 2 effector cells to exert physiological effects in the airways (Akdis et al., 2020). Goblet cells proliferate and increase secretion of mucous into the lumen. Expression of nitric oxide synthase enzymes is increased in respiratory epithelial and endothelial cells (Hoyte et al., 2018), and therefore fractional exhaled nitric oxide (FeNO) increases. Airway smooth muscle proliferates and hypertrophies, in addition to becoming hypersensitive and hypercontractile. There is an increase in blood vessel growth and vascular permeability, promoting infiltration of cells attracted by gradients of type 2 mediators. Fibroblasts proliferate and increase deposition of extracellular matrix (ECM), which can in turn lead to subepithelial airway fibrosis. These changes to non-haematopoietic cells are together loosely referred to as *airway remodelling* (Hirota & Martin, 2013), and are generally considered to be irreversible and damaging to lung health. These physiological changes may damage the barrier integrity, increasing access for the initial trigger. The airway epithelium can be damaged by mast cell protease or basic proteins from eosinophils, increasing its permeability to inhaled particles and therefore setting up a positive feedback loop of inflammation whereby a poorly sealed barrier allows in more triggers. Type 2 inflammation can also antagonise other inflammatory processes (e.g., type 1 and type 17 inflammation), which may be important for antiviral, antitumour, or antifungal immunity. For example, type 2 immune response in mice from helminths inhibits their antiviral response (Actor et al., 1993). However, there is likely to be positive functions of an acute type 2 response in the airway, and it is now appreciated that airway immune response is more nuanced than type-1/2 division (Harker & Lloyd, 2021). One well known example is the role of ILC2 in repairing tissue damaged by influenza infection in mice (Monticelli et al., 2011). However, the physiological effects of type 2 inflammation can manifest in a number of respiratory diseases, including allergic rhinitis, chronic

rhinosinusitis with nasal polyps (CRSwNP), and some presentations of asthma or COPD (Akdis et al., 2020).

1.3 Type 2 lymphocytes and the CRTH2 receptor

1.3.1 Type 2 lymphocyte subsets

Lymphocytes are a class of white blood cells, generally referring to antigen-specific T and B cells of the adaptive immune system, but more recently including innate counterparts called innate lymphoid cells (ILCs) (Kenneth Murphy & Weaver, 2017). T cells are defined by the presence of a T-cell receptor (TCR), of which there are billions of variations within an individual, and can be broadly classified as T helper cells (Th) or cytotoxic T cells (Tc). Th cells recognise peptides presented on class II major histocompatibility complex (MHC) proteins at the surface of professional antigen presenting cells such as DCs, and this interaction is stabilised by the cluster of differentiation 4 (CD4) coreceptor. Their main roles include activation of partner cells (e.g., B cells and Tc cells) and direction of the immune response via cytokine production. Tc cells recognise class I MHC on the surface of any nucleated cell, express the CD8 coreceptor, and have the capacity to directly kill a target cell if necessary. T cells that leave the thymus begin life in a naïve state and acquire effector and/or memory properties with antigen experience, resulting in memory subsets (Mahnke et al., 2013). Naïve T cells also have the potential to differentiate into effector subsets with different cytokine profiles. Classification by cytokine production is best established in the context of Th cells, which have been subdivided into Th1, Th2, type 9 T helper (Th9), type 17 T helper (Th17), regulatory T (Treg) and T follicular helper (Tfh) lineages (J. Zhu et al., 2010).

The subdivision of Th cells by cytokine production into Th1 and Th2 was made in the 1980s (Mosmann & Coffman, 1989) and Th2 cells have been studied intensely since. Th2 cells are associated with production of type 2 cytokines (IL-4, IL-5, IL-9, and IL-13) and direction of the type 2 immune response, including: physiological functions of antihelminth immunity and tissue repair; and pathological roles in allergic and eosinophilic inflammation (Walker & McKenzie, 2018) (Table 3). Differentiation of Th2 cells from naïve T cells is influenced by factors including: strength and duration of the peptide-MHCII-TCR signal; the cytokine milieu, especially IL-4; location; and factors relating to the presenting APC (e.g. DC, macrophage, or basophil), such as alarmin-activation of DCs (Ruterbusch et al., 2020; J. A. Walker & McKenzie, 2018). Compared to other Th subsets, Th2 cells express at their surface the IL-25 receptor subunit IL17RB (Y. H. Wang et al., 2007) and PGD_2 receptor *chemoattractant receptor-homologous molecule expressed on T-Helper type 2 cells* (CRTH2, encoded by the gene *PTGDR2*), also known as PGD_2 receptor 2 (DP2) (Nagata, Tanaka, et al., 1999). Most Th2 cells express the memory marker CD45RO and the IL-7 receptor subunit α (IL7R-α or CD127) (de Ruiter et al., 2020). Their identity is maintained by the master transcription factor GATA3 (W. Zheng & Flavell, 1997), supported by other transcription factors such as IRF4 and STAT6 (Walker & McKenzie, 2018). Two recent concepts in Th2 biology are heterogeneity and plasticity (Walker & McKenzie, 2018). Examples of reported Th2 subsets are *proallergic Th2* (Th2A) cells present in allergic individuals (Wambre et al., 2017), or the *pathogenic Th2* (Tpath2) subset that produces IL-5 and is associated with eosinophilic asthma (reviewed in Nakayama et al., 2017).

Relative to Th cells, there has been less focus on effector subsets of Tc cells. Still, they appear to be capable of differentiation into analogous subsets of Tc1, Tc2, Tc9, Tc17, $CD8^+$ Treg, and $CD8^+$ Tfc cells (M. St. Paul & Ohashi, 2020). The existence of

Table 3 Overview of type 2 lymphocyte subsets

Information derived from recent reviews of Th2 (T. Nakayama et al., 2017; Ruterbusch et al., 2020; J. A. Walker & McKenzie, 2018), Tc2(Hinks et al., 2019; M. St. Paul & Ohashi, 2020), and ILC2 cells (Bal et al., 2020; Rodriguez-Rodriguez et al., 2021; Vivier et al., 2018). ILC2 frequencies were derived from the healthy donor group ($n = 51$) of a recent study (Loyon et al., 2019). Th2 and Tc2 frequencies were derived from the control group ($n = 25$) of a previous study (Tsuda et al., 2001). Abbreviations: APC, antigen presenting cell; CRSwNP, chronic rhinosinusitis with nasal polyps; EoE, eosinophilic esophagitis; ILCP, innate lymphoid cell progenitor; LTE_4, leukotriene E_4 NMU, neuromedin U; PGD_2, prostaglandin D_2; TCR, T-cell receptor; TSLP, thymic stromal lymphopoietin.

	Tc2	**Th2**	**ILC2**
Progenitor	Naïve $CD8^+$ T cell	Naïve CD4+ T cell	ILCP
Induction of development	IL-4		
	TCR triggering factors (e.g., duration, affinity) APC factors (polarisation and co-stimulation)		
		Epithelial alarmin cytokines (IL-25, IL-33, TSLP)	
% of blood lymphocytes	~ 0.6 %	~ 1.0 %	~ 0.05 %
Upstream activators	PGD_2, LTE4, common gamma chain cytokines (e.g., IL-2)		
	p-MHC-I binding + co-stimulation	p-MHC-II binding + co-stimulation	Alarmin cytokines (TSLP, IL-33, IL-25) Neuropeptides (NMU)
Common surface markers	CRTH2 (PGD_2 receptor), IL17RB (IL-25 receptor)		
	CD3, CD45RO, CD127 (IL7R-α)		Lineage-negative $CD161^+$
	CD8	CD4	
Transcription factors	GATA3, STAT6		
		IRF4	IRF4, RORα
Activation products	Type 2 cytokines (IL-4, IL-5, IL-13, IL-9), Amphiregulin		
Physiological function	Type 2 immunity (e.g., anti-helminth response, tissue repair)		
	Adaptive response		Innate response
	Cytotoxicity		
Tissue distribution	Circulatory system, peripheral tissues (e.g., lung, gut, and skin)		
	Lymphatic organs		
Disease associations	Allergic diseases (e.g., asthma, allergic rhinitis, atopic dermatitis) Eosinophilic diseases (e.g., eosinophilic asthma, EoE, CRSwNP)		
Heterogeneity and plasticity	Unknown	Yes	

Tc2 cells was reported soon after the emergence of the Th1/Th2 paradigm, utilising similar techniques like *in vitro* differentiation with IL-4 (M Croft et al., 1994). Many properties of Tc2 are similar to Th2: production of type 2 cytokines after stimulation (Seder & Le Gros, 1995); surface markers of CRTH2 (Nagata, Hirai, et al., 1999), IL17RB (Hilvering et al., 2018), CD45RO and IL7R-α (CD127) (de Ruiter et al., 2020); and the transcription factor GATA3 (Pai et al., 2004). Furthermore, Tc2 cells can functionally contribute to type 2 inflammation in mice (R A Seder & Le Gros, 1995) and humans (de Ruiter et al., 2020), and have been linked to type 2 disease such as asthma (Hinks et al., 2019) and atopic dermatitis (Bangert et al., 2021). There is some evidence of interconversion of Tc subsets (Mittrücker et al., 2014; M. St. Paul & Ohashi, 2020) but little involving Tc2 cells. Likewise, there has been little study of Tc2 cell heterogeneity.

Unlike Th2 cells, Tc2 have cytotoxic capabilities (Cerwenka et al., 1999; Dobrzanski et al., 2004), but this may be reduced vs Tc1 (B. A. Cho et al., 2012; Do et al., 2004). $CD8^+$ T cells are critical effectors of the anti-tumour response, but it is unclear whether Tc2 cells are pro or anti-cancer (M. St. Paul & Ohashi, 2020), despite being the dominant $CD8^+$ T cell population in some tumours (Sheu et al., 2001). Regarding antiviral immunity, it is assumed that a type 2 response is undesirable (Chiu & Openshaw, 2015), and there is some evidence from mouse models that Tc2 cells are less effective than Tc1 at clearing influenza (Cerwenka et al., 1999) and vaccinia (Actor et al., 1993) viruses. Conversely, in the context of autoimmunity Tc2 cells may play a protective role (Gravano & Hoyer, 2013), or at least a less pathogenic one vs Tc1 and Tc17 (Vizler et al., 2000). However, Tc2 cell presence in synovial fluid is associated with progression of rheumatoid arthritis (B. A. Cho et al., 2012). It seems therefore that the role of the Tc2 cell is likely to be nuanced.

The group 2 innate lymphoid cell (ILC2) is the most recently discovered type 2 lymphocyte (Neill et al., 2010) but has rapidly developed a substantial literature (Rodriguez-Rodriguez et al., 2021). ILC2 cells are a subset of the family of ILCs, innate counterparts of T cells that reside in the blood and mucosal tissues where they can respond to environmental cues and prime the adaptive response (reviewed in Vivier et al., 2018). The ILC2 subset is distinguishable by a lack of lineage markers, and presence of classical Th2 markers including: surface CRTH2, the GATA3 transcription factor, and type 2 cytokine production. Though lacking antigen specificity, ILC2 cells are sensitive to epithelial alarmin cytokines (Neill et al., 2010), and the neuropeptide NMU (Cardoso et al., 2017). ILC2 cells exhibit functional plasticity (Bal et al., 2020) and heterogeneity, both between tissue sites (Rodriguez-Rodriguez et al., 2021) but also within them (Bielecki et al., 2021). In mice, ILC2 cells contribute to physiological and pathological type 2 immunity (Ebbo et al., 2017). In humans, the functional role of ILCs in general remains contested (Vély et al., 2016). However, there is evidence that ILC2 numbers and activation status are elevated in the blood and airways of patients with asthma, correlating with allergy and eosinophilia (Rodriguez-Rodriguez et al., 2021, p. 170). In COPD, circulating ILC2 numbers appear to fall with increasing severity of disease (Silver et al., 2016), but it is unclear if that is a consequence of lung migration.

1.3.2 PGD_2 and its receptors DP_1 and DP_2 (CRTH2)

Prostaglandins are lipid mediators derived from the metabolism of arachidonic acid (Ricciotti & Fitzgerald, 2011). Prostaglandin D_2 (PGD_2) is one of five major prostaglandins which have extensive biological functions (Woodward et al., 2011). The clinical relevance of PGD_2 has been suspected for several decades. In the 1970s PGD_2 was identified as a bronchoconstrictor in dogs (Wasserman et al., 1977) and later humans (C. C. Hardy et al.,

1984). PGD_2 was reported to be elevated in the airways of patients with allergic asthma (M. C. Liu et al., 1990), where large quantities are released on exposure to allergen (Murray et al., 1986). However, in a more recent and larger study, PGD_2 in the sputum was not higher in severe asthma compared to healthy controls, although it was higher in severe eosinophilic asthma vs non-eosinophilic asthma (Fricker et al., 2021).

Synthesis of PGD_2 requires PGH_2 input and either the lipocalin-like (encoded by *PTGDS*) or haematopoietic (H-PGDS, encoded by *HPGDS*) synthase enzymes (Yoshihiro Urade & Eguchi, 2002), after which it can be metabolised to other prostanoids such as PGJ_2 (Figure 5). Activated mast cells are the major source of PGD_2 in the lung (S. T. Holgate et al., 1984; Lewis et al., 1982), and levels of PGD_2 synthesising enzyme H-PGDS correlate with mast cell tryptase and severity of asthma (Fajt et al., 2013). However, PGD_2 can also be made by eosinophils (Luna-Gomes et al., 2011), professional APCs (Y Urade et al., 1989), airway epithelial cells (Werder et al., 2018), and any of the type 2 lymphocyte subsets (W. Chen et al., 2021; Maric et al., 2019; K. Tanaka et al., 2000).

Signalling of prostaglandins occurs through seven-transmembrane G protein-coupled receptors (GPCRs) (Woodward et al., 2011). Three of these are known to bind PGD_2: prostaglandin D_2 receptor 1 (DP_1), CRTH2, and thromboxane receptor (TP), which signal through Gαs, Gαi, and Gαq respectively, meaning that DP_1 and CRTH2 have opposing effects on cyclic adenosine monophosphate (cAMP) levels (Pettipher et al., 2007; Woodward et al., 2011). Selective agonists and antagonists (Sandham et al., 2017; Woodward et al., 2011) have facilitated resolution of PGD_2 functions through the different receptors. PGD_2 interaction with TP is least studied due to lower affinity compared with DP_1 and CRTH2, but partially mediates smooth muscle contraction (S. L. Johnston et al., 1995), though TP is expressed

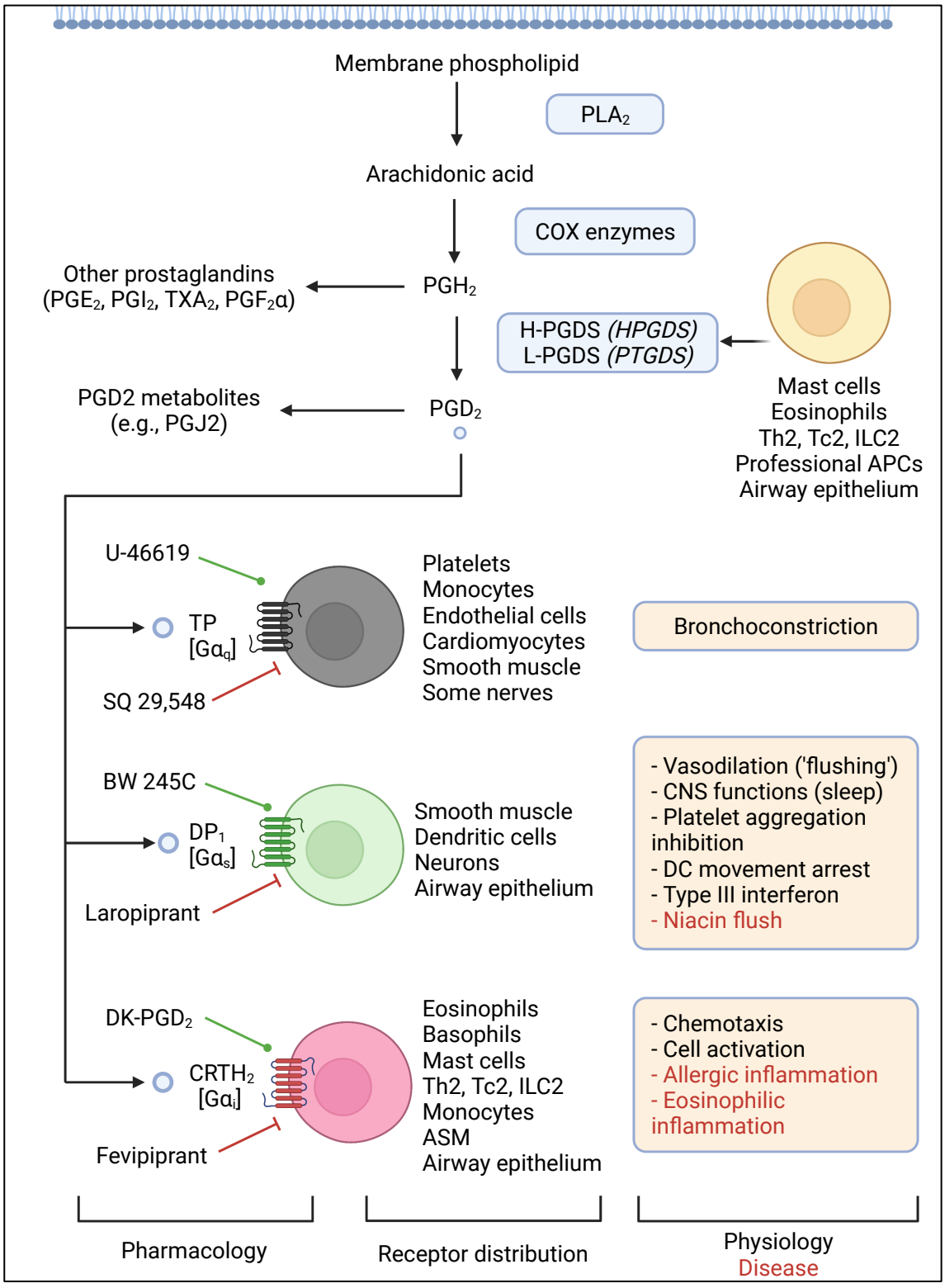


Figure 5 Overview of PGD_2 biology

This diagram is based on similar figures elsewhere (C. Brightling et al., 2021; Domingo et al., 2018; Pettipher et al., 2007). Abbreviations: APC, antigen presenting cell; ASM, airway smooth muscle; COX, cyclooxygenase; DC, dendritic cell; DP_1, prostaglandin D_2 receptor 1; PG, prostaglandin; PGDS; prostaglandin D synthase; PLA2, phospholipase A2; TP, thromboxane receptor; TXA_2, thromboxane A2. Created in Biorender.com.

on a broad range of cell types (Woodward et al., 2011). DP_1 is expressed by: platelets, smooth muscle, dendritic cells, and in the central nervous system (CNS) (Pettipher et al., 2007); and by airway epithelial cells (Werder et al., 2018). The physiological effects of DP_1 signalling include peripheral vasodilation, CNS functions such as sleep, inhibition of platelet aggregation, inhibition of DC migration (Woodward et al., 2011), and type III interferon response (Werder et al., 2018). Clinically, the PGD_2-DP_1 axis is responsible for niacin flush, a side effect of nicotinic acid therapy for dyslipidemia (Cheng et al., 2006).

CRTH2 was first identified in cultured human Th2 cells after comparing their gene expression patterns with Th1 cells (Nagata, Tanaka, et al., 1999). Its expression was later detected on: eosinophils, basophils, and Tc2 cells (Nagata, Hirai, et al., 1999; Nagata, Tanaka, et al., 1999); and later ILC2 cells (Mjösberg et al., 2011), nonclassical monocytes (Wong et al., 2011), airway epithelial cells (Werder et al., 2018) and airway smooth muscle (Saunders et al., 2019). An endogenous CRTH2 agonist was found in the supernatant of activated mast cells (Nagata, Hirai, et al., 1999) and then identified as PGD_2 (Hirai et al., 2001). PGD_2 action through CRTH2 induces chemotaxis (Hirai et al., 2001; Saunders et al., 2019), and activation of eosinophils (Gervais et al., 2001) and type 2 lymphocytes (Hilvering et al., 2018; K. Tanaka et al., 2004; Xue et al., 2005, 2014).

The PGD_2-CRTH2 axis connects elements of the allergic (mast cell), eosinophilic, and type 2 (Th2, Tc2, and ILC2) inflammatory networks. The axis has been implicated in a number of diseases, most notably asthma (C. E. Brightling et al., 2020), but also atopic dermatitis (Yanes & Mosser-Goldfarb, 2018), various allergies (Wambre et al., 2017), aspirin-exacerbated respiratory disease (Cahill et al., 2015), and eosinophilias such as eosinophilic oesophagitis (Straumann et al., 2013). Consequently, there has been intensive exploration of CRTH2 antagonists as therapy (Marone et al., 2019). The most advanced CRTH2

antagonist is fevipiprant, which has superior binding properties to previous compounds (C. Brightling et al., 2021). Preclinical studies showed that fevipiprant inhibits type 2 lymphocyte, eosinophil, and basophil activation, and eosinophil recruitment to the lung (C. Brightling et al., 2021). Early trial results were promising, with reduced airway eosinophilia and improvements in lung function and symptoms (N. Barnes et al., 2012; Gonem et al., 2016). However, the results of two recent phase III trials (Christopher E. Brightling et al., 2021; Castro et al., 2021) showed no significant reduction in asthma exacerbations.

1.4 The role of Tc2 cells in T2-high airways disease

1.4.1 Overview of T2-high asthma and COPD

Evidence for the involvement of airway type 2 inflammation in asthma can be found at every level of study: gross pathology such as airway remodelling (Dunnill, 1960); changes to cellular composition such as infiltration of eosinophils (Herrick, 1911) and Th2 cells (Douglas S. Robinson et al., 1992); and molecular biomarkers such as elevated FeNO (Kharitonov et al., 1994), serum IgE (Burrows et al., 1989), and airway type 2 cytokines (Saha et al., 2008; Christoph Walker et al., 1994). More recent evidence has come from GWAS studies identifying, for example, involvement of the IL-33 axis (Moffatt et al., 2010). However, only around half of patients with asthma exhibit signs of elevated type 2 inflammation (Fahy, 2015), and these patients are distinct from T2-low endotypes in terms of airway gene expression patterns (Woodruff et al., 2009). Thousands of studies have looked at type 2 inflammation in asthma and the emergent knowledge is summarised in a number of recent reviews (Hammad & Lambrecht, 2021; Pavord et al., 2018). Several type 2-targeting therapies for asthma are approved or in trials (M. C. Peters & Wenzel, 2020). Clustering studies identified at least two subsets of type 2 inflammatory profiles in asthma, differing in body mass index (BMI) and submucosal eosinophils (Kuo et al., 2017).

A subset of COPD patients exhibit eosinophilic airway inflammation which cannot be accounted for by comorbidity of asthma (Singh et al., 2014). Approximately one third of COPD patients exhibit persistent blood eosinophilia (Singh et al., 2014), and a smaller fraction have airway eosinophilia at stable state. Roughly one third of COPD exacerbations are associated with airway eosinophilia and these form a mathematically distinct cluster compared to bacterial or virally induced exacerbations (Bafadhel et al., 2011). Indeed, airway eosinophilia is particularly associated with exacerbations of both asthma and COPD (George & Brightling, 2016). The benefits of systemic (Christopher E. Brightling et al., 2000) and inhaled (C. E. Brightling et al., 2005) corticosteroids correlate with airway eosinophilia in COPD. Fewer trials of type 2 biologics have been conducted in COPD (Yousuf et al., 2019), but a large trial of mepolizumab showed it reduces exacerbations in patients with blood eosinophilia (Pavord et al., 2017). However, the mechanisms for airway eosinophilia in COPD are less well understood than for asthma. A large recent study found that airway type 2 cytokines and PGD_2 were elevated in eosinophilic asthma but not eosinophilic COPD (Fricker et al., 2021). It is therefore possible that eosinophilic COPD is driven by nonclassical mechanisms.

1.4.2 The ambiguous role of $CD8^+$ T cells in airways disease

The role of $CD8^+$ T cells in asthma is unclear, with evidence of both protective and pathological roles (Betts & Kemeny, 2009). In animal studies, experiments in which $CD8^+$ T cells were depleted before sensitisation and challenge suggested a protective role for $CD8^+$ T cells (Lloyd & Hessel, 2010). Conversely, adoptive cell transfer studies showed that $CD8^+$ T cells have the potential to contribute to the inflammation seen in rodent models of eosinophilic airway inflammation. This could be due to $CD8^+$ T cells being mostly type 1 at resting state, but having the capacity to convert to type 2 cytokine profile

in the context of allergic inflammation. Generally, phenotyping of the Tc cells in these experiments has been limited (Lourenço et al., 2016).

In patients with asthma, $CD8^+$ T cell counts in bronchoalveolar lavage (BAL) are often elevated in adults (Kelly et al., 1989; Christoph Walker et al., 1991, 1994) or wheezing infants (Arnoux et al., 2001). Evidence of enrichment in biopsies has been less consistent (Bradley et al., 1991; Hirosako et al., 2010). $CD8^+$ T cell count was linked to airway hyperresponsiveness in one study (Sont et al., 1996) but a more recent allergen challenge study found no differences (Ali et al., 2007). Activation of $CD8^+$ T cells has been reported in nonatopic (C. Walker et al., 1992) and severe (Tsitsiou et al., 2012) asthma, but in asthma overall the evidence is mixed (D. S. Robinson et al., 1993; Christoph Walker et al., 1991). A longitudinal study at 7.5 (Van Rensen et al., 2005) and 14-year (Den Otter et al., 2016) time points found $CD8^+$ T cell counts in biopsies were the best predictor of lung function decline but the nature of these Tc cells is an open question (Baraldo et al., 2016).

There is a consensus amongst recent studies that $CD8^+$ T cells are enriched and activated in the airways of patients with COPD (Williams et al., 2021). Smoking, the major risk factor for COPD, is associated with $CD8^+$ T cell infiltration of the airways (Ravensberg et al., 2013). But the reason for this infiltration, and whether $CD8^+$ T cells play a protective or pathogenic role, remains unclear. Expression of residency markers (CD103) is lower, and activation markers (CD69 and CD25) higher (Leckie et al., 2003). Attempts to phenotype $CD8^+$ T cells in COPD have shown mixed results for Tc1 enrichment, but Tc2 were generally enriched (Williams et al., 2021).

Respiratory viruses are a major precipitator of exacerbation of both asthma and COPD (Kurai et al., 2013), which in turn can lead to death. There is also a body of evidence (Mikhail & Grayson, 2019) that specific viruses, especially respiratory syncytial virus (RSV)

and rhinoviruses, are linked to the development of asthma in children. $CD8^+$ T cell infiltration of the lung has been detected in fatal asthma (Faul et al., 1997) but the finding was not reproduced in a later study (De Magalhães Simões et al., 2005). There is evidence of $CD8^+$ T cell activation in fatal asthma, in terms of surface CD25 (O'Sullivan et al., 2001) and granzyme A/B expression (Annoni et al., 2015). The conversion of Tc1 cells to a Tc2 phenotype remains a theory to explain the vulnerability of patients with asthma to viral infections (Lloyd & Hessel, 2010).

In summary, a broad role for $CD8^+$ T cells in asthma or COPD has been elusive and the literature contains examples of irreproducible and inconsistent findings. More detailed phenotyping of both patients and the $CD8^+$ T cells being studied may clarify their role.

1.4.3 Observations of Tc2 cells in airways disease

The role of Th2 (Hammad & Lambrecht, 2021) and ILC2 (Rodriguez-Rodriguez et al., 2021) cells in the pathogenesis of T2-high asthma has been studied extensively. However, the role of Tc2 cells has been the subject of much less study and debate (Hinks et al., 2019). This is despite a growing appreciation that the type 2 immune response is heterogenous (Gause et al., 2020). The source of type 2 cytokines in the airways has not always been shown despite the dominant focus on Th2 cells (Hinks et al., 2019).

In ovalbumin sensitisation-challenge models of asthma, native $CD8^+$ T cells produce type 2 cytokines and contribute to airway eosinophilia and hyperresponsiveness (Hamelmann et al., 1996; Miyahara, Takeda, et al., 2004; Sawicka et al., 2004; Stock et al., 2004). These are effector $CD8^+$ T cells (Miyahara, Swanson, et al., 2004) that require help from antigen specific Th2 cells (Koya et al., 2007) and the action of leukotriene B_4 (Miyahara et al., 2005; Taube et al., 2006). The contributing Tc2 cells are not necessarily antigen specific (Coyle et

al., 1995). Adoptive transfer of *in vitro*-differentiated Tc2 cells into CD8 depleted mice has similar effects (Jia et al., 2013) which can be enhanced by treating the Tc2 cells with steroids before transfer (Ohnishi et al., 2008). Conversely, using adoptive transfer or vaccination (with T1-skewing adjuvant) to induce interferon gamma (IFNγ) producing $CD8^+$ T cells partially abrogates airway eosinophilia (Hansen et al., 2000; Takeda et al., 2009; Tang et al., 2012; Wells et al., 2007). Recently, Tc2 (but not Th2) cells were identified as a major source of type 2 cytokines in a different model based on sensitisation to house dust mite (L. Wang et al., 2021), and Tc2 also have a role in the cockroach antigen (Schaller et al., 2005) and RSV infection (Azzeddine Dakhama et al., 2005) models. Overall, the animal data suggests that Tc2 promote T2 inflammation in multiple models, whilst Tc1 can play a balancing role by counteracting this (Betts & Kemeny, 2009).

Studies of human asthma support a role for Tc2 cells but there is not yet conclusive evidence of what that role is. Tc2 ($IL\text{-}4^+$ or $IL\text{-}5^+$) have been detected alongside Th2 in bronchial biopsies in nonatopic and atopic asthma (Ying et al., 1997), and were enriched and activated ($CD25^+$) in fatal asthma (O'Sullivan et al., 2001). Further indirect biopsy evidence includes $CD8^+$ infiltration of the submucosa being especially high in T2-high asthma, although it is not clear if those cells are Tc2 (Kuo et al., 2017). In BAL, the proportion of $CD8^+$ T cells that were Tc2 as defined by IL-13 (A. Dakhama et al., 2013) or IL-4 (Christopher E. Brightling, Symon, et al., 2002) expression was higher in asthma vs health, and correlated with disease markers such as basement membrane thickness (A. Dakhama et al., 2013). Bulk expanded BAL $CD8^+$ T cells produced more IL-5 if derived from atopic asthmatics vs atopic or nonatopic controls (Till et al., 1995). However, another BAL study looking at all three type 2 cytokines found no difference in Tc2 (Krug et al., 2001). In sputum, the proportion of Tc2 ($IL\text{-}4^+$ or $IL\text{-}5^+$) was higher in atopic asthmatics

vs controls (S. H. Cho et al., 2005), whereas Tc1 potency was reduced in severe asthma (Hamzaoui et al., 2005). Furthermore, $CD8^+$ gene signatures were downregulated in sputum from patients with T2-low asthma (M. C. Peters, Ringel, et al., 2019). In blood, bulk sorted $CD8^+$ T cells from asthmatics produced more IL-4 but not more IFNγ (Stanciu et al., 1997). A circulating $IL6R^+$ Tc2 ($IL\text{-}5^+$ or $IL\text{-}13^+$) population was enriched in asthma, but it correlated negatively with airway eosinophilia (N. Lee et al., 2014). Circulating Tc2 ($IL\text{-}5^+$) but not Th2 were associated with blood eosinophilia (Stoeckle & Simon, 2013)

There are fewer studies of Tc2 in COPD but these tend to suggest enrichment (Williams et al., 2021). A study of BAL from patients with COPD found increased proportion of Tc2 ($IL\text{-}4^+$ or $IL\text{-}13^+$), but no difference in Tc1 (Barcelo et al., 2006). Another study expanded BAL T cells and found an increased proportion of these expanded cells were Tc2 ($IL\text{-}4^+$) in COPD vs health (Barczyk et al., 2006). In blood, one study found no difference in Tc2 ($IL\text{-}4^+$) between health and COPD (Paats et al., 2012), but two larger study found elevated Tc2 ($IL\text{-}4^+$) cells (Shirai et al., 2010; X. Zhu et al., 2009).

The starting point of this DPhil project was a dataset collected by members of our group in two separate patient cohorts (Hilvering et al., 2018). In a cohort of patients seen in Oxford, blood Tc2 ($CRTH2^+$) cells (but not Th2) were higher, in both absolute and relative terms, in severe eosinophilic asthma vs healthy controls or severe non-eosinophilic asthma. In a separate cohort of patients seen in Southampton, blood Tc2 cells ($IL\text{-}13^+$) were elevated in asthma generally, and severe asthma, and associated with nasal polyps, a feature of T2-high asthma. They also were associated with smoking history, but patients with COPD were not assessed. Also, the patient groups were not matched in terms of age, sex, or BMI, and such factors remain plausible confounders. The proportion of Tc2 in $CD8^+$ T cells varied depending on the marker used, the range being up to ~1% of $CD8^+$ T cells when Tc2 were

defined by IL-13 production, but up to ~20% when defined by CRTH2 expression. This raises the question: why is there a large difference in the proportion of Tc2 using different marker-based definitions? In the airways, Tc2 (IL-13$^+$) but not Th2 were higher in eosinophilic asthma vs non-eosinophilic asthma or healthy controls, in contrast to the large body of data supporting Th2 elevation and activation in asthma (Hammad & Lambrecht, 2021). Tc2 (CRTH2$^+$) and Th2 from peripheral blood mononuclear cells (PBMCs) were studied *in vitro*, confirming their ability to migrate towards PGD_2 and to become activated to produce type 2 mediators in response to PGD_2-CRTH2 interactions and/or interactions between LTE_4 and cysteinyl leukotriene receptor 1 (CysLT1), or by TCR triggering with anti-CD3 and anti-CD28 antibodies.

1.5 Aims of this thesis

The goal of this work is to increase our understanding of CRTH2$^+$ CD8$^+$ T cells and their role in airways diseases. It involves a mixture of *in vitro* and *ex vivo* study of human T cells. Specifically, the following questions will be addressed.

1. What are the clinical and biological correlates of CRTH2$^+$ T cells in the blood and airways of patients with asthma or COPD? Are these cells responsive to therapy?

2. What is the relationship between CRTH2 expressed at the surface and the capacity of a cell to produce the type 2 cytokines IL-4, IL-5, and IL-13?

3. What is the biological significance of multiple CRTH2$^+$ CD8$^+$ T cell activation pathways? Do TCR-dependent and TCR-independent pathways have redundant or specific functions?

Chapter 2

The determinants of circulating CRTH2^{+} T cell counts

2.1 Introduction

The most widely cited discovery in the field of asthma immunology is that type 2 T cells are enriched in the airways of patients with atopic asthma (Douglas S. Robinson et al., 1992). This initial paper identified type 2 T cells by combined surface CD3 expression and internal messenger ribonucleic acid (mRNA) encoding type 2 cytokines IL-4 and IL-5.

In the succeeding decade it emerged that labelling of surface CRTH2 identified a significant proportion, if not all, of the type 2 T cell compartment (Cosmi et al., 2000). For convenience, the remainder of this chapter refers to $CRTH2^+$ T cells as T2, and subsets as Th2 ($CD4^+CRTH2^+CD3^+$), Tc2 ($CD8^+CRTH2^+CD3^+$), or DNT2 ($CD4^-CD8^-CRTH2^+CD3^+$). I will use these abbreviations to indicate the absolute cell count for these subsets. For relative CRTH2 expression (the proportion of T cells expressing CRTH2) I use the term rT2, and the subsets are similarly denoted rTh2, rTc2, and rDNT2 (e.g., rTh2 of 10% would mean that 10% of $CD4^+$ T cells expressed surface CRTH2). It is important to remember that in this chapter the '2' stands for CRTH2 expression and not type 2 cytokine production.

The two premises that: type 2 T cell frequencies are elevated in the asthmatic airway; and surface CRTH2 identifies type 2 T cells, naturally led to the study of $CRTH2^+$ T cells in asthma. The first such report found that BAL rT2 is increased in asthma, (Mutalithas et al., 2010). Subsequent papers reproduced this finding and linked rT2 to disease severity in BAL (Fajt *et al.*, 2013) and biopsy specimens (Stinson, Amrani and Brightling, 2015). These findings supported the initial Robinson *et al* study, extending its observations to include nonatopic asthma. Overall, the available evidence agrees that $CRTH2^+$ T cells are enriched in the airways of many patients with asthma.

However, there is less consensus regarding the role of CRTH2$^+$ T cells circulating in the blood. The initial 2010 study of CRTH2$^+$ T cells in asthma found no difference in blood rT2 between health and asthma (Mutalithas et al., 2010), but later studies report differences in T2 subsets in specific disease contexts. RTh2 (Campos Alberto et al., 2012) and absolute counts of Th2 and Tc2 (Boonpiyathad et al., 2020) have been linked to allergic asthma, despite previous evidence showing no relationship with atopy or serum IgE (Mutalithas et al., 2010), and a more recent paper which does not reproduce this claim (Wiest et al., 2018). In terms of severe asthma, one study reported rTh2 increased compared to health (N. S. Palikhe et al., 2016), but two other investigators did not replicate this finding (Hilvering et al., 2018; Wiest et al., 2018). Finally, a recent paper from our own group found a relative and absolute increase in Tc2 specific to severe eosinophilic asthma (Hilvering et al., 2018).

I could not find data regarding CRTH2$^+$ T cells specifically in the context of COPD. However, eosinophilic inflammation has been observed in subsets of COPD patients and is linked to worse disease and positively with steroid responsiveness (Bafadhel, 2017; Singh et al., 2014). So, it is likely that a role exists for type 2 T cells, and therefore CRTH2$^+$ T cells, in the circulation and/or the airways.

In this chapter, I present data collected from samples of healthy volunteers and patients seen at the RMU in Oxford. The chapter primarily aims to address the inconsistencies in the literature regarding enrichment of blood CRTH2$^+$ T cells in asthma. I hope to achieve this by presenting both relative and absolute data for CRTH2$^+$ T cells and their subsets, for a larger number of participants than previous studies. Secondly, I have compared the CRTH2$^+$ T cell data with a range of clinical and biological observations. Circulating type 2 T cell counts have been linked to normal ageing (Mansfield et al., 2012), cigarette smoking (Hilvering et al., 2018), and obesity (Van Der Weerd et al., 2012), but these factors are not

necessarily controlled for in studies of asthma. It is important to determine what factors beyond airways disease might influence CRTH2$^+$ T cell counts, in order to control confounding variables when studying airways disease.

In summary, the following questions will be addressed:

1. Are the previously reported findings regarding CRTH2$^+$ T cell counts in asthma reproducible in a larger cohort?

2. What are the major clinical and biological determinants of blood and airway CRTH2$^+$ T cell counts?

3. Are CRTH2$^+$ T cell counts responsive to therapy?

2.2 Methods

2.2.1 Study participants and categorisation

Participants were recruited at the Respiratory Medicine Unit (RMU) of the Nuffield Department of Medicine (NDM), University of Oxford. The data presented in this chapter derive from two study cohorts: the Brightling study (Research Ethics Committee (REC) reference: 08/H0406/189) and the Oxford Airways Study (OAS) (REC reference: 18/SC/0361). Both are observational studies including patients with asthma, a smaller number of patients with COPD, and healthy volunteers. The core aim of these studies is biomarker development, with a long-term goal of personalised therapy.

Diagnosis of asthma was made by a doctor using Global Initiative for Asthma (GINA) guidelines (Global Initiative for Asthma, 2020). Briefly, a patient can be diagnosed with asthma if they: present with symptoms of wheeze, shortness of breath, chest tightness

and/or cough; and experience variable airway obstruction measured by flow of exhaled air. Asthma exacerbation was defined in line with the GINA (Global Initiative for Asthma, 2020) and/or American Thoracic Society (ATS)/European Respiratory Society (ERS) definitions (Reddel et al., 2009). Exacerbations involve a worsening of the patient's condition beyond their normal variability, usually requiring some type of action by the patient or their clinicians. This dataset considers exacerbation events in the 12 months preceding the visit at which the sample was taken. COPD was diagnosed in accordance with Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (Global Initiative for Chronic Obstructive Lung Disease, 2021). Patients with COPD present with progressive shortness of breath and fixed reduction in lung function, which may be accompanied by cough and sputum production, and is associated with smoking.

Patients with asthma were further categorised as severe or non-severe (mild-moderate asthma or MMA) by the clinical team using the ERS/ATS guidelines (Chung et al., 2014). This classification takes into account a patient's treatment regimen, symptoms scores, frequency of asthma attacks, and spirometry. Those with severe asthma were subcategorised by endotype (eosinophilic or non-eosinophilic) using criteria established by the Dose Ranging Efficacy And safety with Mepolizumab in severe asthma (DREAM) trial for mepolizumab (Pavord et al., 2012). A patient was considered eosinophilic if at visit they had: blood eosinophil count $\geq 0.3 \times 10^9$/L; FeNO ≥ 50 ppb; or sputum eosinophil fraction ≥ 3 %. Patients currently treated with systemic steroids (e.g. oral prednisolone) or mepolizumab could not be categorised this way because these treatments eliminate eosinophils (Pavord et al., 2012; K. M. West et al., 1960), and were therefore considered in a separate subcategory of severe asthma treated with eosinophil-depleting therapy (STA) (Figure 6).

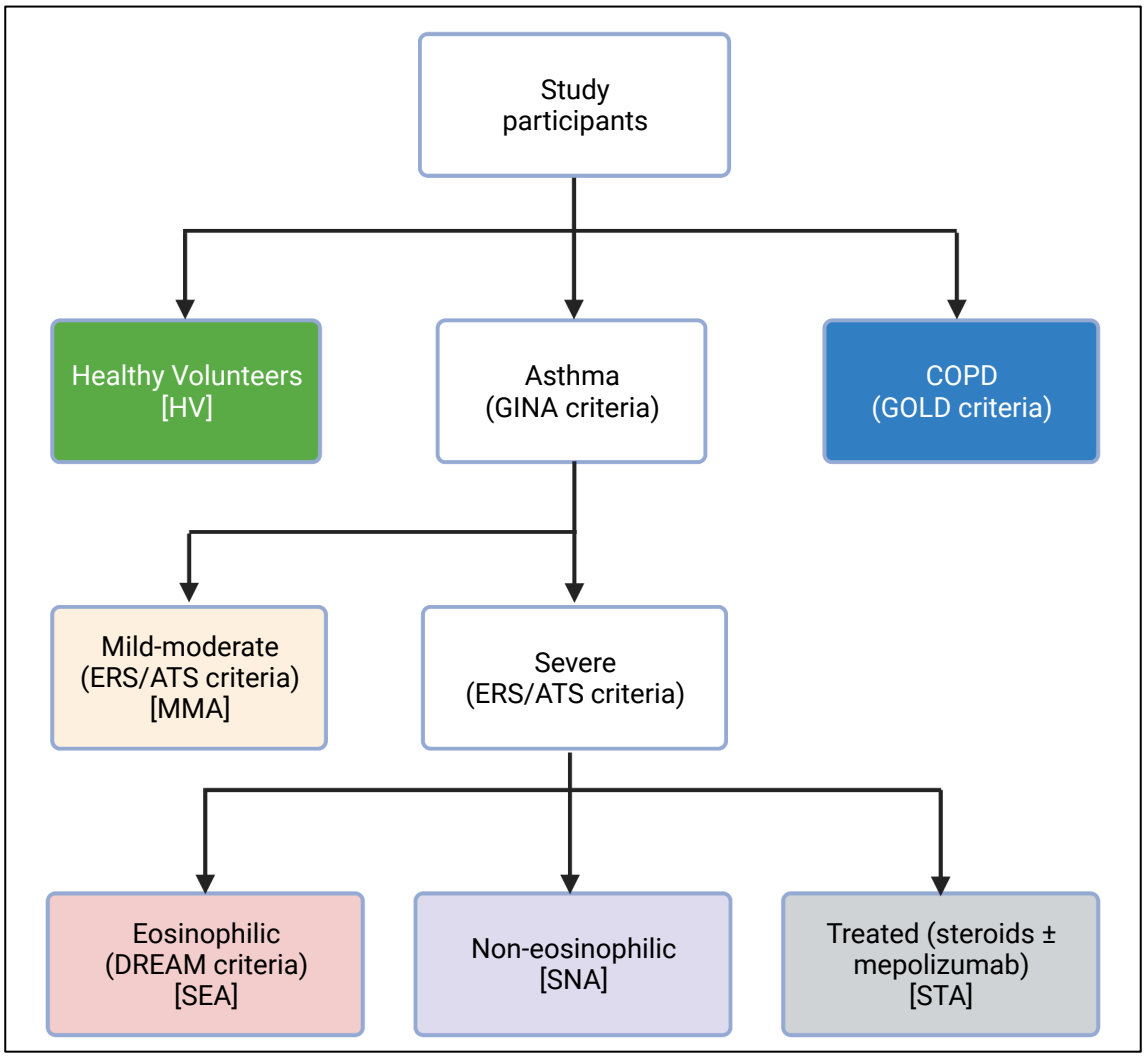


Figure 6 Overview of participant classification

Abbreviations: ATS, American Thoracic Society; COPD, chronic obstructive pulmonary disease; DREAM, Dose Ranging Efficacy And safety with Mepolizumab in severe asthma; GINA, Global Initiative for Asthma; GOLD, Global initiative for Obstructive Lung Disease; MMA, mild-moderate asthma; SEA, severe eosinophilic asthma; SNA, severe neutrophilic asthma, STA, severe asthma treated with eosinophil-depleting therapy. Created in Biorender.com.

Participants were classified as ever-smokers if they had any smoking history, others were considered never-smokers. Ever-smokers were further categorised as current or previous (ex-smokers). Smoking history was quantified by pack-year history (PYH), where one PYH is the equivalent of 20 cigarettes a day for one year. Individuals with PYH ≥ 10 were considered heavy smokers (Neumann et al., 2013). Classification by comorbidity (e.g., nasal polyposis) was made by the clinician taking the medical history at visit.

2.2.2 Clinical procedures

Clinical procedures were carried out by clinical staff within Oxford University Hospitals (OUH) NHS Foundation Trust. Phlebotomy was performed by clinical staff, and blood collected into lithium heparin coated tubes (Greiner Bio-One, 455084). Spirometry measures the flow of inhaled and exhaled air over time and was conducted by clinical staff following ERS/ATS guidelines (Miller, 2005) using a Vitalograph Pneumotrac spirometer and Spirotrac software. For measurement of FEV1 (the volume of air forcibly exhaled in one second), the best reading of at least three successive readings with values within 100 mL of each other was taken. FEV1 % predicted is a general measurement of lung function impairment which expresses a patient's FEV1 relative to what would be expected of someone the same age, size, and sex (Pellegrino, 2005). Predicted values were calculated automatically by the spirometer using Global Lung Function Initiative (GLI) 2012 reference values (Quanjer et al., 2012).

Control of asthma was measured using a short version (Elizabeth F. Juniper et al., 2005) of the Asthma Control Questionnaire (ACQ) (E. F. Juniper et al., 1999). The ACQ asks patients to describe their symptoms in terms of night-time waking, morning symptoms, activity limitation, shortness of breath, and wheeze. An overall score is given as the mean average score per question, ranging from zero (totally controlled) to six (extremely poorly controlled) (E. F. Juniper et al., 1999). A value less than or equal to 0.75 can be considered well controlled, whilst a value of 1.5 or greater implies uncontrolled symptoms (Elizabeth F. Juniper et al., 2006). The ACQ covers symptoms experienced in the last 7 days, so it reflects current level of symptom control, something which varies over time in line with the characteristic features of asthma.

Fractional exhaled nitric oxide (FeNO) is a measure of airways inflammation associated with eosinophilia and steroid responsiveness (Dweik et al., 2011) and reflects the level of IL-13 induced inflammation of the airway epithelium. It was measured using a Niox Mino or Niox VERO set to a flow rate of 50 ml/sec. To perform the measurement, a seated participant exhales completely into the device at the set flow rate and the device confirms correct sampling.

Full Blood Counts (including blood eosinophils, basophils, neutrophils and lymphocytes) were performed as a standard clinical test by the OUH Haematology Laboratories Department using an automated haematology analyser, which can identify different cell types by their light scatter properties (flow cytometry) and electrical impedance (the Coulter principle). Counts are given in units of 10^9/L unless indicated otherwise.

Participants were considered as atopic or allergic if any of the following criteria were met: patient-reported allergies; patient was designated atopic or allergic by a clinician; or a positive radioallergosorbent test (RAST) for specific IgE to a common allergen. The RAST test is a standard clinical test carried out by the Clinical Immunology Laboratory within OUH. It uses a radioimmunoassay, in which bound antigen is incubated with patient serum, and specific IgE identified and quantified using radiolabelled anti-human IgE. The allergens tested were: house dust mite, mixed grasses, common fungi and moulds, cat, and dog. Total IgE was quantified as a standard clinical test by the same laboratory using turbidimetry, in which anti-IgE antibodies bound to microparticles are incubated with human serum. In the presence of IgE, antibody-antigen complexes are formed which absorb laser light.

Sputum induction was conducted by the clinical team following ERS guidelines (Chanez et al., 2002; Djukanovic et al., 2002). Participants were premedicated with salbutamol.

Increasing concentrations of saline (3, 4, and 5 %) were inhaled for five minutes each by the participant using an ultrasonic nebuliser (Flaem Nuova Easyneb II, E212E21). At each saline concentration, the subject attempted to cough up sputum which is collected in a pot. Sputum was stored on ice or in a fridge until it could be processed at the RMU sputum lab.

Sputum processing followed a previously described protocol (Pizzichini et al., 1996) and was conducted by a research assistant. 'Plugs' from the lower airways were manually separated from saliva with blunt forceps, and disaggregated with 4 x plug volume of 0.1 % dithiothreitol (DTT) for 15 minutes on ice to homogenise the sputum by reducing mucoprotein disulphide bonds. The sample was filtered through 48-micron gauze paper and centrifuged at 400 g for 5 minutes, then resuspended in phosphate-buffered saline (PBS). 10 μL of the sample was stained with trypan blue and counted by haemocytometer, the remainder resuspended at 1 million cells per ml. 75 μL of this sample (75 000 cells) was centrifuged on a Cytospin 4 (Thermo) at 450 rpm (23 rcf) for six minutes to generate a slide. Slides were fixed in methanol, then stained with RAPI-DIFF II (Atom scientific, RRSK1-1000). The RAPI-DIFF II stain contains a basic thiazine dye, an acidic eosin dye, and polychromed methylene blue, which stains acidic parts of cells including nuclei. Remaining cells were pelleted and stored, or analysed by flow cytometry.

2.2.3 Extracellular staining and flow cytometry

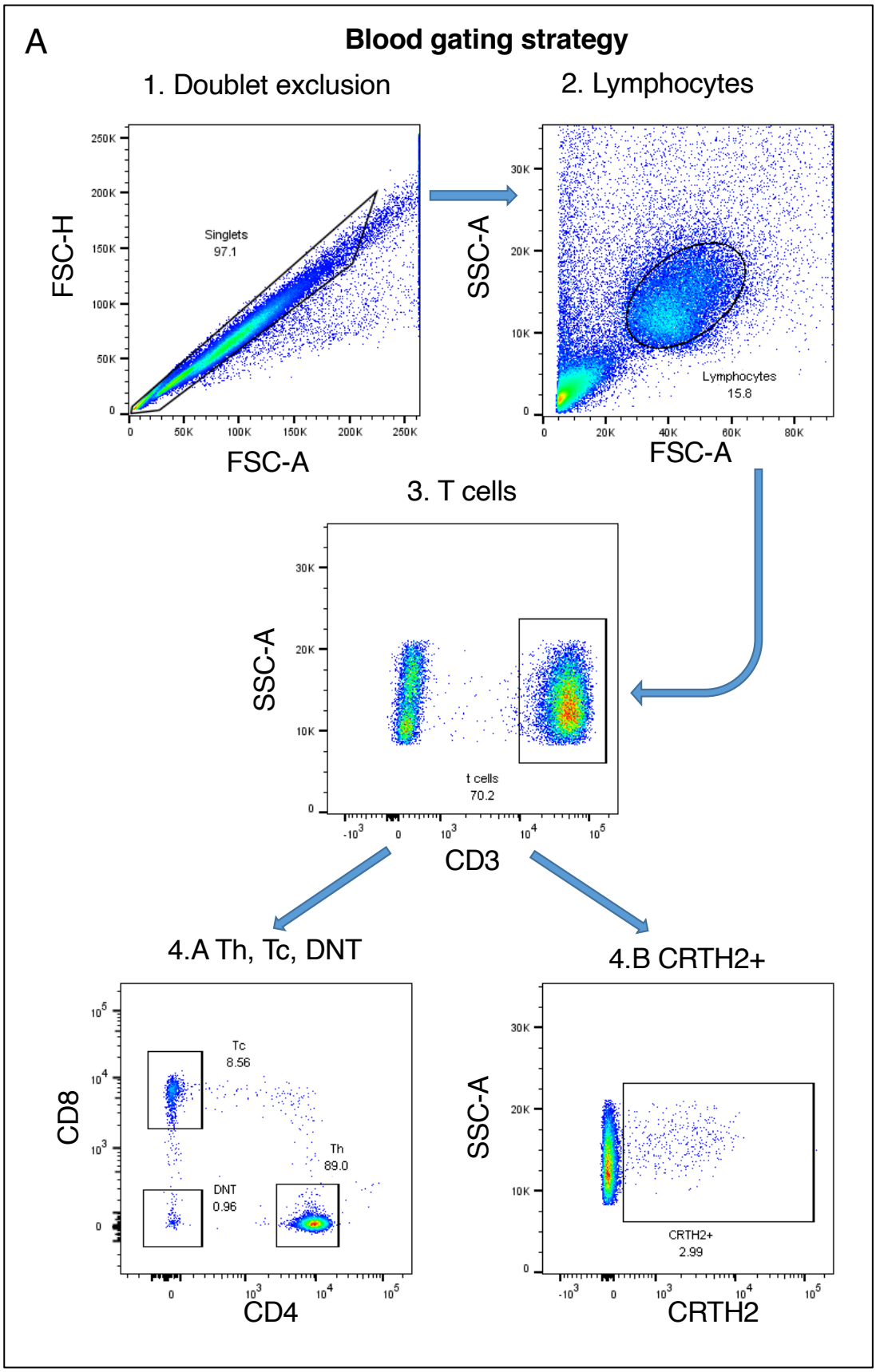
Samples of whole blood (50 μL), or cell pellets left over from sputum processing and resuspended in PBS (50 μL), were stained directly with an antibody cocktail (final volume 100 μL) as outlined in Table 4, for 30 minutes at 4 °C. Stained blood was fixed and lysed for 15 minutes in fluorescence-activated cell sorting (FACS) lysing solution (BD, 349202, diluted to working concentration in deionised H2O). Fixed and lysed blood samples were washed and spun (5 minutes at 400 g) three times in 2 mL of PBS and then analysed the

Table 4 Antibodies used for flow cytometry

Antigen	Clone	Fluorochrome	Supplier	Number	End dilution
Blood panel					
CD3	OKT3	FitC	BioLegend	317306	100
CD4	RPA-T4	PE	BioLegend	300508	800
CD8	RPA-T8	PB	BioLegend	301026	1000
CRTH2	BM16	AF647	BioLegend	350104	50
Sputum panel					
CD3	OKT3	PE-ef610	eBioscience	61-0037-42	50
CD4	RPA-T4	PE	BioLegend	300508	800
CD8	RPA-T8	PB	BioLegend	301026	1000
CRTH2	BM16	AF647	BioLegend	350104	50
Fixable viability dye		Zombie Aqua	BioLegend	423102	400

same or next day. Stained sputum samples were washed twice in PBS and then resuspended in 200 μL of a 1:1 mix of PBS and intracellular fixation buffer (eBioscience, 00-8222-49) overnight, then filtered with filter FACS tubes (Fischer, 10585801) and analysed. Notably, I found that washing the sputum samples at least twice after staining but before fixation was critical for achieving good separation in the CRTH2 stain.

Flow cytometry was performed on Becton Dickinson and Company (BD) LSRI II or LSR Fortessa flow cytometers at the NDM Experimental Medicine Flow Cytometry Facility using BD FACSDiva™ software. Measurement consistency between experiments was ensured using rainbow control beads (BioLegend, 422905). Compensation was performed using compensation control beads (BD, 552843 and 552844). Flow cytometry data were analysed in FlowJo (Becton Dickinson and Company, 2019). The gating strategies are given in Figure 7. Data were exported to Microsoft Excel (Microsoft, 2021b) to be prepared for statistical analyses. Relative expression of CRTH2 on T cells and subsets are given as percentages. Absolute counts of T2 cells and subsets were calculated by multiplying their relative proportion in lymphocytes by the total lymphocyte count, and are given as cells/mL unless indicated otherwise.


A
Blood gating strategy
1. Doublet exclusion
2. Lymphocytes
FSC-H
FSC-A
Singlets
97.1
SSC-A
Lymphocytes
15.8
3. T cells
t cells
70.2
CD3
4.A Th, Tc, DNT
4.B CRTH2+
CD8
CD4
Tc
8.56
Th
89.0
DNT
0.96
CRTH2+
2.99
CRTH2

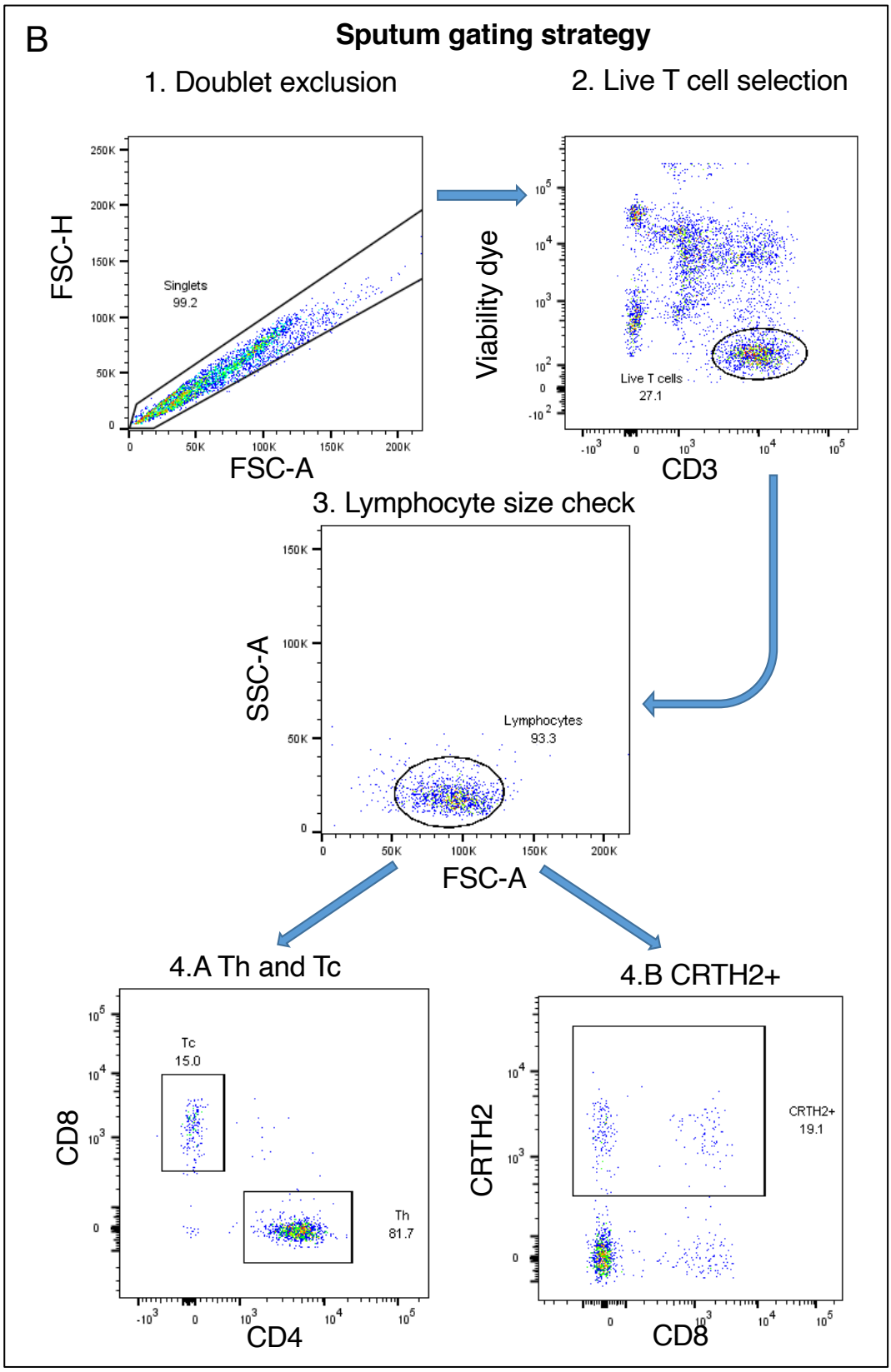


Figure 7 Representative gating strategy for identification of T2 cells and subsets

(A) blood gating strategy and (B) sputum gating strategy. Abbreviations: FSC-A, forward scatter-area, FSC-H, forward scatter-height; SSC-A side scatter-area.

2.2.4 Data handling and statistical analyses

Clinical data were recorded by hand and entered into the Brightling or OAS databases on Microsoft Access (Microsoft, 2021a). From here, data were exported to Microsoft Excel where they were paired with corresponding flow cytometry data of T2 cells, exported from FlowJo. Data were rearranged in Microsoft Excel for downstream analyses. All statistics were calculated in Prism (GraphPad Software Inc, 2021).

My analyses involved a mixture of parametric and nonparametric tests. Parametric tests make assumptions including a normal distribution. For scale variables, distribution was assessed by histograms and quantile-quantile (Q-Q) plots (M. B. Wilk & Gnanadesikan, 1968). The Q-Q plot is a scatterplot that compares sample data values against a theoretical normal distribution of the same mean and standard deviation. Well-matched distributions will produce a straight line. Histograms and Q-Q plots for normally distributed variables are shown in Supplementary Figure 1.

If the histogram of a variable suggested a lognormal distribution, then I assessed lognormality using histograms and Q-Q plots of data transformed by the equation $f(x) = \log(x)$ (Gaddum, 1945). Lognormal data were analysed in the log space, and back-transformed to report the geometric mean and confidence intervals. If data contained zero values, a small constant was added to all values before transformation (McDonald, 2014). Histograms and Q-Q plots for lognormally distributed variables are shown in Supplementary Figure 2.

Minor deviations from normal distributions were tolerated because parametric tests are robust to this (McDonald, 2014; Rasch & Guiard, 2004). I avoided formal normality tests because the literature suggests that they lack power at small sample sizes, and are irrelevant

at large sample sizes due to the central limit theorem (Fagerland, 2012; Lumley et al., 2002; Rochon et al., 2012).

A second assumption of parametric tests is equal spread of data between groups (homoscedasticity). For parametric variables, I tested for heterogeneity of variance by examining scatter plots, standard deviations, and by using the Bartlett test (Bartlett, 1937). This test involves computing individual group variance and comparing it to overall pooled variance. A test statistic *B* is generated which can be compared to a table of critical values for the Chi-square distribution.

To compare means of two parametric groups I used the *t*-test (Student, 1908), which calculates the ratio between group mean difference, over within-group variances and sample size to generate a *t*-statistic and corresponding *p*-value. For heteroscedastic data I used Welch's adapted *t*-test (Welch, 1947), which uses the specific sample standard deviations rather than estimated common variance.

To compare means between more than two parametric groups I used one-way analysis of variance (ANOVA) (Fisher, 1934) which similarly involves the ratio of mean differences between groups over variability within groups and sample size to generate a test statistic (*F*-statistic*)* and *p*-value. For heteroscedastic data I used Welch's ANOVA(Welch, 1951), which is an extension of Welch's adapted *t-test.* For specific *post hoc* comparisons to a healthy control group I used Dunnett's test (Dunnett, 1964). This test considers sample size, variation within groups, and degrees of freedom, to calculate the minimum significant difference between the two means. For *post hoc* tests between all possible combinations of groups I used the Tukey-Kramer method (Kramer, 1956). This test also calculates the minimally significant difference between two means, considering sample sizes, number of comparisons, and variation within groups, accounting for difference in sample sizes.

Some scale variables exhibited nonparametric distribution (Supplementary Figure 3). There is ongoing debate about whether such data should be tested with parametric or nonparametric statistics. I chose to use nonparametric statistics for two reasons. Firstly, my sample are small and the central limit theorem is not applicable (Lumley et al., 2002), secondly because this is increasingly the convention in medical sciences (Fagerland, 2012).

To compare two nonparametric groups, I used the Mann-Whitney U test (Mann & Whitney, 1947). This test involves ranking data by magnitude and calculating the *U*-statistic based on where each data point is ranked relative to the other group. The *U*-statistic is then used to generate a *p*-value. For more than two groups I used the Kruskal-Wallis test (Kruskal & Wallis, 1952), which extends the Mann-Whitney U test and generates a *H*-statistic. Individual comparisons were then made using Dunn's test (O. J. Dunn, 1964), which compares the difference in rank sum between two groups with the expected difference, accounting for sample size and number of groups.

The remaining variables were nominal (e.g., severe asthma endotype) and of these most were dichotomous (e.g., sex). In this case I tested for differences between groups using the chi-squared test (K. Pearson, 1900). This test considers the observed frequencies in a contingency table, compared with what would be expected under a null hypothesis. This information is used to generate a χ^2-statistic which can then be compared to a table of probabilities for the χ^2-distribution to determine if a critical value has been reached. For specific pairwise comparisons I used Fisher's exact test because the sample sizes were small, applying the Bonferroni correction for multiple comparisons (McDonald, 2014). This test considers the probability of proportions being at least as extreme as observed, under the null hypothesis that the proportions are the same, and using the hypergeometric distribution to generate *p*-values.

My analyses include tests of association between all the different types of variable considered. To correlate two parametric variables I used Pearson's correlation coefficient (P. K. Pearson, 1895). This generates value r by considering the sum of the products of z-scores (how many standard deviations each point (x, y) is away from the sample means $(\bar{x}, \bar{y})$ for each point (x, y). Significance was then calculated using a t-test. To compare one parametric and one dichotomous variable, I used point-biserial correlation (Tate, 1954). This test is a special case of Pearson's correlation and generates an analogous statistic r_{pb}. To compare two binary variables, I used the phi coefficient r_φ which again is a special case of Pearson's (Kaltenhauser & Lee, 1976).

In some cases, I tested a correlation between two parametric variables whilst accounting for a third parametric variable. In this context I used multiple linear regression. This method calculates an equation to predict an outcome variable based on linear functions of input variables (McDonald, 2014). The p-value generated indicates whether a given input variable has significantly improved the predictive model.

To associate nonparametric variables with scale variables, I used Spearman's rank correlation (Spearman, 1904), which is equivalent to the Pearson correlation between two variables' rank values, and generates a value r_s. Finally, to test association between one nonparametric and one dichotomous variable, I used rank biserial correlation (Cureton, 1956), which is equivalent to Spearman's rank correlation and is denoted r_{rb}.

The correlograms to summarise the results of tests of association were generated in RStudio (RStudio Team, 2020), an environment for working with R (R Core Team, 2020). Correlation and p-value matrices were generated by Prism, and converted into a correlogram using the corrplot package (T. Wei & Simko, 2017).

P-values, the probability that a similar result would be found with randomly generated data of the same average and spread, are indicated as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, and **** ≤ 0.0001. Referring to *r* values, I indicated magnitude by terms: weak < |0.3|, |0.3| < moderate < |0.6|, and strong > |0.6|. For test results concerning normally distributed variables, mean difference is denoted as *MD*. For results concerning lognormal variables, mean ratio is denoted *MR*. Confidence intervals are all 95 % and are reported as *CI* = [lower limit] to [upper limit]. For lognormal data, confidence intervals refer to ratios of geometric means rather than absolute difference between arithmetic means. For nonparametric test results, I have given only the *p*-value and not the mean rank difference, because it is not biologically meaningful.

2.3 Results

2.3.1 Comparisons between participant groups

One hundred and twenty-seven participants were included in a cross-sectional analysis in this chapter from sections 2.3.1 through to 2.3.9. Data were collected at the participant's initial 'baseline' visit, or the visit at which the most contextual data were collected (blood counts, lung function tests etc.).

A summary of participant group statistics is given in Table 2. All comparisons were made to the healthy volunteer group. Patients with MMA had higher BMI (*MD* = 4.6 kg/m^2, *CI* = 0.6 to 8.7, *p* = 0.008) and FeNO (*MR* = 1.9, *CI* = 1.2 to 3.2, *p* = 0.002). Patients with SNA were older (*MD* = 16.3 years, *CI* = 1.2 to 32, *p* = 0.03), and had lower FEV1 % predicted (*MD* = -24.2 %, *CI* = -50 to 2, *p* = 0.04). Patients with SEA had lower FEV1 % predicted (*MD* = -33.4 %, *CI* = -47 to-20, *p* < 0.0001); higher BMI (*MD* = 3.5 kg/m^2, *CI* = 0.1 to 6.9, *p* = 0.02); FeNO (*MR* = 2.6, *CI* = 1.4 to 4.6, *p* < 0.0001); IgE (*MR* = 2.6, *CI* 1.4

to 4.6, $p < 0.0001$); blood leukocytes (*MR* = 1.4, *CI* = 1.1 to 1.7, $p = 0.004$) including eosinophils (*MR* = 2.8, *CI* = 1.3 to 6.3, $p = 0.002$), basophils (*MR* = 1.7, *CI* = 1.2 to 2.6, $p = 0.002$) and monocytes (*MR* = 1.5, *CI* = 1.1 to 2.0, $p = 0.002$); and sputum eosinophils ($p = 0.005$). Patients with STA were older (*MD* = 12.7 years, *CI* = 0.7 to 25, $p = 0.03$); had reduced FEV1 % predicted (*MD* = -26.6 %, *CI* = -44 to -9, $p = 0.0003$); higher FeNO (*MR* = 2.5, *CI* = 1.4 to 4.6, $p = 0.0003$); BMI (*MD* = 4.1 kg/m^2, *CI* = 0.7 to 7.6, $p = 0.005$); and blood leukocytes (*MR* = 1.5, *CI* = 1.2 to 1.9, $p < 0.0001$) including blood neutrophils (*MR* = 1.9, *CI* = 1.4 to 2.5, $p < 0.0001$).

Patients with COPD were older (*MD* = 23.8 years, *CI* = 3 to 44, $p = 0.02$); more likely to have ever smoked ($p = 0.005$), and smokers had higher pack-year histories ($p = 0.003$); more likely to have cardiovascular comorbidities ($p = 0.02$); and had higher lymphocyte counts (*MR* = 1.4, *CI* = 1.1 to 1.8, $p=0.03$).

2.3.2 Overview of associations between all measured variables

The aim of this chapter is to understand relationships between blood CRTH2$^+$ T cells and

Table 5 Summary statistics of patient groups compared to healthy volunteers

For dichotomous variables, difference between group proportions was detected by the Chi-squared test, and individual comparisons to healthy volunteers made by Fisher's exact test. For normal/lognormal variables, difference in group mean/geometric mean was determined by one-way ANOVA or Welch's ANOVA (if failed Bartlett test), and specific comparisons to health were made with Dunnett's test. For nonparametric variables, a difference between groups was detected by Kruskal Wallis test and specific comparisons to health made with Dunn's test. Blue arrows (↑) indicate a significant increase in that group compared to health as calculated by the relevant *post hoc* test, and red arrows (↓) a decrease. The *p*-value is from the overall group analysis (e.g., one-way ANOVA) rather than specific *post hoc* tests. Therefore, it is possible for the overall p-value to be significant but without any of the individual *post hoc* tests reaching significance. Exacerbations refers to the past year. In terms of data distributions, dichotomous data are given in the 'Number (%)' format, normally distributed data are shown in the 'mean (SD)' format, lognormally distributed data in the 'gMean (SD)' format, and non-parametric data shown in the 'Median (IQR)' format. Abbreviations: ACQ, Asthma Control Questionnaire; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; gMean, geometric mean; IQR, interquartile range; MMA, mild-moderate asthma; PYH, pack-year history; SD, standard deviation; SEA, severe eosinophilic asthma; SNA, severe non-eosinophilic asthma; STA, severe asthma treated with eosinophil-depleting therapy.

Variable	Format	Healthy	MMA	SNA	SEA	STA	COPD	p-value
N (sample size)		17	29	12	30	34	5	
Allergy or Atopy	Number (%)	8 (47)	16 (55)	7 (58)	19 (63)	21 (61)	0 (0)	0.15
CVD	Number (%)	1 (6)	2 (7)	2 (17)	5 (17)	8 (24)	3 (60) ↑	0.044
Ever Smoker	Number (%)	4 (24)	9 (31)	2 (17)	7 (23)	12 (35)	5 (100) ↑	0.016
Male	Number (%)	8 (47)	14 (48)	4 (33)	18 (60)	17 (50)	2 (40)	0.73
Nasal Polyps	Number (%)	2 (12)	1 (3)	5 (42)	4 (13)	13 (38)	1 (20)	0.0049
Age	Mean(SD)	45 (17)	55 (17)	61 (11) ↑	56 (14)	57 (17) ↑	69 (7) ↑	0.027
BMI	Mean (SD)	24 (2.8)	29 (6.2) ↑	31 (7.7)	28 (4.7) ↑	28 (5.4) ↑	27 (5.3)	0.007
FEV1 % predicted	Mean (SD)	97 (9)	89 (20)	73 (24) ↓	64 (21) ↓	71 (23) ↓	63 (42)	<0.0001
Platelets	Mean (SD)	252 (51)	286 (83)	277 (56)	284 (80)	288 (67)	292 (36)	0.67
Blood Leukocytes	gMean (SD)	5.7 (1.6)	6.5 (2.1)	6.9 (2.7)	7.7 (1.9) ↑	8.7 (3.1) ↑	8.1 (1.4)	<0.0001
Blood Eosinophils	gMean (SD)	0.16 (0.22)	0.18 (0.25)	0.12 (0.09)	0.44 (0.41) ↑	0.10 (0.28)	0.25 (0.13)	0.0004
Blood Neutrophils	gMean (SD)	3.2 (1.5)	4.1 (1.7)	4.3 (2.1)	4.1 (1.1)	6.1 (2.8) ↑	4.7 (1.2)	<0.0001
Blood Lymphocytes	gMean (SD)	1.7 (0.43)	1.7 (0.65)	1.5 (0.73)	2.1 (1.0)	1.7 (0.9)	2.5 (0.29) ↑	0.0002
Blood Basophils	gMean (SD)	0.04 (0.02)	0.04 (0.02)	0.05 (0.02)	0.06 (0.03) ↑	0.05 (0.03)	0.06 (0.03)	0.01
Blood Monocytes	gMean (SD)	0.44 (0.15)	0.50 (0.11)	0.57 (0.15)	0.65 (0.19) ↑	0.61 (0.51)	0.57 (0.21)	0.0055
FeNO (ppb)	gMean (SD)	20 (11)	38 (29) ↑	23 (13)	51 (70) ↑	49 (65) ↑	10 (5)	<0.0001
IgE (kU/L)	gMean (SD)	39 (141)	113 (1099)	168 (340)	233 (768) ↑	197 (737)	315 (186)	0.08
ACQ	Median (IQR)	N/A	1.4 (2.0)	2.2 (3.2)	2.2 (1.4)	1.8 (2.1)	N/A	0.41
Exacerbations	Median (IQR)	N/A	1 (3)	4 (3)	4.5 (5.5)	2 (6)	N/A	0.0014
PYH (if smoker)	Median (IQR)	2.8 (5.6)	14 (26.0)	4 (0)	4.6 (10.5)	12 (17.6)	42.5 (23.0) ↑	0.0082
Sputum Eos %	Median (IQR)	0.1 (0.2)	0.5 (7.6)	0.6 (1.7)	30.7 (55.2) ↑	0.95 (10.75)	3 (5.8)	0.0043
Sputum Neutros %	Median (IQR)	55 (75)	54 (46.8)	84.9 (81.1)	27 (46)	58.2 (49.6)	91 (10.5)	0.0337

selected clinical and biological variables. This requires an understanding of how those variables relate to each other. One way to analyse relationships between many variables is with a correlation matrix. Therefore, I computed a matrix of correlation coefficients for all combinations of variables, and plotted individually statistically significant associations in a correlogram (Figure 8). This matrix of associations is further summarised in the form of a Venn diagram of the clinical subgroups and their associated variables (Figure 9).

The correlogram suggests associations exist between blood T2 cells and several variables under investigation. Positive associations were detected between T2 cells (or subsets of T2 cells) and: smoking history; cardiovascular comorbidity; COPD; age; allergy; sex; and blood cell counts including lymphocytes, basophils, platelets, and monocytes. A negative association was detected with steroid use. These relationships will be explored in detail in the subsequent sections of this chapter. But first, I will report the associations between other variables.

Compared to participants with either asthma or COPD, health correlated with: FEV1 % predicted (r_{pb} = 0.34, p = 0.0005); lower FeNO (r_{pb} = -0.29, p = 0.0013) and IgE (r_{pb} = -0.33, p = 0.0066); and fewer blood leukocytes (r_{pb} = -0.31, p = 0.0006) including eosinophils (r_{pb} = -0.21, p = 0.047), basophils (r_{pb} = -0.24, p = 0.006), neutrophils (r_{pb} = -0.25, p = 0.0055), and monocytes (r_{pb} = -0.27, p = 0.0035) . Healthy participants tended to be younger (r_{pb} = -0.27, p = 0.0026) with a lower BMI (r_{pb} = -0.26, p = 0.0037). Healthy smokers had a lower pack-year history than those with disease (r_{rb} = -0.41, p = 0.015). In healthy sputum there were relatively fewer eosinophils (r_{rb} = -0.30, p = 0.0029).

Asthma in general was associated with elevated FeNO (r_{pb} = 0.41, p < 0.0001), and weakly with allergy (r_{ϕ} = 0.18, p = 0.043), IgE (r_{pb} = 0.26, p = 0.03), BMI (r_{pb} = 0.26, p = 0.0047),

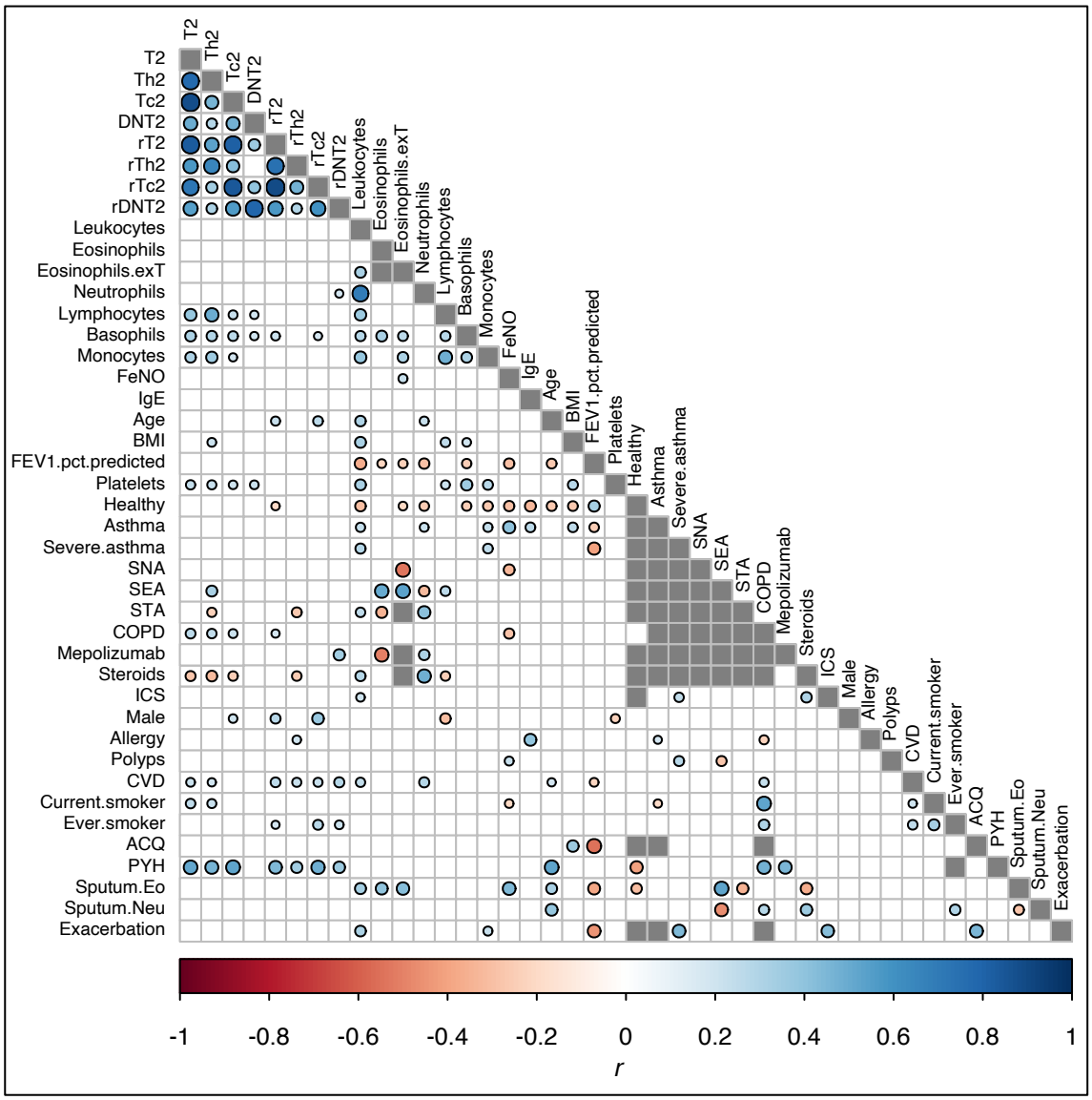


Figure 8 Correlogram of associations between all measured variables.

Associations below threshold $p = 0.05$ are shown. The size of each circle and its colour are proportional to the *r* value between those two variables. Nonsignificant associations are white boxes. Mutually exclusive (e.g., health vs COPD) or meaningless (e.g., STA vs steroid use) comparisons are grey boxes. Data from all participants ($N = 127$) for all variables was analysed with the following exceptions: the effects of PYH were only considered within ever-smokers; eosinophil counts for the STA group were excluded in the 'Eosinophils.exT' variable because of artificial reduction in their eosinophil counts; the effects of severe asthma endotype (SNA, SEA, or STA), mepolizumab use, or steroid use were only considered within severe asthma patients; the effect of ICS use was considered within patients with asthma or COPD; the effects of severe asthma, ACQ score, and number of exacerbations were only considered within patients with asthma. The statistical tests are as follows: two parametric variables were compared by Pearson's correlation; one parametric and one dichotomous by point biserial correlation; two dichotomous by phi coefficient; one nonparametric and one parametric, or two nonparametric, by Spearman's rank correlation; and one nonparametric and one dichotomous by rank biserial correlation, full details in 2.2.4. Cell counts refer to blood except for sputum eosinophils ('Sputum.Eo') and sputum neutrophils ('Sputum.Neu'), which refer to the percentage of those cells in sputum slide counts. 'Exacerbation' refers to number of asthma exacerbations in the past 12 months. Abbreviations are as described in earlier text and figures.

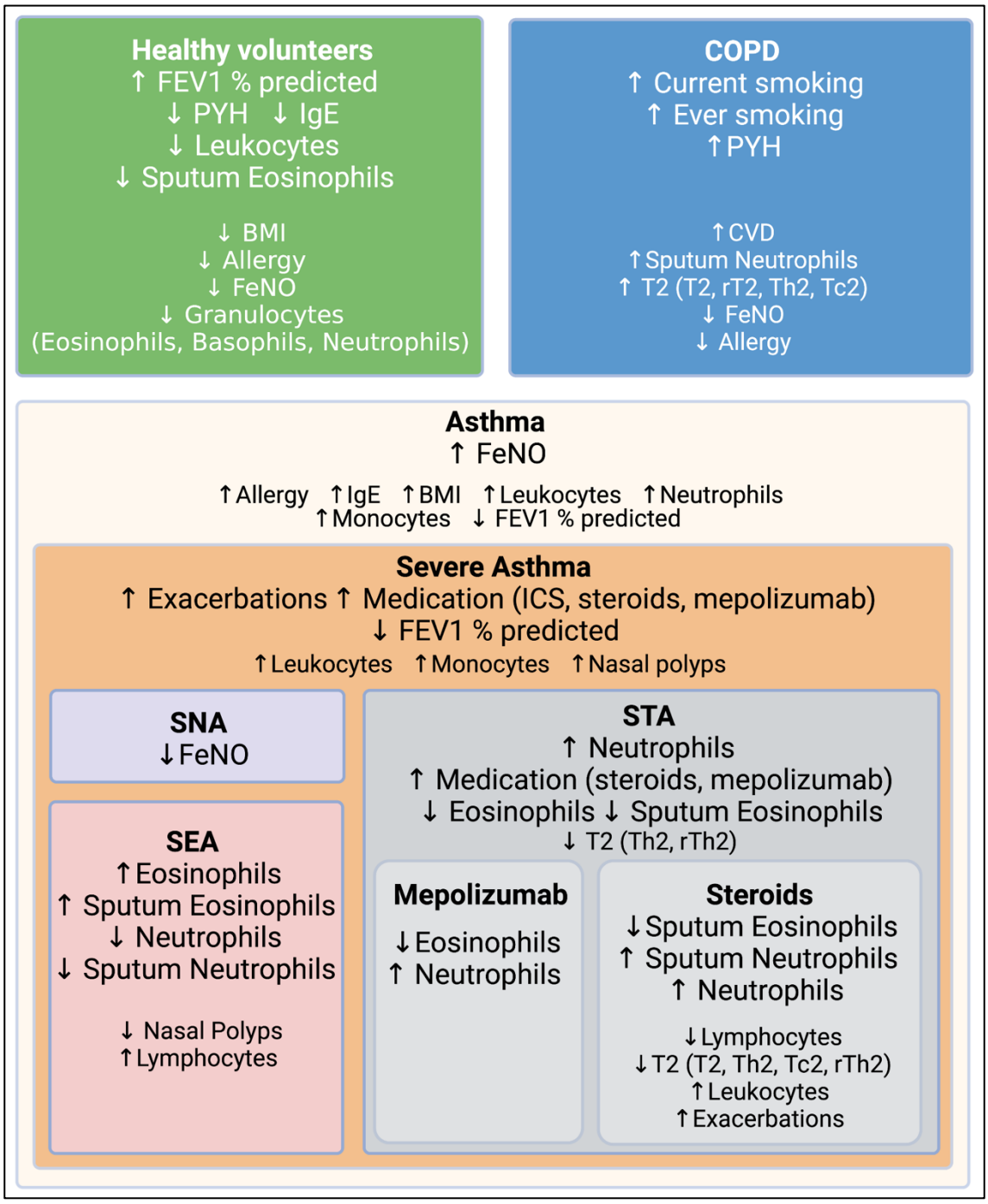


Figure 9 Venn diagram of associations observed for each patient group

Moderate and strong associations ($r > 0.3$) are shown in larger typeface relative to weak associations in smaller typeface. Health, COPD, and asthma are compared with each other. Within asthma, severe and non-severe (MMA) are compared. Within severe asthma, SNA, SEA, and STA are compared to each other. Use of mepolizumab or systemic steroids specifically were considered within severe asthma. Exact *r* and *p* values are found in the text. Named cells are assumed to refer to circulating cells unless specified as sputum. This figure summarises the results of the correlation analysis shown in Figure 8, in which all variables were associated with all other variables in an unbiased way. It is therefore different to the analysis in Table 5, in which the averages of specific variables in each patient group were compared to the healthy volunteer group. Abbreviations are as described in earlier text and figures. Created in Biorender.com.

and reduced lung function (r_{pb} = -0.25, p = 0.012). The disease correlated weakly with increased leukocytes (r_{pb} = 0.24, p = 0.009), including neutrophils (r_{pb} = 0.22, p = 0.017) and monocytes (r_{pb} = 0.23, p = 0.011). Considering only asthmatics, exacerbation frequency in the past 12 months was associated with reduction in FEV1 % predicted (r_s = -0.45, p < 0.0001), ACQ score (r_{rb} = 0.46, p < 0.0001), current prescription of inhaled corticosteroids (ICS) (r_{rb} = 0.43, p < 0.0001), leukocytes (r_s = 0.32, p = 0.005), and weakly with monocytes (r_s = 0.23, p = 0.0046). ACQ score was further associated with reduction in FEV1 % predicted (r_s = -0.54, p < 0.0001) and BMI (r_s = 0.36, p = 0.0005). Across all participants, FeNO was generally associated with sputum eosinophils (r_s = 0.45, p = 0.0009), and weakly with blood eosinophils (excluding STA) (r = 0.23, p = 0.036), reduction in lung function (r = -0.29, p = 0.003) and nasal polyps (r_{pb} = 0.22, p = 0.016).

Severe asthma was associated with exacerbations (r_{rb} = 0.45, p < 0.0001) and reduction in FEV1 % (r_{pb} = -0.41, p = 0.0001), and weakly with nasal polyps (r_ψ = 0.28, p = 0.0044), leukocytes (r_{pb}= 0.28, p = 0.0045), monocytes (r_{pb} = 0.26, p = 0.001) and current ICS use (r_ψ = 0.24, p = 0.013), compared to MMA. When the three endotypes of severe asthma (SNA, SEA, and STA) were considered together (excluding other participants), differences were detected between them. The SNA endotype correlated negatively with FeNO (r_{pb} = -0.33, p = 0.0056) and eosinophils (excluding STA) (r_{pb} = -0.54, p = 0.0056). SEA was associated with relatively more blood (r_{pb} = 0.54, p = 0.0002) and sputum (r_{rb} = 0.53, p = 0.00023) eosinophils, and fewer blood (r_{pb}= -0.31, p = 0.0077) and sputum (r_{rb} = -0.46, p = 0.0070) neutrophils, and weakly with more lymphocytes (r_{pb}= 0.26, p = 0.023) and fewer cases of nasal polyps (r_ψ = -0.28, p = 0.015). STA was associated with fewer blood (r_{pb} = -0.34, p = 0.003) and sputum eosinophils (r_{rb} = -0.35, p = 0.047), and more blood neutrophils (r_{pb}= 0.42, p = 0.0003).

Within severe asthma, current steroid use associated with blood neutrophils (r_{pb}= 0.48, p < 0.0001) which resulted in increased leukocytes (r_{pb} = 0.29, p = 0.016) despite a slight fall in lymphocytes (r_{pb}= -0.25, p = 0.037). Steroid prescription linked to reduced sputum eosinophil percentages (r_s = -0.37, p = 0.037) and concomitant increase in sputum neutrophils (r_s = 0.37, p = 0.040). These patients were more likely to also use ICS (r_ψ = 0.30, p = 0.009) and mepolizumab (r_ψ = 0.30, p = 0.009), and had more recent exacerbations (r_{pb} = 0.27, p = 0.017). Mepolizumab use was associated with a decrease in eosinophils (r_{pb}= -0.51, p < 0.0001) and with increased neutrophils (r_{pb}= 0.31, p = 0.008). Mepolizumab users who had smoked, had higher pack-year histories (r_{rb} = 0.45, p = 0.046). Steroid and mepolizumab use was considered only within severe asthma data because nobody in the healthy, COPD, and MMA groups were taking these drugs at the time of their visit.

COPD was associated with current smoking (r_ψ = 0.53, p < 0001), ever smoking (r_ψ = 0.30, p = 0.0005), pack-year history (r_ψ = 0.48, p = 0.0038), cardiovascular disease (r_ψ = 0.24, p = 0.0074), and sputum neutrophils (r_{rb} = 0.29, p = 0.036). It was weakly negatively associated with allergy (r_ψ = -0.23, p = 0.010) and FeNO (r_{pb} = -0.29, p = 0.0017). Cardiovascular disease was in turn weakly associated with age (r_{pb} = 0.19, p = 0.032), current smoking (r_ψ = 0.20, p = 0.024), ever smoking (r_ψ = 0.26, p = 0.004), fall in FEV1 % predicted (r_{pb} = -0.21, p = 0.033), leukocytes (r_{pb} = 0.24, p = 0.008) and neutrophils (r_{pb}= 0.28, p = 0.0018). Ever smoking was associated with sputum neutrophils (r_{rb} = 0.31, p = 0.025) and current smoking with reduction in FeNO (r_{pb} = -0.21, p = 0.024). Older smokers had higher pack-year history (r_s = 0.52, p = 0.0013)

Other factors associated with reduced lung function were leukocytes (r = -0.37, p = 0.0001), age (r = -0.27, p = 0.007), blood eosinophils (r = -0.24, p = 0.032) and sputum

eosinophils, (r_s = -0.39, p = 0.009) basophils (r = -0.24, p = 0.014), and neutrophils (r = -0.29, p = 0.003).

Several further changes were observed with age. Leukocyte counts increased (r = 0.31, p = 0.0007), including blood (r = 0.23, p = 0.014) and sputum (r_s = 0.38, p = 0.004) neutrophils, and sputum eosinophils (r_s = 0.33, p = 0.017). Participant BMI correlated with leukocytes (r = 0.33, p = 0.0003), weakly with basophils (r= 0.20 p = 0.026), platelets (r = 0.28, p = 0.003) and lymphocytes (r= 0.25, p = 0.006). Men had fewer platelets (r_{pb} = -0.22, p = 0.017) and lymphocytes (r_{pb}= -0.31, p = 0.0007). Participants with allergies tended to have higher circulating IgE (r_{pb} = 0.39, p = 0.0011).

Total leukocyte count is the sum of white blood cells and correlated strongly with the number of neutrophils (r = 0.70, p < 0.0001), moderately with lymphocytes (r =0.36, p < 0.0001), monocytes (r = 0.38, p < 0.0001), eosinophils (excluding STA) (r = 0.32, p = 0.002) and sputum eosinophils (r_s = 0.34, p = 0.016), and platelets (r = 0.32, p = 0.0003), and weakly with basophils (r = 0.29, p = 0.0016). Excluding STA (patients with artificially depleted eosinophils), blood eosinophils were linked to blood basophils (r = 0.33, p = 0.0003), monocytes (r = 0.31, p = 0.003), and sputum eosinophils (r_s = 0.32, p = 0.005). Lymphocyte count was positively correlated with monocytes (r= 0.49, p < 0.0001), and weakly with basophils (r = 0.27, p = 0.002) and platelets (r = 0.21, p = 0.017). Basophils were associated with platelets (r = 0.36, p < 0.0001) and monocytes (r = 0.32, p = 0.0004). Monocytes were also associated with platelets (r = 0.36, p < 0.0001). Finally, sputum eosinophil fraction was negatively associated with sputum neutrophil fraction (r_s = -0.28, p = 0.045).

2.3.3 Blood CRTH2+ T cells were higher in COPD but not in asthma compared to health

The primary question of this study was to determine the relationship between circulatory T2 cell counts and asthma. Overall, there was no significant difference between asthma and health for absolute or relative T2 cells and their subsets, although mean numbers were higher in asthma (Figure 10). However, there was a significant increase in both absolute (*MR* = 4.2, *CI* = 1.7 to 10.7, $p = 0.0013$) and relative (*MR* = 2.9, *CI* = 1.3 to 6.8, $p = 0.01$) T2 cells in COPD compared to health. This included an increase in both absolute (*MR* = 3.1, *CI* = 1.4 to 6.7, $p = 0.0028$) and relative (*MR* = 2.0, *CI* = 1.0 to 3.9, $p = 0.039$) Th2 cells, and absolute (*MR* = 6.6, CI = 1.5 to 28.1, $p = 0.0088$) and relative (*MR* = 4.9, CI = 1.2 to 19.5, $p = 0.021$) Tc2 cells.

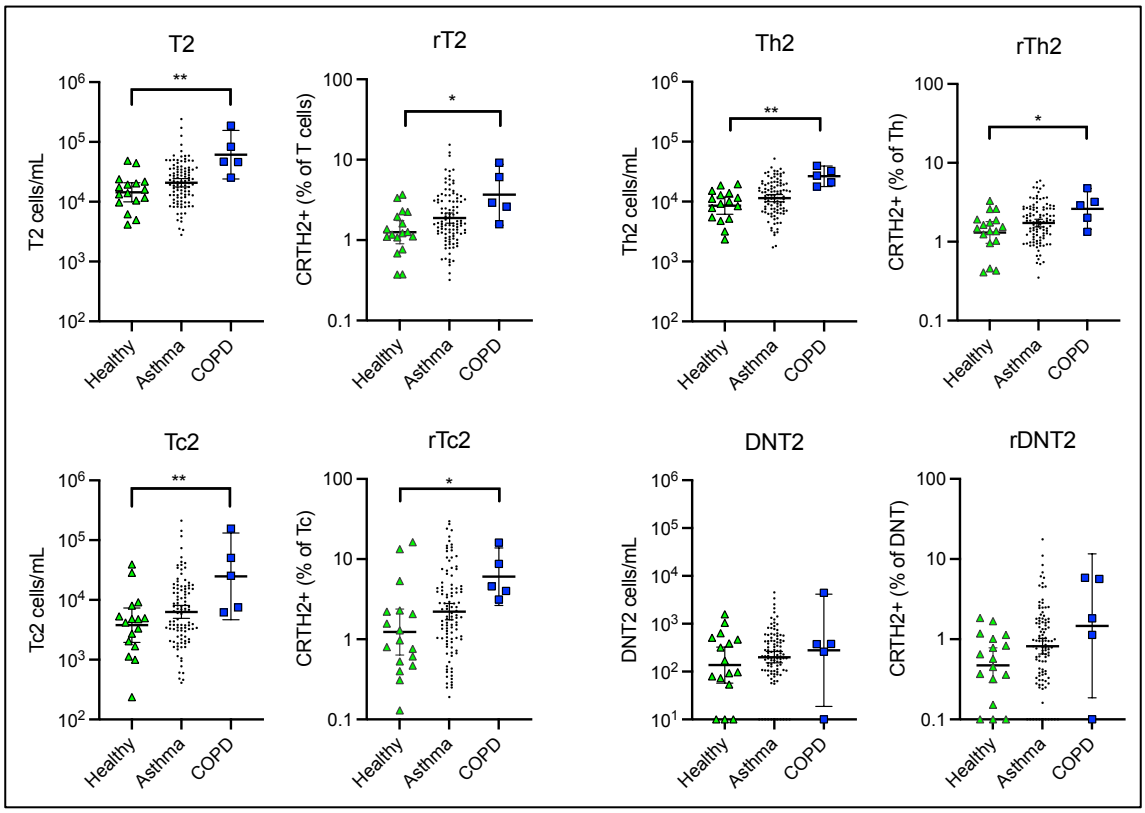


Figure 10 T2 cell counts were higher in COPD but not asthma compared to health.

Log transformed data was analysed by one-way ANOVA. Specific comparisons to healthy volunteers were made with Dunnett's test. Bars show geometric mean and 95 % confidence interval. Sample sizes healthy = 16, asthma = 103, COPD = 5.

2.3.4 Smoking and cardiovascular disease were associated with blood CRTH2⁺ T cells

The correlogram suggests associations between smoking, cardiovascular disease, and CRTH2⁺ T cells, so I investigated these factors in detail. Patients with cardiovascular comorbidities had higher absolute (*MR* = 1.6, *CI* = 1.1 to 2.4, $p = 0.016$) and relative (*MR* = 1.7, *CI* = 1.2 to 2.4, $p = 0.0027$) T2 cells (Figure 11). This included relative increase in all subsets: rTh2 (*MR* = 1.4, *CI* = 1.1 to 1.9, $p = 0.0097$), rTc2 (*MR* = 2.1, *CI* = 1.2 to 3.7, $p = 0.011$) and rDNT2 (*MR* = 2.4, *CI* = 1.4 to 4.1, $p = 0.0013$), and an absolute increase in the Th2 subset (*MR* = 1.4, *CI* = 1.0 to 2.0, $p = 0.033$).

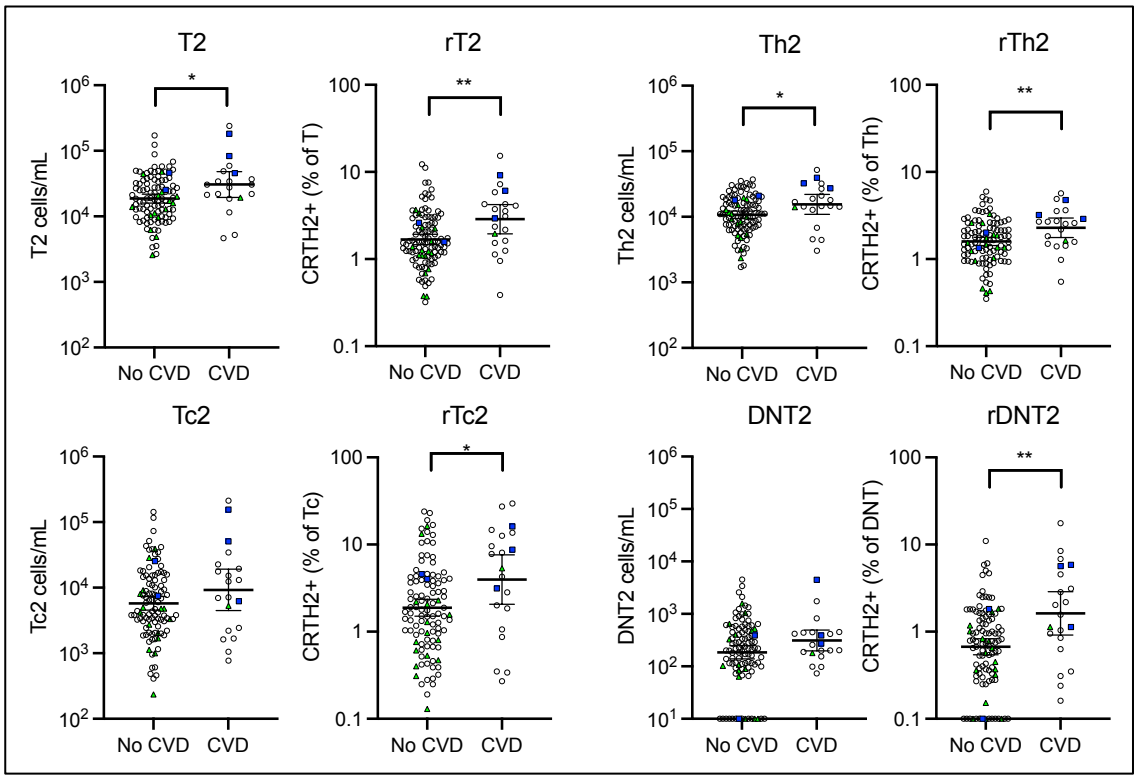


Figure 11 Cardiovascular comorbidity was associated with CRTH2⁺ T cell counts

Log-transformed data were analysed by two-tailed *t*-test. Bars show geometric mean and 95 % confidence interval. Sample sizes: no CVD = 104, CVD = 20. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares.

Smoking can be classified in different ways, and this affected observed increases in CRTH2$^+$ T cells. Comparing ever-smokers to never-smokers (Figure 12) showed increased relative expression of CRTH2 on T cells or rT2 (*MR* = 1.3, *CI* = 1.0 to 1.8, p = 0.042), including rTc2 (*MR* = 2.1, *CI* = 1.3 to 3.2, p = 0.0021) and rDNT2 (*MR* = 1.7, *CI* = 1.1 to 2.6, p = 0.017) subsets. Current smokers specifically had higher absolute T2 cell numbers (*MR* = *CI ratio* 1.1 to 7.0, p = 0.020), and ex-smokers higher rTc2 (*CI ratio* 1.1 to 3.5, p = 0.017) (Supplementary Figure 4). By dichotomising smokers into heavy (PYH > 10) and light (PYH < 10) smokers (Figure 13), there appeared to be relatively higher CRTH2 expression on T cells (rT2) in heavy smokers (*MR* = 1.6, *CI* = 1.0 to 2.5, p = 0.027) including rTc2 (*MR* = 2.9, *CI* = 1.5 to 5.6, p = 0.0012) and rDNT2 subsets (*MR* = 2.1, *CI*

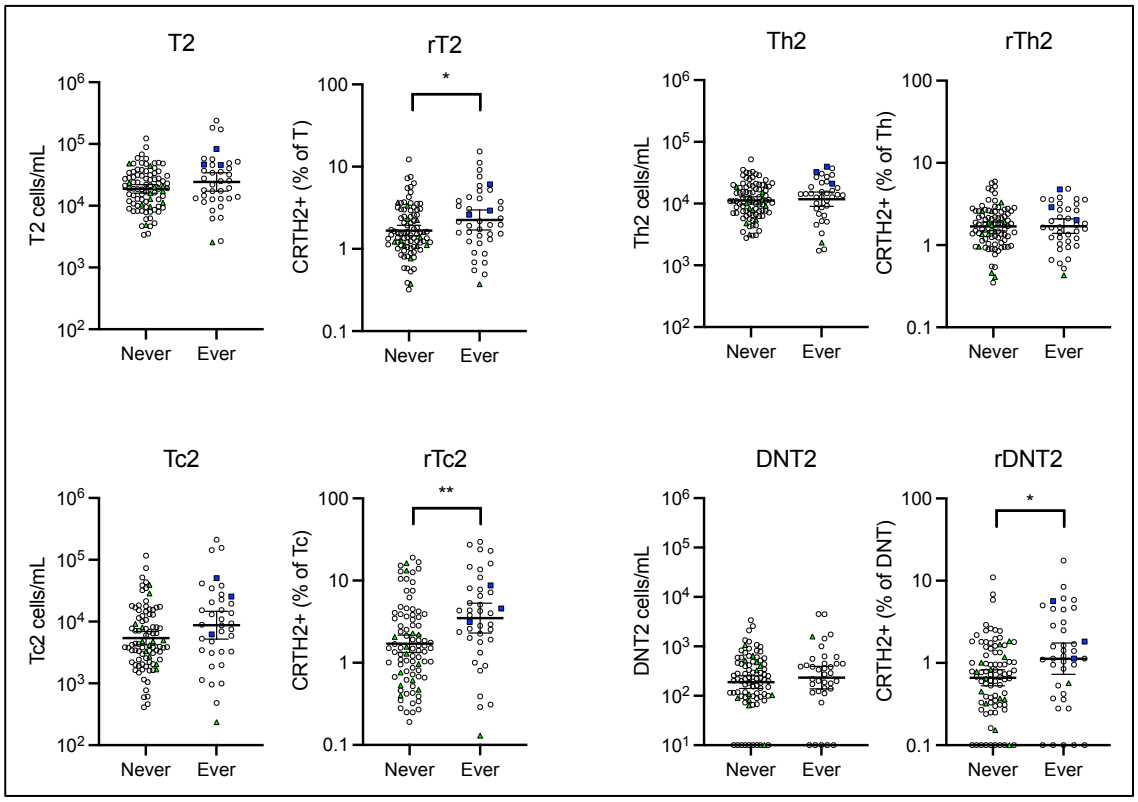


Figure 12 Ever smokers had higher proportions of Tc2 cells than never smokers

Log-transformed data were analysed by two-tailed *t*-test. Bars show geometric mean and confidence interval. Sample sizes: never = 87, ever = 37. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares.

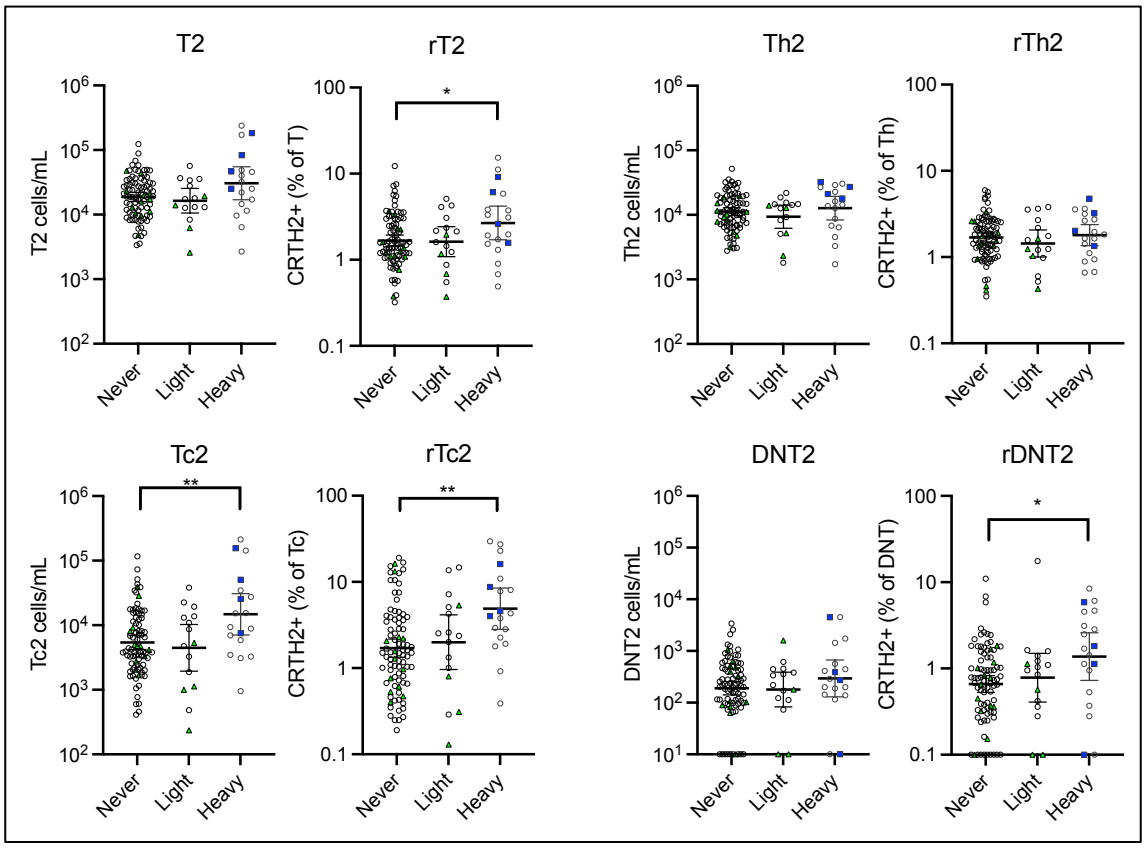


Figure 13 Elevation of CRTH2+ Tc cells in smokers was driven by heavy smokers

Log-transformed data were analysed by one-way ANOVA. Specific comparisons to never smokers were made by Dunnett's test. Bars show geometric mean and confidence interval. Sample sizes never = 87, light = 15, heavy = 18. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD with blue squares.

1.1 to 3.9, $p = 0.021$) compared to non-smokers. There were also more Tc2 cells in heavy smokers vs non-smokers ($MR = 2.7$, $CI = 1.3$ to 5.7, $p = 0.0049$).

If smoking is linked to CRTH2$^+$ T cells, one imagines a relationship with the magnitude of cigarette consumption. Therefore, I analysed scatterplots of T2 cells and PYH (one pack year = 7300 cigarettes) (Figure 14). This revealed a relationship in the data between PYH and both absolute ($r_s = 0.52$, $p = 0.0020$) and relative ($r_s = 0.45$, $p = 0.0071$) T2 cell counts, including absolute Th2 ($r_s = 0.46$, $p = 0.0067$) and Tc2 ($r_s = 0.53$, $p = 0.0016$) subsets, and relative CRTH2 expression on all subsets: Th2 ($r_s = 0.35$, $p = 0.042$), Tc2 ($r_s = 0.49$, $p = 0.003$), and DNT2 ($r_s = 0.36$, $p = 0.032$).

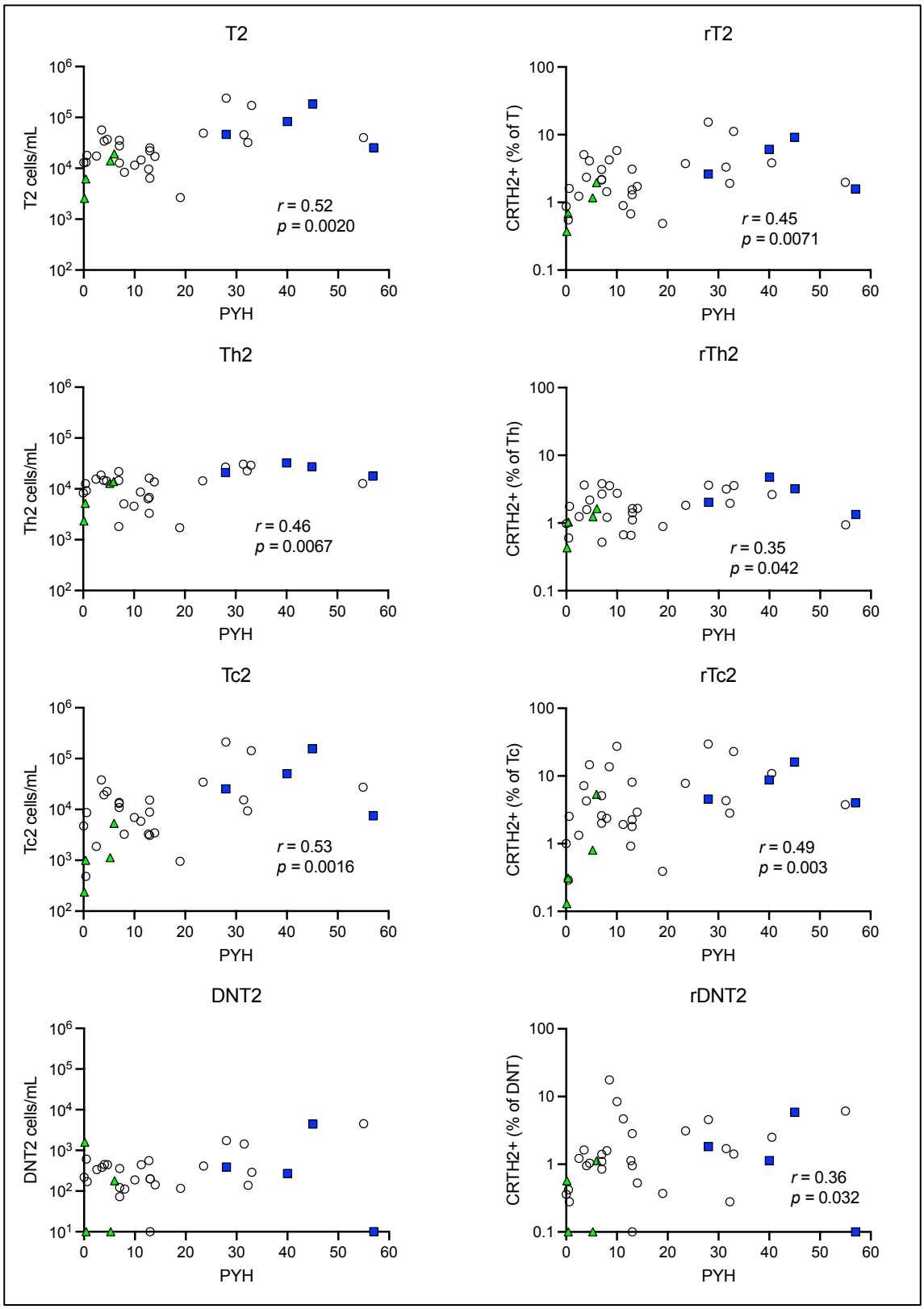


Figure 14 Pack-year history was associated with T2 cell counts

Variables were compared by Spearman correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. *N* = 35 ever smokers with PYH data. Only significant associations are shown.

One disadvantage of pack-year history as a measurement, is that it does not account for smoking cessation. If smoking is really linked to increased CRTH2$^+$ T cells, one might expect smoking cessation to be followed by a decrease in CRTH2$^+$ T cells. Most of the heavy smokers in this data set are ex-smokers of variable quit dates. Therefore, I plotted (for heavy smokers) the number of years since cessation against T2 cell counts. This revealed a fall in absolute (r_s = -0.89, p = 0.0003) and relative (r_s = -0.52, p = 0.028) T2 cells over time (Figure 15), including falls in all subsets of Th2 (r_s = -0.49, p = 0.0063), Tc2 (r_s = -0.75, p = 0.0008), and DNT2 (r_s = -0.49, p = 0.048).

2.3.5 Blood CRTH2$^+$ cytotoxic T cells were higher in men and weakly associated with age

The immune system differs between the sexes (Klein & Flanagan, 2016) and changes with age (Simon et al., 2015), so I checked whether these factors also affect T2 cell counts. Men had a higher proportion of CRTH2 expression on T cells than women (*MR* = 1.5, *CI* = 1.2 to 2.0, p = 0.0020) (Figure 16). This included elevation in absolute (*MR* = 1.7, *CI* = 1.1 to 2.7, p = 0.026) and relative (*MR* = 2.5, *CI* = 1.7 to 3.8, p < 0.0001) numbers of Tc2 cells. From the correlogram (Figure 8) we can see that men were not significantly overrepresented in terms of smoking or cardiovascular disease.

Plotting age against T2 cells showed a weak correlation with relative CRTH2 expression on T cells (r = 0.23, p = 0.0098), including the Tc2 subset (r = 0.26, p = 0.0030) (Figure 17). From the correlogram (Figure 8) we know that age was also associated with cardiovascular disease, pack-year history, and negatively with health. If smokers and participants with CVD are excluded, the associations between age and rT2 (p = 0.14) or rTc2 (p = 0.10) were no longer significant. There was also a weak correlation between BMI and absolute Th2 cell count (r = 0.21, p = 0.020) (Supplementary Figure 5). However, the correlogram

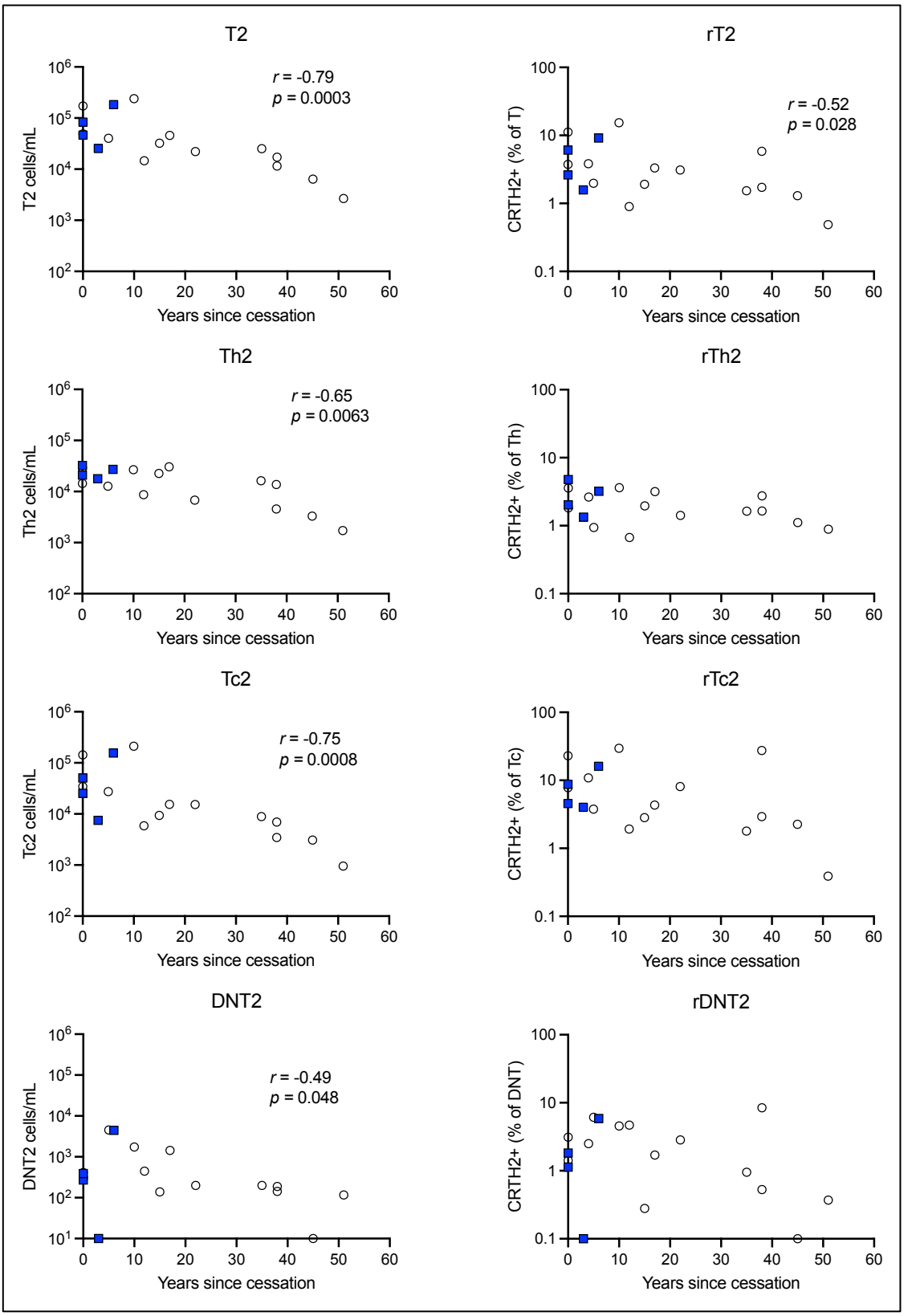


Figure 15 Smoking cessation was associated with a reduction in T2 cell counts

Variables were compared by Spearman correlation. Data points from patients with asthma are indicated with white circles, and COPD with blue squares. *N* = 18 heavy smokers. Only significant associations are shown

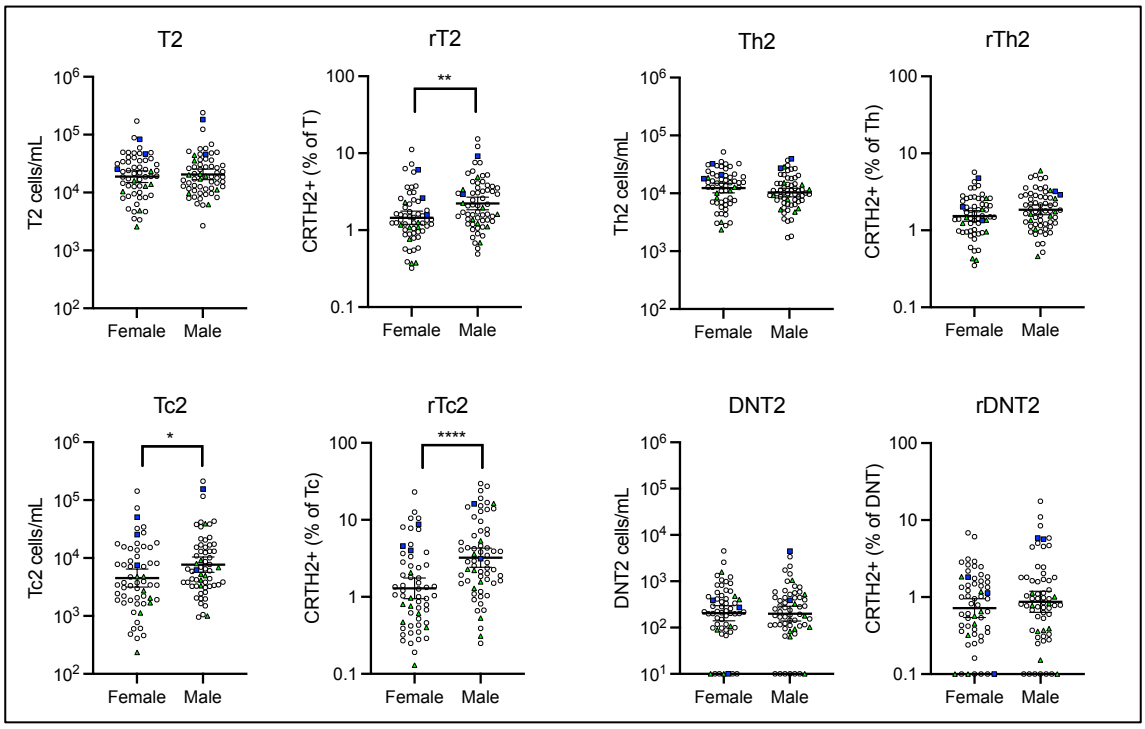


Figure 16 Males had higher absolute and proportional Tc2 cell counts than females.

Log-transformed data were analysed by two-tailed *t*-test. Bars show geometric mean and confidence interval. Sample sizes: female = 58, male = 61. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares.

(Figure 8) indicates that both BMI and Th2 cell count are also related to the general lymphocyte count. In a multiple regression analysis of BMI, lymphocyte count and Th2, there was no link between BMI and Th2 ($p = 0.27$).

2.3.6 The proportion of CRTH2⁺ Th cells in blood was higher in allergic asthma

Asthma is heterogeneous and can be classified in myriad ways. To determine if T2 cells were related to a specific type of asthma, I analysed the data using different asthma classifications. Relative quantities of Th2 cells were higher in allergic asthma vs healthy controls ($MR = 1.5$, $CI = 1.1$ to 2.1, $p = 0.021$) and the same was true for relative quantities of T2 cells overall ($MR = 1.6$, $CI = 1.0$ to 2.5, $p = 0.037$) (Figure 18). However, absolute Th2 counts were not different. Participants with allergy of any description (ignoring airways disease) also had a higher proportion of CRTH2$^+$ Th cells ($MR = 1.3$, $CI = 1.0$ to

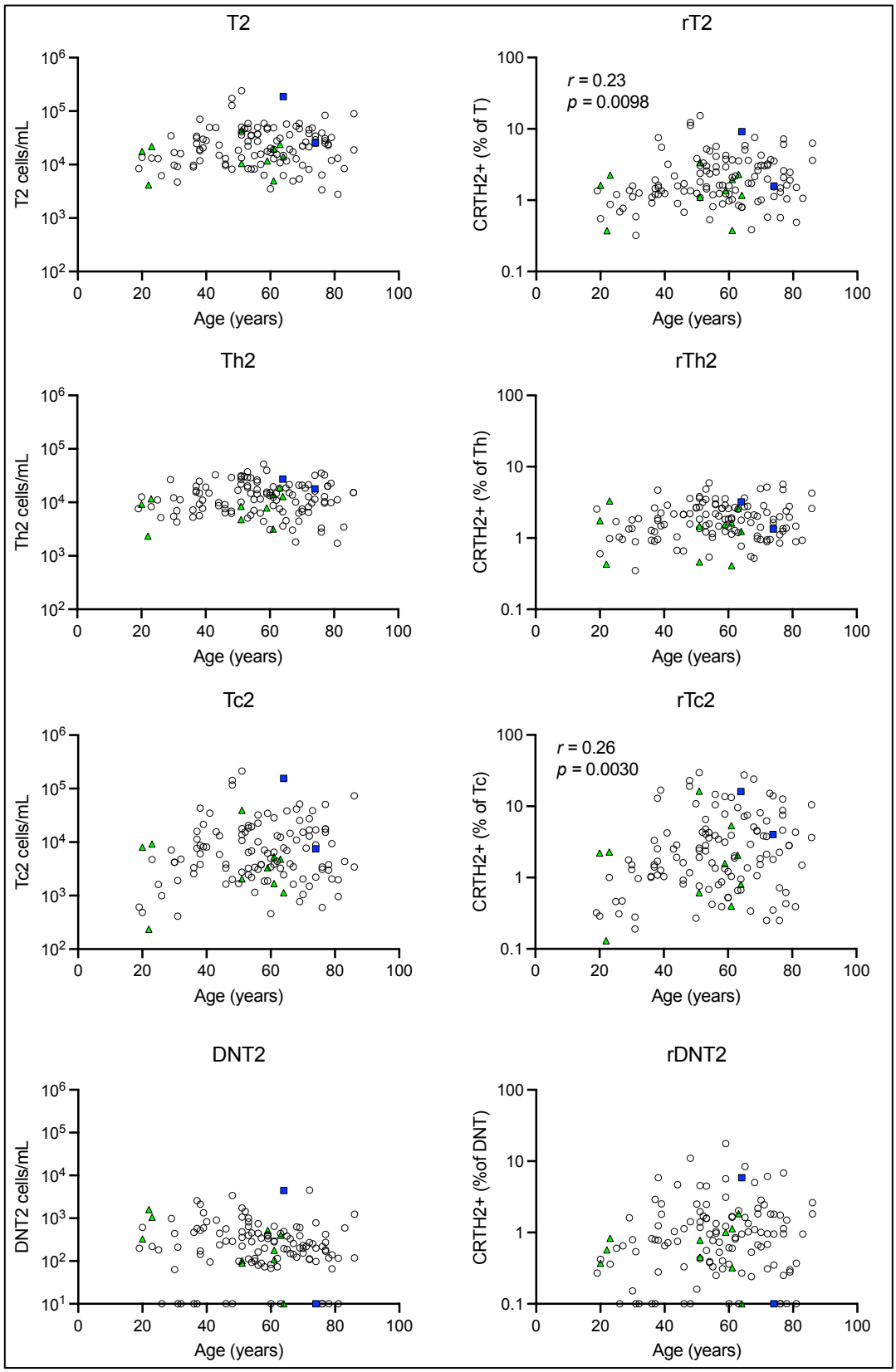


Figure 17 Age was weakly associated with the proportion of Tc2 cells

Variables compared by Pearson correlation. Data points from healthy volunteers are indicated by green triangles, asthma patients by white circles, and COPD by blue squares. *N* = 127.

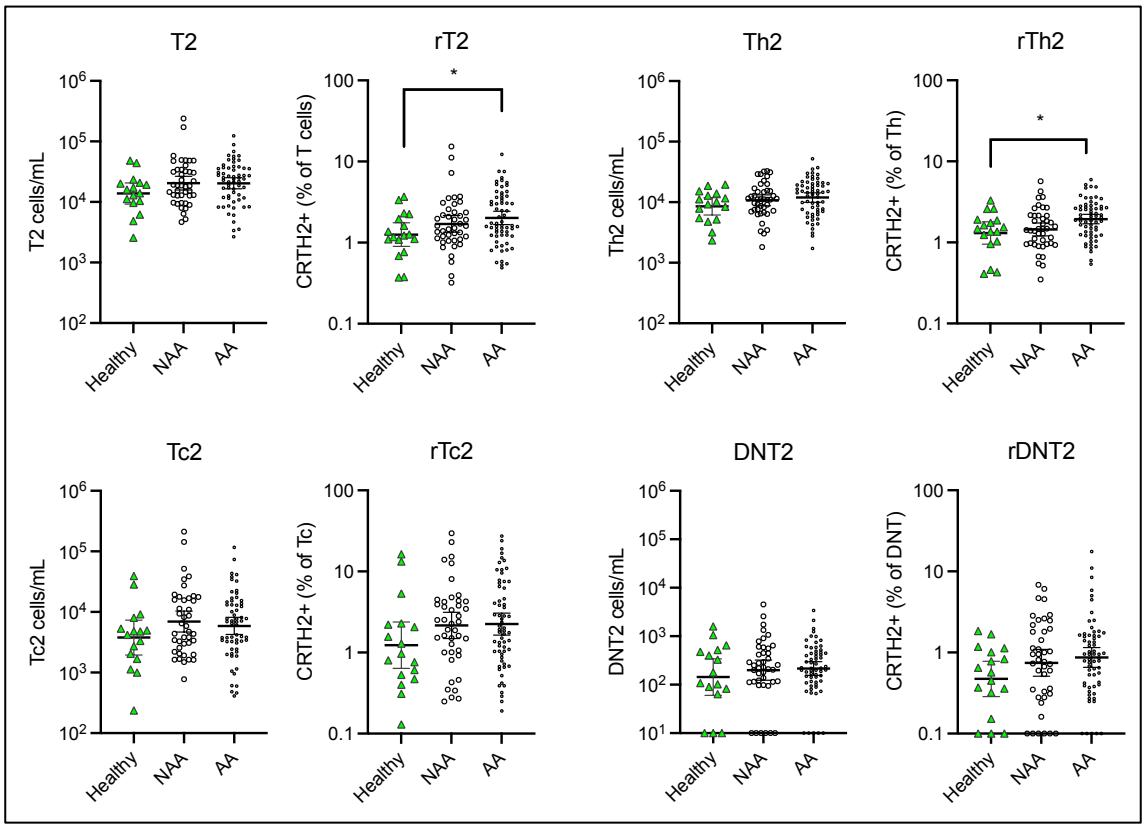


Figure 18 The proportion of CRTH2⁺ T cells and Th cells was higher in allergic asthma

Data were analysed by one-way ANOVA in the log space. Pairwise comparisons to the healthy group were made by Dunnett's test. Bars show geometric mean and confidence interval. Sample sizes: healthy = 16, NAA = 42, AA = 61. Abbreviations: NAA, nonallergic asthma; AA, allergic asthma.

1.6, $p = 0.022$) (Supplementary Figure 6). However, no relationships were found with serum IgE (Supplementary Figure 7), another key component of the allergic response.

2.3.7 Asthma severity was not associated with blood CRTH2⁺ T cells

There were no differences between severe vs non-severe (MMA) asthma, and neither of these groups were different to health (Figure 19). If the number of circulating CRTH2⁺ T cells is related to asthma severity, one might expect a relationship to exist with measures such as lung function (Supplementary Figure 8), asthma exacerbations (Supplementary Figure 9), and/or symptoms (as measured by ACQ score) (Supplementary Figure 10). No relationships were found between T2 cells and any of these variables.

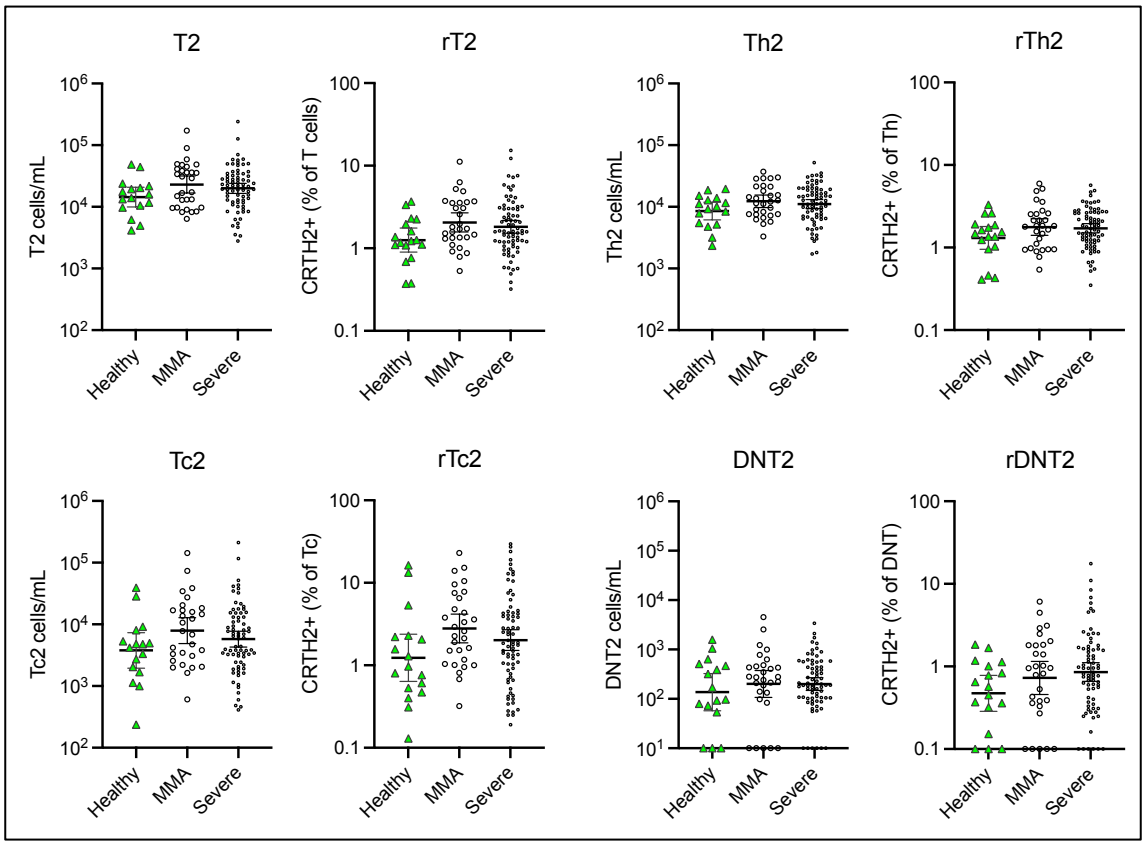


Figure 19 No difference in T2 cell counts between mild/moderate and severe asthma.

Log-transformed was analysed by one-way ANOVA. Specific comparisons to the healthy group were made with Dunnett's test. Bars show geometric mean and confidence interval. Sample sizes: healthy = 16, MMA = 29, severe asthma = 74. Abbreviations: MMA, mild-moderate asthma (non-severe asthma).

2.3.8 Asthma endotype was not associated with blood CRTH2+ T cells

Within severe asthma, I considered the endotypes SNA, SEA, and STA. The only difference detected was elevated Th2 cells in the SEA group vs healthy controls (*MR* = 1.7, *CI* = 1.0 to 2.9) (Figure 20). Furthermore, there were no relationships detected between $CRTH2^+$ T cells and measures associated with T2-high asthma, such as blood eosinophil count (Supplementary Figure 11), sputum eosinophil percentage (Supplementary Figure 12), presence of nasal polyps (Supplementary Figure 13), or FeNO (Supplementary Figure 14). Similarly, there was no evidence of a link to neutrophilic inflammation of the blood (Supplementary Figure 15) or sputum (Supplementary Figure 16).

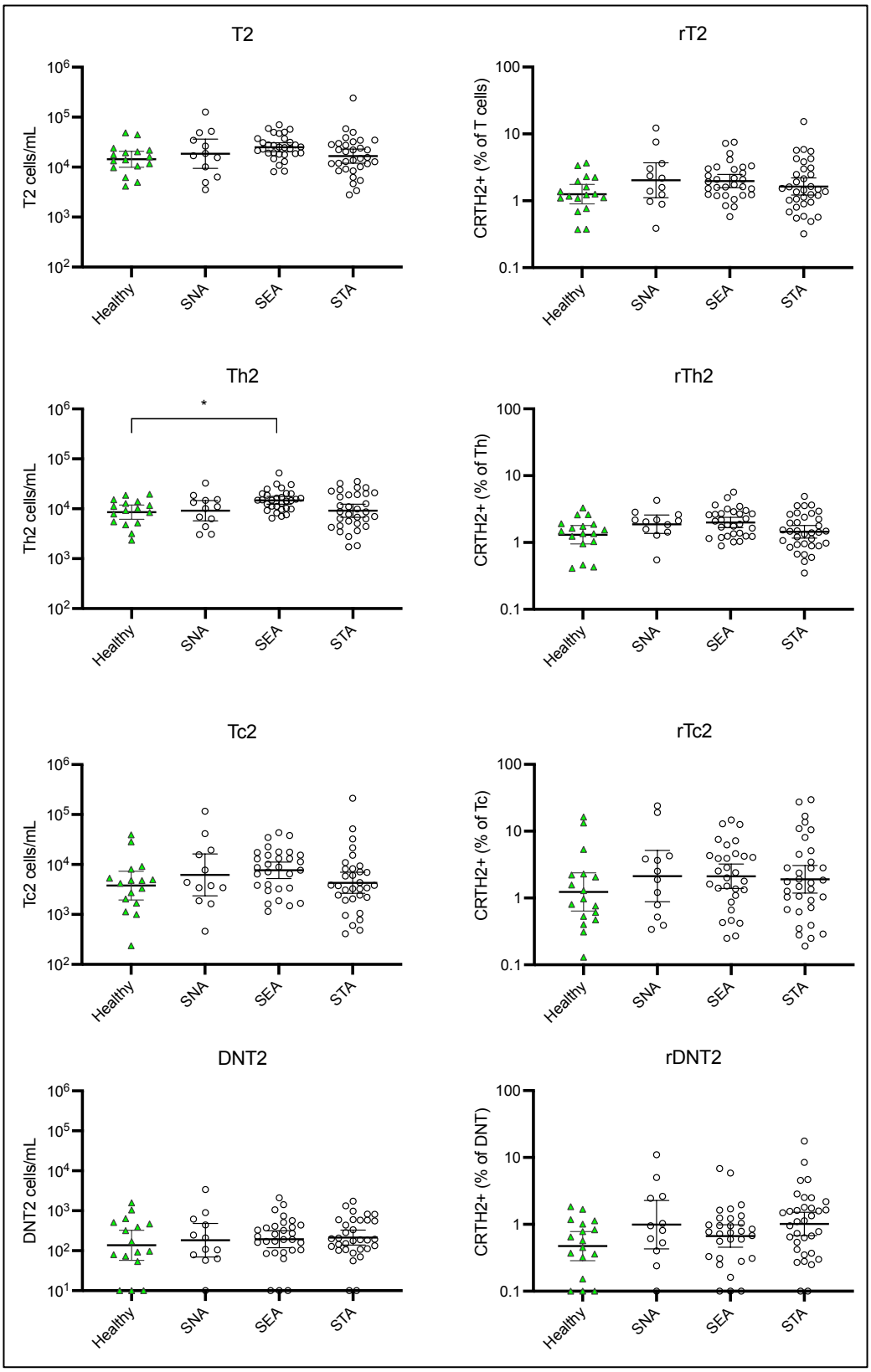


Figure 20 Endotype of severe asthma did not affect T2 cell counts

Log-transformed data were analysed by one-way ANOVA. Specific comparisons to healthy control were made by Dunnett's test. Bars show geometric mean and confidence interval. Sample sizes: healthy = 16, SNA = 12, SEA = 30, STA = 32.

2.3.9 Basophils were associated with blood CRTH2$^+$ T cells

Although no relationships were observed with eosinophils or neutrophils, CRTH2$^+$ T cell counts were also compared against numbers of other cell types in the blood. T2 cells and subsets did not associate with total leukocyte counts (Supplementary Figure 17). Absolute T2 counts correlated with the total number of lymphocytes ($r = 0.39$, $p < 0.0001$) (Figure 21); this was most true for Th2 cells ($r = 0.49$, $p < 0.0001$) and Tc2 to a lesser degree ($r = 0.22$, $p = 0.014$) and DNT2 ($r = 0.20$, $p = 0.030$) subsets. Relative CRTH2 expression was unrelated to lymphocyte count.

Basophils were associated with total T2 cells ($r = 0.32$, $p = 0.0003$) including all subsets: Th2 ($r = 0.30$, $p = 0.0008$), Tc2 ($r = 0.26$, $p = 0.0031$) and DNT2 ($r = 0.19$, $p = 0.031$) (Figure 22). There were also weak correlations with rTc2 ($r = 0.18$, $p = 0.040$) and rT2 ($r = 0.21$, $p = 0.017$.) However, the correlogram (Figure 8) shows that basophil number was also related to the overall lymphocyte count ($r = 0.27$, $p = 0.0019$) (Supplementary Figure 18). Using multiple linear regression, I tested the significant correlations reported in Figure 22, accounting for total lymphocytes. In this analysis, a significant correlation was still observed between basophils and T2 cells ($p = 0.0078$), Th2 ($p = 0.031$), and Tc2 ($p = 0.016$) subsets, but not DNT2 ($p = 0.10$).

Absolute T2 cell counts correlated with monocytes ($r = 0.31$, $p = 0.0007$), including the Th2 ($r = 0.35$, $p < 0.0001$) and Tc2 ($r = 0.19$, $p = 0.042$) subsets (Supplementary Figure 19). However, monocytes also correlated with overall lymphocyte counts ($r = 0.49$, $p < 0.0001$) (Supplementary Figure 18). Using multiple linear regression to account for the association between monocyte and lymphocyte counts, there were no longer associations between monocytes and T2 ($p = 0.14$), Th2 ($p = 0.17$), or Tc2 ($p = 0.34$) counts.

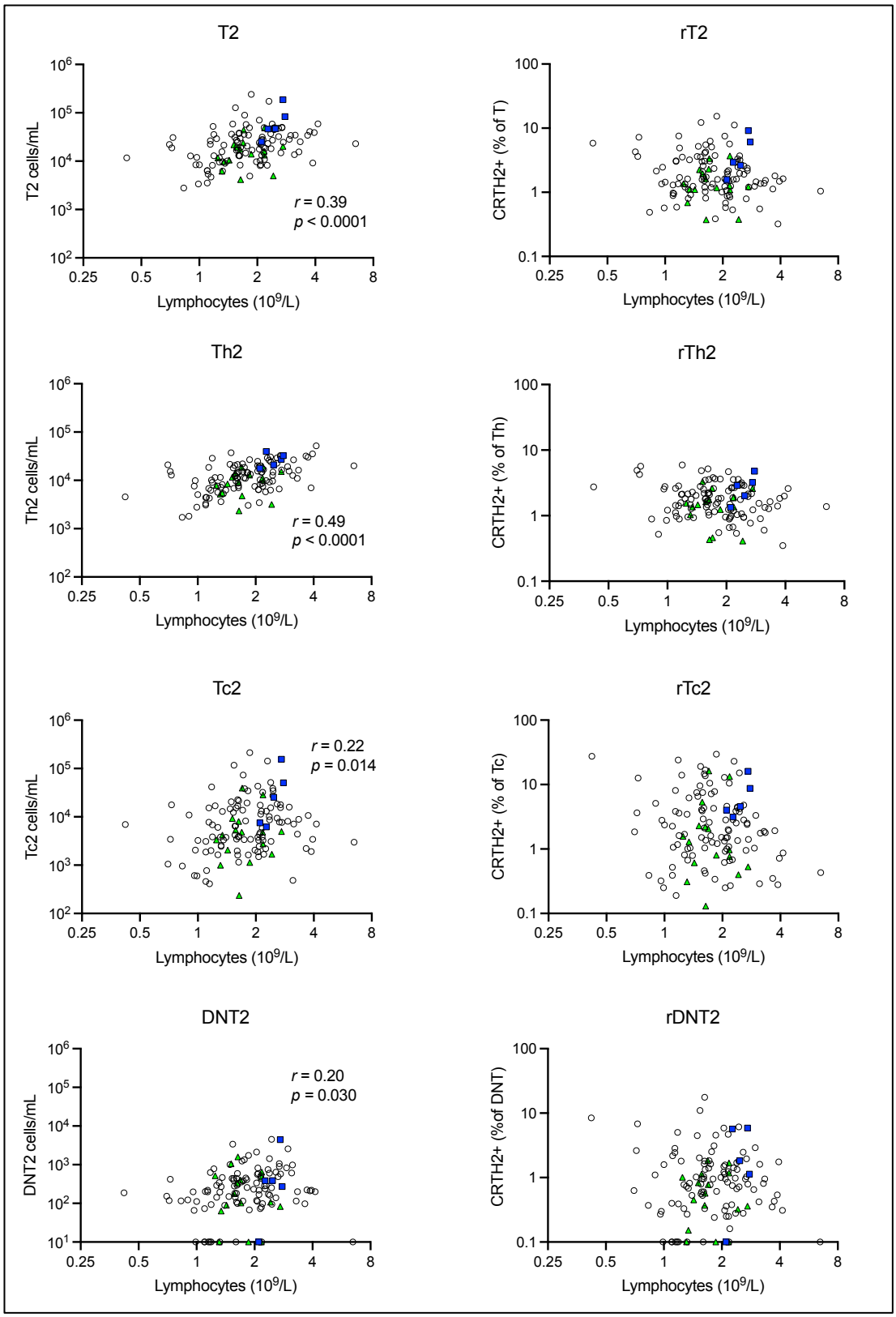


Figure 21 Absolute numbers of T2 cells are associated with total lymphocyte count

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. *N* = 121.

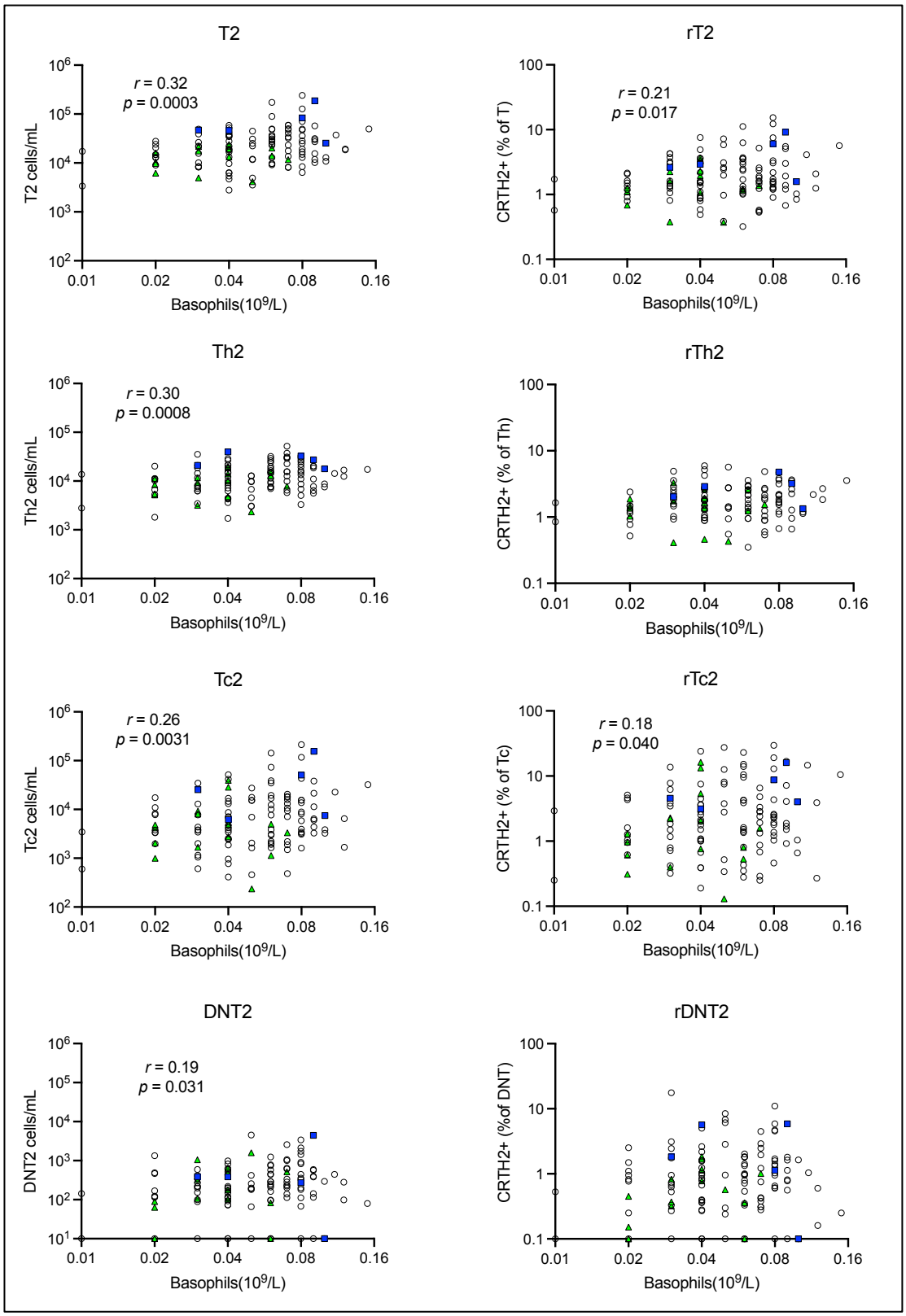


Figure 22 Basophil count was associated with T2 cell counts

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. *N* = 121.

Similarly, platelet counts were associated with T2 cells ($r = 0.24$, $p = 0.0003$), including all T2 subsets: Th2 ($r = 0.30$, $p = 0.0008$), Tc2 ($r = 0.26$, $p = 0.0031$), and DNT2 ($r = 0.19$, $p = 0.031$) (Supplementary Figure 20). There was also a weak correlation with rDNT2 ($r = 0.19$, $p = 0.043$). Again, platelets were linked to the overall number of lymphocytes ($r = 0.22$, $p = 0.017$) (Supplementary Figure 18). Accounting for this with a multiple linear regression model, there was no significant association between platelets and T2 cells ($p = 0.067$), including Th2 ($p = 0.14$), Tc2 ($p = 0.079$), or DNT2 ($p = 0.056$) subsets.

2.3.10 Treatments for asthma did not affect blood CRTH2$^+$ T cell counts

The mechanism of action for the most common asthma therapies, inhaled and systemic corticosteroids (oral corticosteroids (OCS), or injected), are complex and poorly understood (P. J. Barnes & Adcock, 2003). However, these drugs do exert widespread effects on immune responses. Therefore, I asked whether three different asthma therapies (ICS, systemic steroids, or mepolizumab) could influence the number of blood T2 cells.

In the cross-sectional dataset, some differences were observed between patients based on current treatment regimen (Supplementary Figure 21 to Supplementary Figure 23). However, it was unclear whether these differences were due to the treatment or another confounding variable. To control for these external variables, I collected longitudinal data across multiple patient visits, before and after therapeutic interventions.

In the longitudinal dataset, there was no change observed in T2 cells after treatment with ICS (Figure 23). To check for adherence and effect of medication I looked at FeNO, which is known to fall after treatment with ICS (Dweik et al., 2011). There was a reduction in FeNO with treatment ($MR = 0.5$, $CI = 0.26$ to 1.00, $p = 0.050$), indicating the treatment had the expected effect.

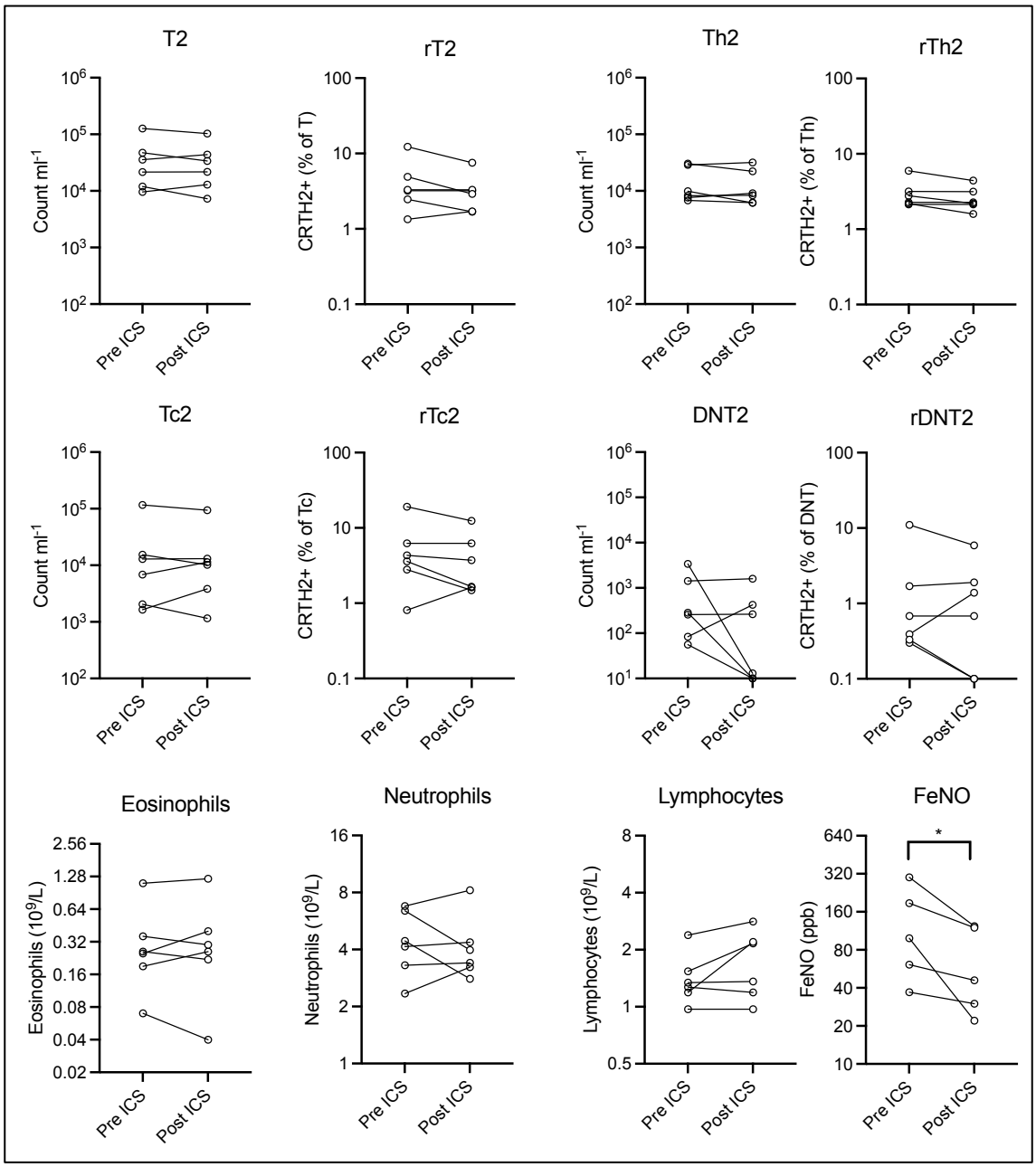


Figure 23 No change in T2 cell counts after treatment with ICS

Measurements before and after were compared by a two-tailed paired *t*-test. The time between pre and post measurements varied between one to eight weeks depending on patient visit dates. The medications used were Seretide (fluticasone/salmeterol) ($n = 3$) or beclomethasone ($n = 3$) at a dose decided by the clinician.

Similarly, no change was observed after treatment with systemic corticosteroids (Figure 24). To check for adherence and effect of medication I looked for a decrease in eosinophils ($MR = 0.27$, $CI = 0.09$ to 0.82, $p = 0.025$) and increase in neutrophils ($MR = 1.5$, $CI = 1.15$ to 1.95, $p = 0.0070$), known effects of these drugs (Stanbury & Graham, 1998).

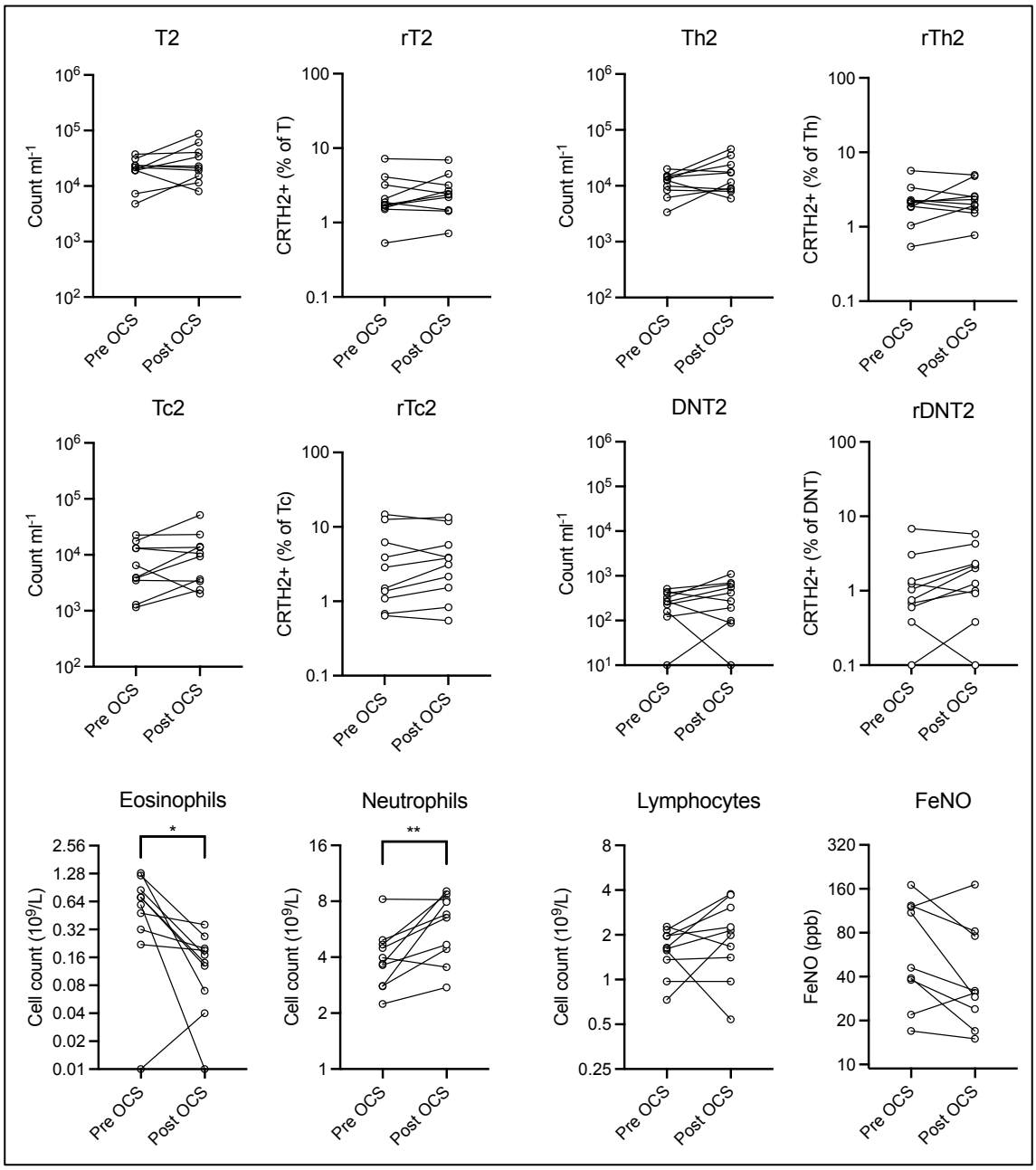


Figure 24 No change in T2 cell counts after treatment with systemic steroids

Measurements before and after were compared by a two-tailed paired *t*-test. The time between pre and post measurements ranged from one to four weeks depending on patient visit dates. The medications used were injected triamcinolone (n = 2) or oral prednisolone (n = 8) at a dose decided by the clinician.

Finally, there was no change observed after treatment with the anti-IL5 monoclonal antibody, mepolizumab (Figure 25). To confirm activity of the drug I confirmed a decrease in eosinophils (*MR* = 0.29, *CI* = 0.10 to 0.80, *p* = 0.021) in line with evidence from clinical trials (Pavord et al., 2012).

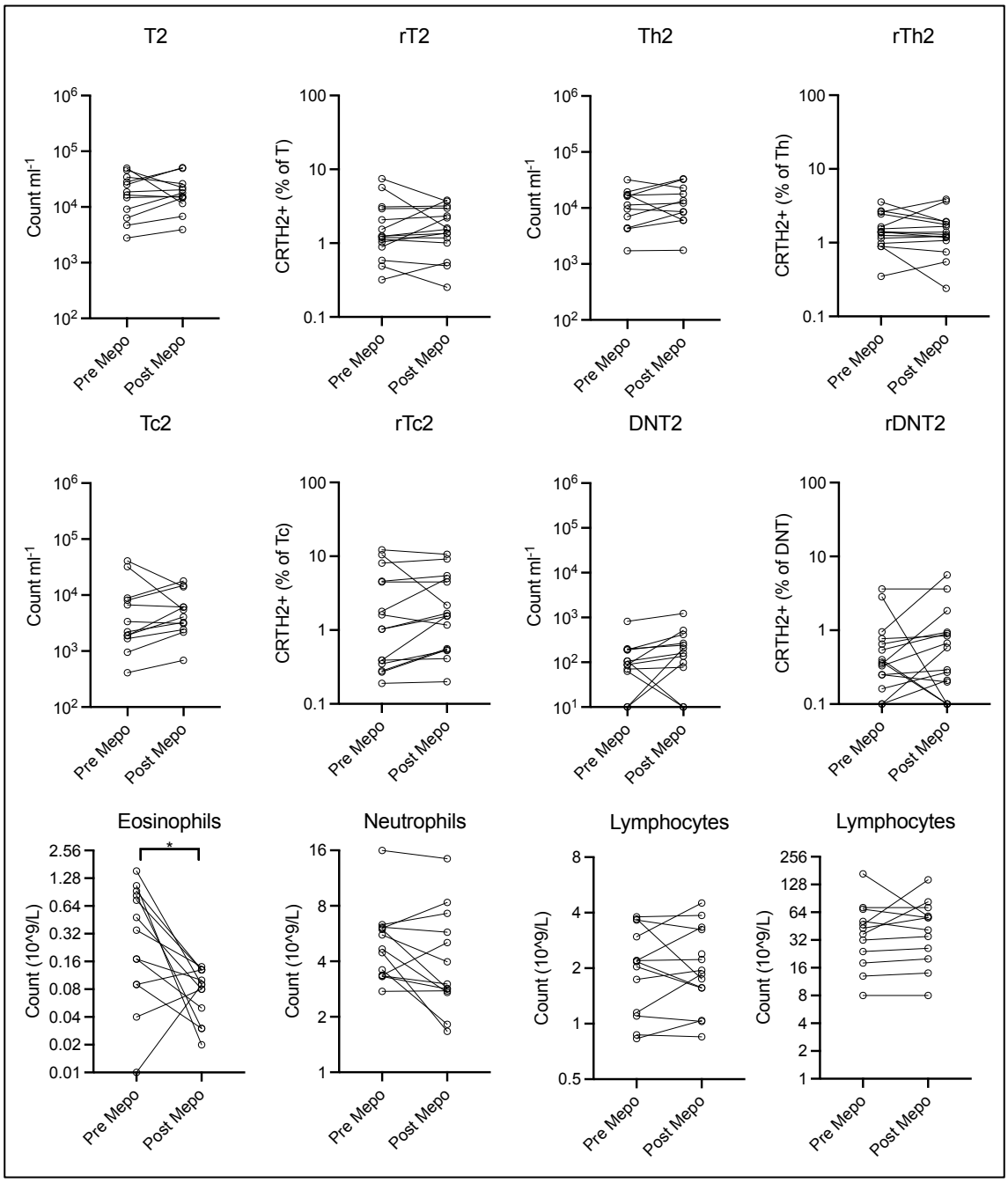


Figure 25 No change in T2 cell counts after treatment with mepolizumab

Measurements before and after were compared by a two-tailed paired t-test. The time between pre and post measurements was approximately four weeks. N = 16.

2.3.11 Sputum CRTH2+ T cells were associated with airway eosinophils and reduced in patients taking steroids

Across all of the patient visits I have studied (encompassing the cross-sectional, longitudinal, and other uncategorised visits), a small fraction have resulted in sputum

samples that were successfully analysed by flow cytometry. Nevertheless, it has been possible to identify some potential relationships involving sputum CRTH2$^+$ T cells. There were thirteen samples for which I was able to obtain acceptable sputum data, with a range of accompanying contextual depending on the patient visit. To look for patterns, I computed another correlogram (Supplementary Figure 24).

Expression of CRTH2 on sputum T cells overall was associated with BMI ($r = 0.65$, $p = 0.028$) and blood Th2 cells count ($r = 0.68$, $p = 0.001$) (Figure 26). Expression was lower in ever smokers ($MR = 0.18$ $CI = 0.039$ to 0.85, $p = 0.033$) and patients currently taking systemic steroids ($MR = 0.21$, $CI = 0.044$ to 0.98, $p = 0.047$).

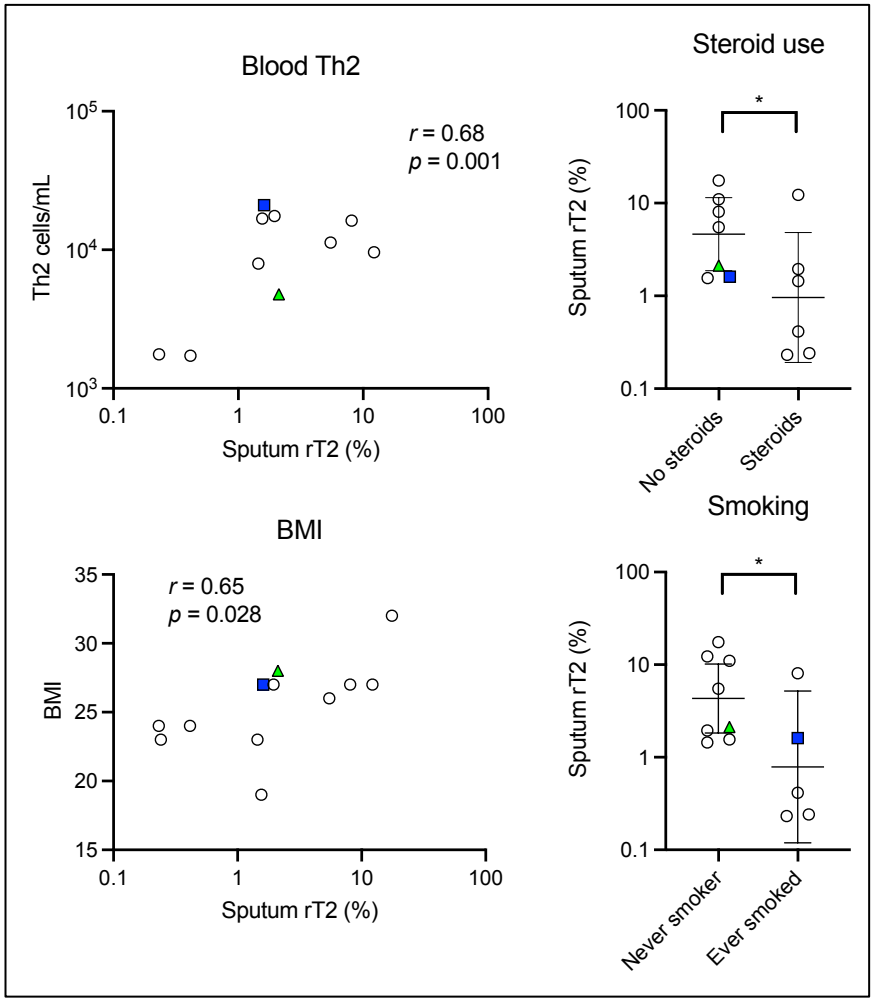


Figure 26 Sputum T2 cell proportion was associated with smoking and systemic steroids

Sputum rT2 and BMI were compared by Pearson correlation. Sputum rT2 and Blood Th2 were compared by first log-transforming the data and then by Pearson correlation. To assess the effect of steroids and smoking on sputum Tc2, data were log-transformed and compared by two-tailed *t*-test. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 10 to 13.

Expression of CRTH2 on sputum cytotoxic T cells was associated with BMI ($r = 0.81$, $p = 0.003$), and was lower in patients currently taking steroids (*MR = CI ratio* 0.017 to 0.57, $p = 0.014$) (Figure 27). Expression of CRTH2 on sputum Th cells was associated with: sputum eosinophils ($r_s = 0.77$, $p = 0.012$), sputum CRTH2 expression on Tc cells ($r = 0.63$, $p = 0.030$), blood Th2 cells in both absolute ($r = 0.73$, $p = 0.013$) and relative terms ($r = 0.61$, $p = 0.036$), and blood monocytes ($r = 0.75$, $p = 0.012$) (Figure 28).

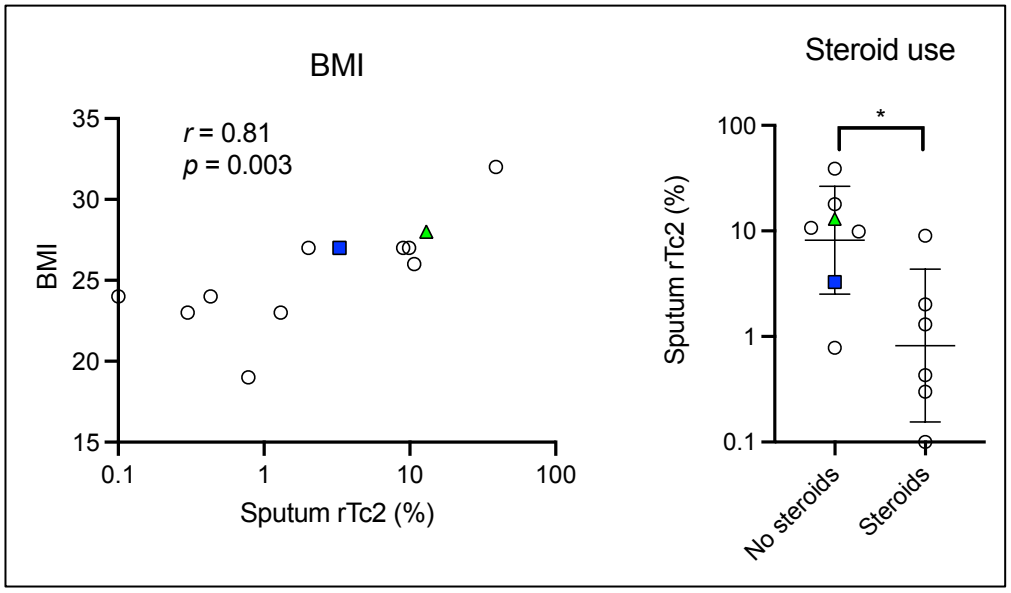


Figure 27 Sputum Tc2 proportion was associated with BMI and was lower in patients taking steroids

Sputum Tc2 and BMI were compared by Pearson correlation. To assess the effect of steroids on sputum Tc2, data were log-transformed and compared by two-tailed *t*-test. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 12 (BMI) or 13 (steroid use).

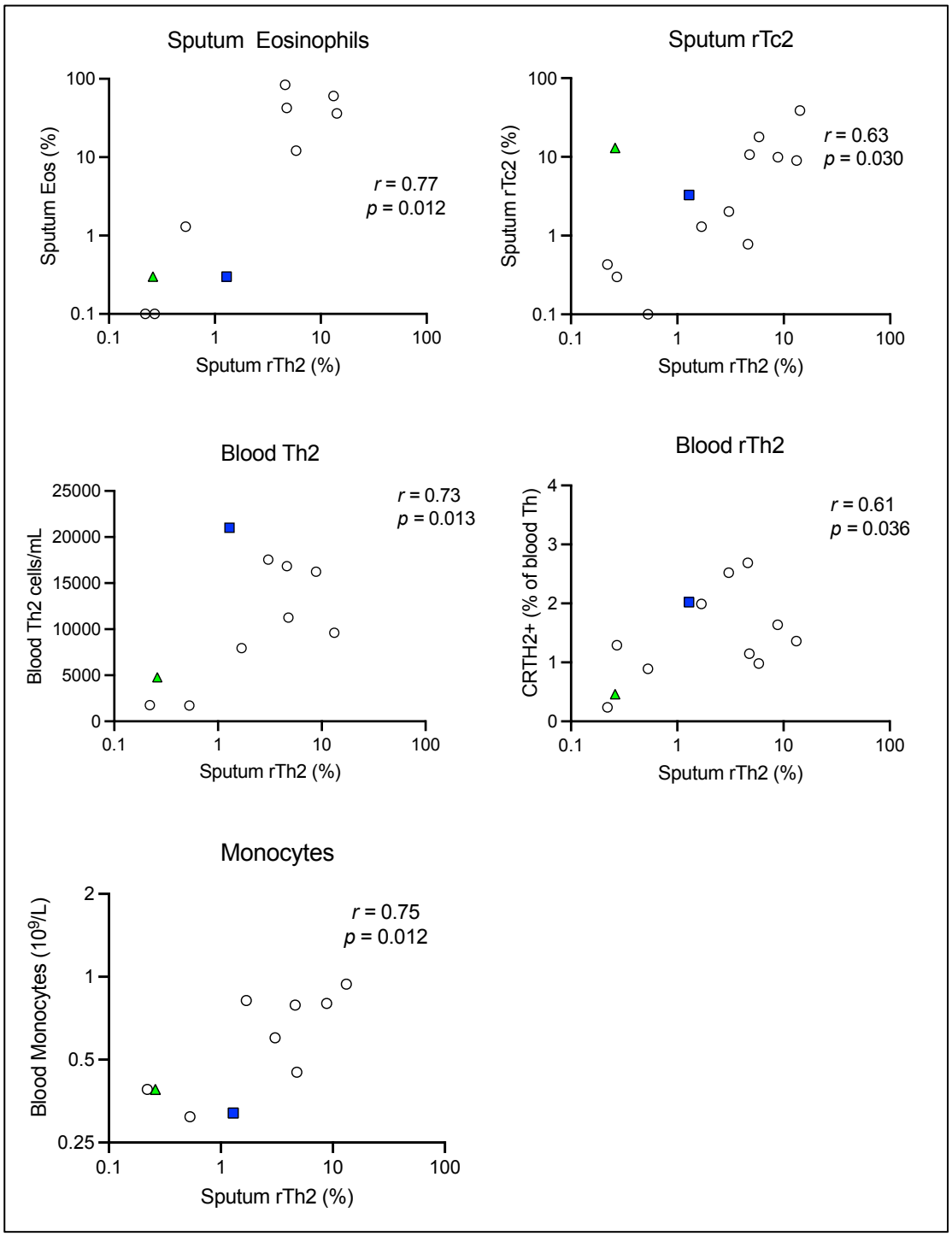


Figure 28 Sputum Th2 fraction associated with sputum eosinophils and other T2 cells

Sputum Th2 and sputum eosinophils were compared by Spearman correlation. For other variables, log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 10 to 13.

2.4 Discussion

2.4.1 Strengths and limitations of this dataset

This dataset is the largest (N = 127) to look specifically at blood CRTH2$^+$ T cell counts in patients with chronic airways disease and healthy controls. The minimal number of surface markers detected ensured consistent and accurate flow cytometry gating throughout (CRTH2 was the only marker on the red laser with essentially zero fluorescence overlap with other markers). The participants studied have been thoroughly phenotyped as part of the OAS study and this information has been included in the analysis. As a result, it has been possible to assess the reproducibility of previous claims in the literature, and to identify possible confounding variables to be considered in future studies. The downside of this amount of information is that a large number of statistical comparisons have been made without statistical correction. Many multiple comparison correction methods exist but were deliberately not included because I prioritised sensitivity (low type II error) rather than specificity (low type I error). Therefore, the results of this study can only be considered tentative pending subsequent confirmatory study.

To achieve specificity there are two possible options for future studies. First, specific variables of interest (e.g., smoking and Tc2 cell counts) could be tested alone in smaller follow-up studies, thus avoiding the multiple comparisons problem. These studies may also wish to match patients in groups by factors such as age and sex. Alternatively, a multivariate regression analysis could be conducted. However, this strategy must be approached carefully, and may only hint at underlying relationships rather than confirm them (McDonald, 2014). In this dataset, 42 variables were measured: the recommended sample size for just ten predictor variables is over one thousand participants, and my the sample size in this dataset is just below even the threshold recommended for a multiple

regression analysis with two predictor variables (Maxwell, 2000). Therefore, a multiple regression strategy is unlikely to be realistic except by the very largest clinical studies, although it may be appropriate in a follow-up study of a small number of variables.

2.4.2 Links between circulating CRTH2$^+$ T cell counts and asthma severity, endotype, or therapy, were not reproduced

The key observation of this study is that circulatory CRTH2$^+$ T cell counts were not related to asthma or any of its subclassifications. These results support the null findings of the original study by Mutalithas *et al* of CRTH2$^+$ T cells in asthma (Mutalithas et al., 2010), which found no difference in the proportion of blood CRTH2$^+$ T cells in asthma.

This dataset did not replicate observations from Palikhe *et al* linking the proportion of CRTH2$^+$ Th cells to severe asthma (N. S. Palikhe et al., 2016). This previous study compared cohorts of MMA and SA patients, well-matched in terms of age, sex, and smoking history. Both of our studies show clear gating of the CRTH2$^+$ population, so it is not obvious to me why this association was not reproduced. One difference between the studies is that the prior one quantified CRTH2$^+$ Th cells as a proportion of the total leukocyte count. It would be therefore be possible that observed changes in CRTH2$^+$ Th cells as a proportion of total leukocytes were influenced by a change in overall leukocyte numbers, rather than expansion of the CRTH2$^+$ T cells. However, in my data leukocyte counts were not reduced in severe asthma, so this is unlikely to have been responsible for their observed increase in CRTH2$^+$ Th cell proportion. The authors also observed a relationship between proportion of CRTH2$^+$ Th cells and lung function reduction and asthma exacerbations in the past year. Neither of these associations were reproduced in my dataset. Instead, my data reproduces the null findings of Hilvering *et al* (Hilvering et al., 2018), who found no difference in absolute or relative CRTH2$^+$ Th cells between health

and severe eosinophilic or non-eosinophilic asthma, and Wiest *et al* (Wiest et al., 2018), who observed no increase in the proportion of central memory CRTH2$^+$ Th cells in severe asthma vs non-asthmatic controls. Since blood CRTH2$^+$ T cell counts were not associated with lung function decline, asthma symptoms, or number of recent exacerbations, and were unaffected by therapy, it is unlikely that they are related closely to severity of asthma.

A second recent study from the same authors reported that an elevated proportion of Th cells express CRTH2 during asthma exacerbation (Palikhe et al., 2021). This study included a cross sectional analysis comparing exacerbating patients presenting at an emergency department with stable patients with asthma, and found that the proportion of CRTH2 expressing Th cells was higher. The exacerbation group contained one-third current smokers and the control group was entirely non-smokers, but the authors state in the discussion that similar results were observed when smokers were excluded. The authors suggest that CRTH2$^+$ Th cells might be a predictive marker for exacerbation. Unfortunately, my dataset cannot add support to this idea. Currently, variables known to predict asthma exacerbation include: disease severity by definition (Jean Bousquet et al., 2010) and symptom scores such as ACQ (Meltzer et al., 2011); previous exacerbation (Al-Ani et al., 2013); blood (Price et al., 2015) and sputum (Green et al., 2002) eosinophil counts; and FeNO (Busse et al., 2021). Asthma exacerbation is also related to lung function decline (T. R. Bai et al., 2007). In my dataset, none of these variables were significantly associated with relative or absolute CRTH2$^+$ T cell counts. If blood CRTH2$^+$ T cell counts can really predict asthma exacerbations, it is unclear how they could be unrelated to other variables that are established predictors of exacerbation.

The same study also includes longitudinal analysis of ten patients who were given a five day course of OCS, and then sampled ten days after treatment end. The proportion of

CRTH2$^+$ Th cells fell after steroid treatment whilst the blood eosinophil count remained unchanged. This finding is supported by a recent study from our group in which steroid treatment reduced absolute Th2 and Tc2 cell counts (J. Luo et al., 2019). In that study, patients were sampled immediately before and after a period of 7-33 days of treatment with OCS. My cross sectional data supports both these claims, as steroid use was associated with lower absolute T2 counts and the proportion of CRTH2$^+$ Th cells.

My longitudinal data does not reproduce the effects observed in either study. This is despite similar time intervals between sampling in my data of 7-23 days, and the same number of donors (n = 10) in each study. Two of the three datasets (including mine) show a fall in eosinophils and rise in neutrophils, confirming steroid activity (Shoenfeld et al., 1981). One of the differences in our data is that I have included both oral and injected systemic steroids (as opposed to prednisone only), but both drugs are glucocorticoids with very similar structures. The main difference is perhaps that adherence with injected triamcinolone is guaranteed (Schuckman et al., 1998). It has also been suggested to me that the effect only occurs in steroid-naïve patients, and I cannot prove that the patients I observed were taking their first ever course of systemic steroids. At least one of the previous studies focussed on steroid-naïve patients (J. Luo et al., 2019).. However, if steroids only reduce circulating type 2 T cell counts on their first use, and yet subsequent courses are still effective, then it is unclear how CRTH2$^+$ T cell depletion can be an important part of their mechanism. In my dataset, longitudinal study of patients taking ICS or the anti-IL5 agent mepolizumab, also showed no effect on circulating T2 cell counts. This is unsurprising given that ICS targets the lung and mepolizumab acts downstream of type 2 lymphocytes. The only conclusion I can draw is that if any of these therapies reduce

$CRTH2^+$ T cell counts, then the effect is not reliable, and is unlikely to be responsible for the robust clinical benefits observed.

The importance of longitudinal study in general is highlighted by some of the differences between cross sectional and pre/post findings in this data. For example, mepolizumab use was associated with neutrophilia in the cross sectional data, but the paired analysis shows that mepolizumab does not cause neutrophilia. Therefore, some third variable is probably driving this. Patients with mixed granulocytic inflammation may have worse symptoms and disease, and be more likely to be prescribed mepolizumab for their eosinophilia. Alternatively, patients taking mepolizumab may also be prescribed steroids, which increases neutrophil counts (Shoenfeld et al., 1981) (Figure 24).

Finally, this data also does not reproduce the reported link by Hilvering *et al* between Tc2 and severe eosinophilic asthma (Hilvering et al., 2018). In that study, the healthy control group was younger (mean of 36.6 years in health, 53.7 years in SEA) and had significantly fewer male participants (15% male in health, 50% in SEA), and the smoking history is not listed. My dataset suggests these variables may have been responsible for the elevated Tc2 counts in the SEA group compared to healthy controls. It is, however, notable that at the time of their study mepolizumab was not being regularly prescribed, whereas it is now an adjunct option for severe eosinophilic asthma. Therefore, some of the most severe patients may now be taking eosinophil-reducing biological therapy, and the groups may therefore no longer be equivalent between our studies. Given that blood $CRTH2^+$ T cell counts did not associate with blood or airway eosinophils, or FeNO, it is unlikely that they are elevated in eosinophilic disease.

2.4.3 A link between CRTH2$^+$ Th cells and allergy was reproduced

A small increase in the proportion CRTH2$^+$ blood Th cells was observed in the allergic asthma group, and it is encouraging that this agrees with two previous studies (Boonpiyathad et al., 2020; Campos Alberto et al., 2012). This may be a general property of allergy and is not necessarily a feature of allergic asthma. There were also no associations observed with IgE, which should be an indicator of allergic asthma (Gina Science Committee, 2021), in agreement with previous data (Mutalithas et al., 2010). Furthermore, there was no increase in absolute numbers of CRTH2$^+$ Th cells, so it is unclear whether this observation is of any biological consequence. This finding was not reproduced in a study by Wiest *et al* (Wiest et al., 2018) who looked at CRTH2$^+$ CD45RO$^+$ memory Th cells. Their cohort included 22 controls and 43 patients with allergic asthma, a comparable sample size to this dataset. Therefore, it seems that the link between allergic asthma and CRTH2$^+$ expression on circulating T cells still needs further investigation.

2.4.4 Links between Tc2 cells and smoking were reproduced, and CRTH2$^+$ T cells were associated with COPD and related factors

Blood T2 cell counts were associated with a range of variables summarised in Figure 29. Both Th2 and Tc2 were associated with smoking. Smoking has previously been linked to CRTH2 mRNA in blood (N. S. Palikhe et al., 2016) and circulating IL-13$^+$ CD8$^+$ T cells (Hilvering et al., 2018). Smoking has now been linked to CD8$^+$ T cell responses in general, and circulating Tc2 counts, in multiple studies. There are several possible explanations for this, including direct activation of CD8$^+$ T cells, or indirect activation through APCs or as a result of background infection and/or inflammation. Current smoking, and COPD, were slightly negatively linked to FeNO, implying a lower activity of type 2 inflammation at the airways, despite evidence of more T2 cells in the circulation. This was further supported by a lower CRTH2$^+$ T cell proportion in the airways of smokers. It is known that smoking has

widespread effects on the epigenome (Zong et al., 2019), so it may be worthwhile to study methylation patterns or chromatin accessibility on $CD8^+$ T cells, especially in regions related to activation, expansion or type 2 differentiation. It is possible that two concurrent effects happen in smoking: one in the lung that suppresses type 2 inflammation, and another in the vasculature that promotes it. Another possibility is that airway recruitment is impaired by smoking, which could also contribute to compromised airway defence.

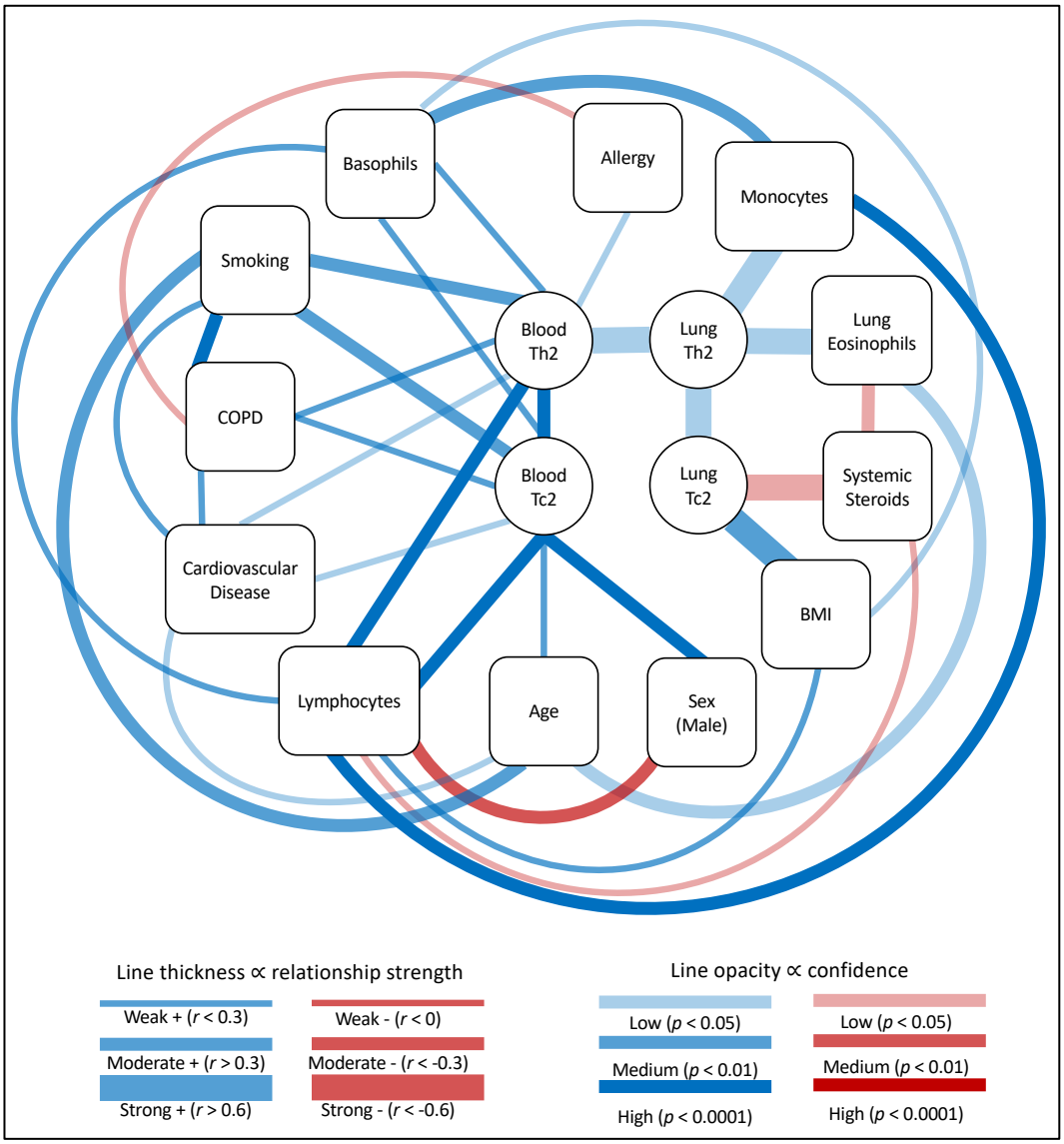


Figure 29 Network diagram of relationships observed involving T2 cells in this study

T2-related nodes are shown as circles, other variables as rectangles. Made in Microsoft PowerPoint. Abbreviations: CV, cardiovascular; COPD, chronic obstructive pulmonary disease; BMI, body mass index.

It is unsurprising that CRTH2$^+$ T cells were also linked to COPD and heart disease in this dataset, which are correlated with each other and with smoking (Rabe et al., 2018). Tc2 were higher in males and increased slightly with age: these are two of the primary predictors for cardiovascular disease (Pencina et al., 2009) and COPD is more common in older men (Mannino & Buist, 2007). Male sex is also a risk factor for adult-onset eosinophilic asthma (De Groot et al., 2016). Together, these observations suggest a link between smoking, associated diseases and risk factors, and circulating CRTH2$^+$ T cells. Specific follow up study would be needed to confirm the reproducibility of these observations. If true, this may stand in contrast to existing literature which argues that the Th1/Th2 balance shifts towards Th1 in the context of COPD (Majori et al., 1999) and cardiovascular diseases such as myocardial infarction (Engelbertsen et al., 2013), chronic heart failure (Cai et al., 2016), and coronary artery disease (Madhumitha et al., 2014) However, it may be important that these studies tend to focus on IL-4 production by Th cells, to the exclusion of Tc cells and other markers such as IL-5, IL-13, and CRTH2, which may be part of the complete picture. Indeed, in the context of damage to the cardiovascular system or blood vessels, such as that induced by smoking, it is plausible that a type 2 response could be induced, given its role in tissue repair. Finally, COPD is a heterogeneous disease and my COPD cohort was too small to dissect sub-groups. Therefore, it might be worth studying Tc2 cells in defined COPD phenotypes.

A previous study from Mansfield *et al* of the blood of four hundred participants found that CRTH2$^+$ Th cells were associated with age (Mansfield et al., 2012). This observation did not occur in my smaller dataset, and I instead observed a small increase in CRTH2 expression on Tc cells. However, the Mansfield cohort was very different to the patients in this study, because severe respiratory or cardiac disease were excluded from their study,

whereas the cohort presented here mostly have severe respiratory disease. Furthermore, the association of Tc2 and age disappeared when smokers and patients with heart disease were excluded. Therefore, it is possible that our findings are compatible, and that CRTH2 expression on Th cells increases with normal aging, but CRTH2 expression on Tc cells could be associated with advanced disease states.

2.4.5 A plausible link between blood CRTH2$^+$ T cells and basophils

The link observed between circulating T2 cells and basophils is consistent with basophil involvement in type 2 inflammation (Siracusa et al., 2013). The role of basophilic inflammation in asthma has received less attention than eosinophilic inflammation, despite their enrichment in biopsy (Macfarlane et al., 2000), sputum (Gauvreau et al., 2000), and whole lung (Kepley et al., 2001) specimens. However, the accuracy of basophil counts by automated haemocytometers has been called into question (Amundsen et al., 2012), so any further work on this link may wish to confirm basophil counts by flow cytometry. Future studies could address whether airway T2 cells relate to airway basophils, and whether the blood and airway compartments are related. There is a body of evidence that, as well as being APCs, basophils can produce IL-4 to differentiate Th2 cells (Karasuyama et al., 2018). It is therefore plausible that increased basophils drives increased differentiation of T cells towards a type 2 phenotype.

2.4.6 Low sputum acquisition success rate impaired the study of airway CRTH2$^+$ T cells

In the airways, strong relationships were observed between sputum CRTH2$^+$ T cells and several variables (Figure 29), but with low confidence due to low sample numbers. Th2 cells in sputum closely correlated with eosinophils. This observation is in line with the established consensus that type 2 immune responses underpin eosinophilic airway

inflammation in asthma (Lambrecht & Hammad, 2015). However, to my knowledge this is the first report of a direct relationship between airway eosinophils and $CRTH2^+$ T cells.

Sputum Th2 cells were also associated with circulating monocytes. The role of monocytes in asthma and type 2 inflammation has not been extensively explored. However, a study of lung tissue samples from children who died of asthma found that airway eosinophils and monocytes were enriched, and correlated with each other (Eguíluz-Gracia et al., 2018). Other studies suggest a role for monocytes in type 2 induction: for example, monocytes were crucial for induction of Th2 responses to diesel fumes in mice (Provoost et al., 2012). A link between monocytes and type 2 airway inflammation is possible, but would need further study. Sputum Tc2 cells were associated with BMI. The relationship between BMI and asthma is well established, with 'obese asthma' considered by some as a distinct syndrome (U. Peters et al., 2018). Obese asthmatics may be resistant to inhaled (Boulet & Franssen, 2007) or systemic steroids (E. R. Sutherland et al., 2008), and even to mepolizumab (Harvey et al., 2020). It is therefore interesting that in my dataset, steroid use was associated with reduced airway Tc2. The effect of asthma therapies on airway T2 cells could be addressed in a larger, longitudinal study.

Lack of airways data has been a limitation of this work. From 127 patient visits, sputum slides for microscopy were successful on 53 occasions, approximately 40% of the time. Only a fraction of these samples were suitable for further analysis due to low cell numbers and/or viability. Sputum is challenging to analyse by flow cytometry due to high levels of debris, cell death, and autofluorescence, and CRTH2 is a lowly expressed protein. The end result is that I could not fully explore the relationship between airway Th2 and Tc2 cells, and the contextual variables discussed throughout this chapter.

2.5 Conclusion

This chapter investigated the relationships between CRTH2$^+$ T cells and a range of factors that can be involved in chronic respiratory illness. The primary aim was to determine the significance of CRTH2 expression on circulating T cells in asthma. The primary conclusion of this chapter is that the data does not reproduce previously reported links between CRTH2$^+$ Th cells and severe asthma, lung function reduction, or asthma exacerbation. Neither does this data reproduce the association between blood CRTH2$^+$ Tc cells and severe eosinophilic asthma. It remains plausible that these cells contribute to asthma through their actions, rather than their numbers, or carry out a role at the tissue site.

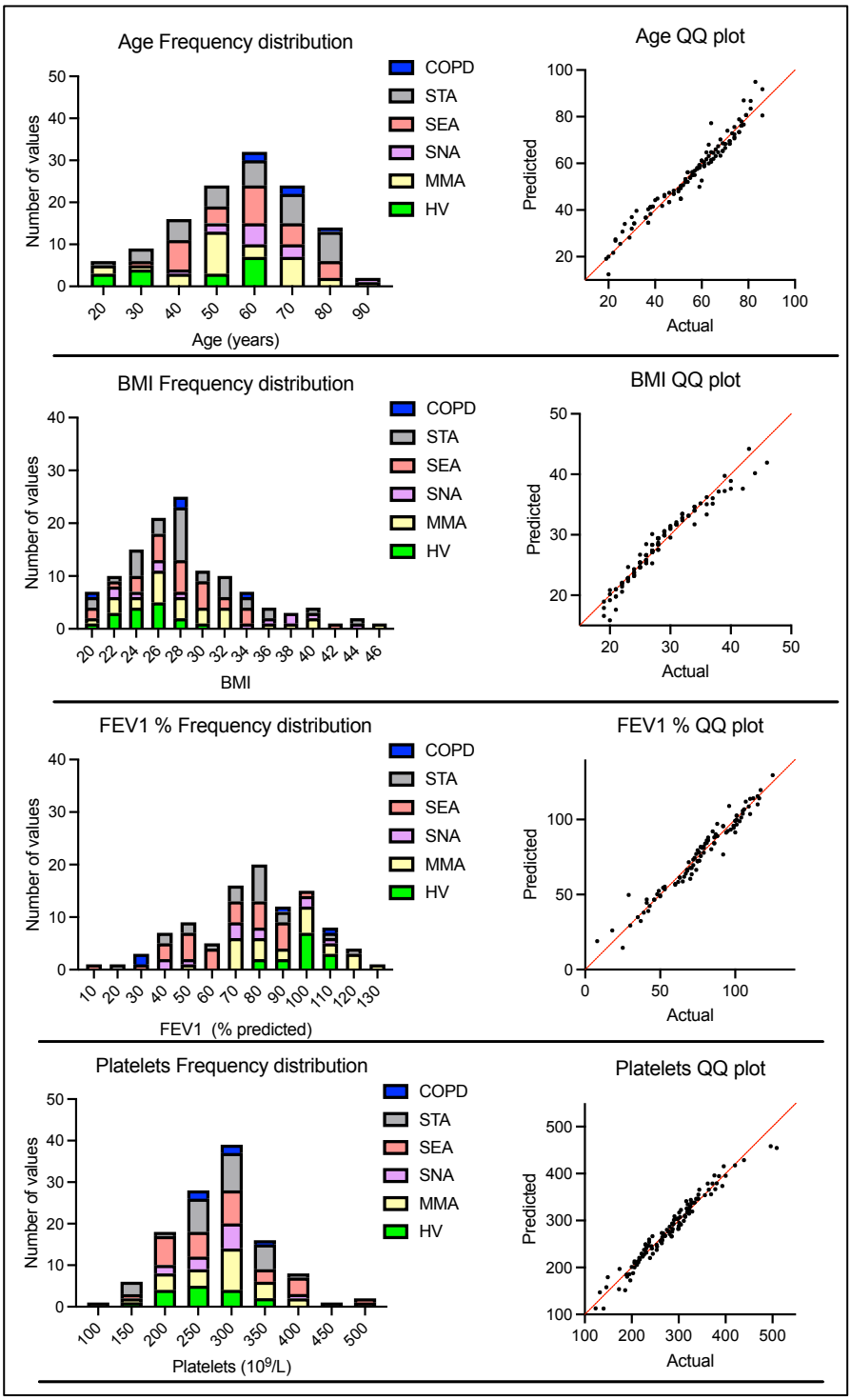
However, blood CRTH2$^+$ T cell counts may be related to other processes including cardiovascular disease and COPD. An association between Tc2 cells and smoking has been reproduced. Researchers wish to study this biomarker in more detail, to explore these relationships fully, and to assess clinical utility.

The relative expression of CRTH2 on airway T cells is related to eosinophilic inflammation of the airways. Future studies of airway CRTH2$^+$ T cell dynamics in patients with asthma are merited. However, this is contingent on reliable airway sampling for large patient cohorts.

In all contexts, CRTH2 expression and cell count were highly variable between individuals, the data spreads tend to span multiple logs. If any differences between health and asthma do exist they are likely to be small and unpredictable. Considering these two statements together, I think it is unlikely that counting CRTH2$^+$ T cells in the blood has clinical utility in asthma. However, their activation state, or numbers and activity in the lungs, may be of importance.

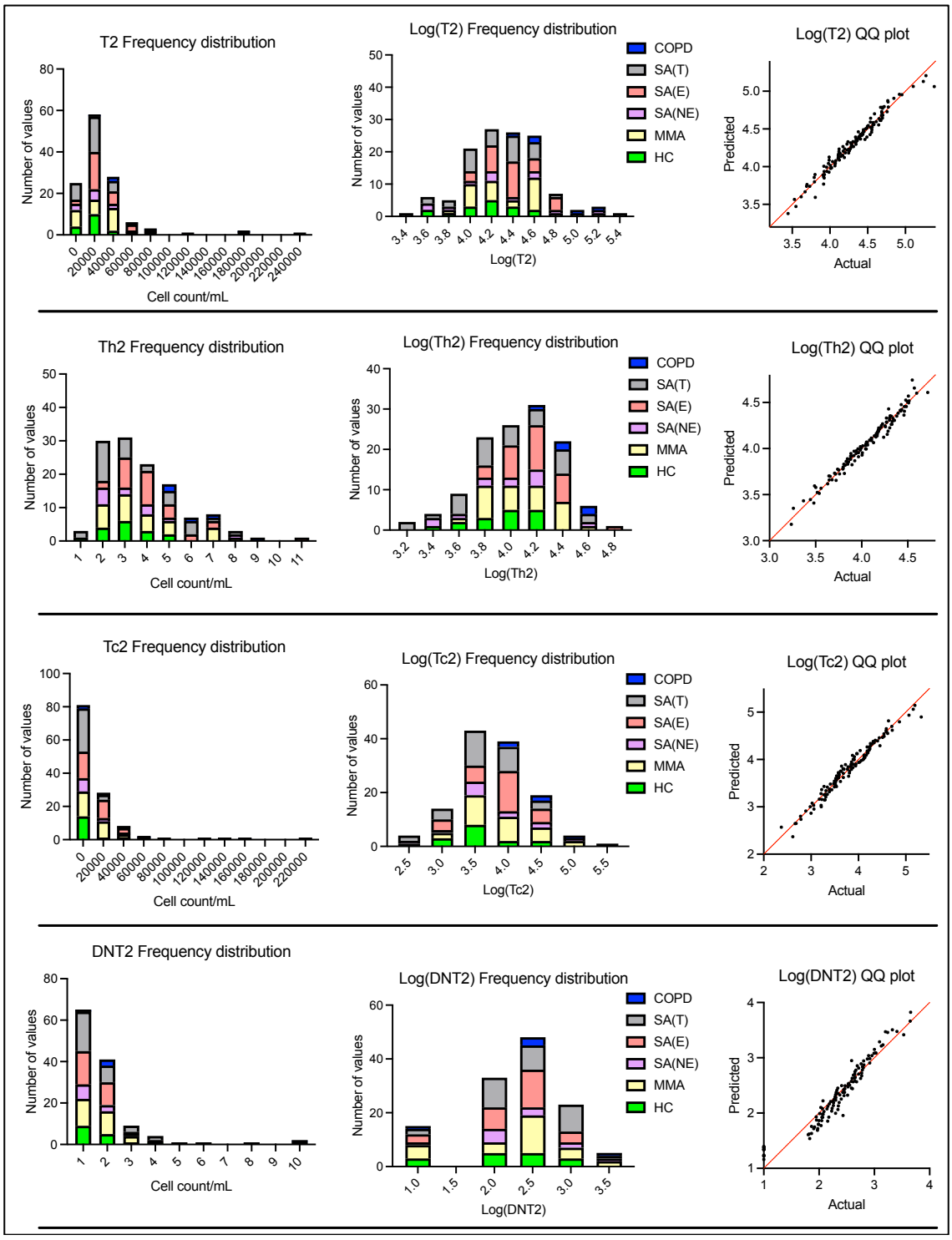
2.6 Appendix

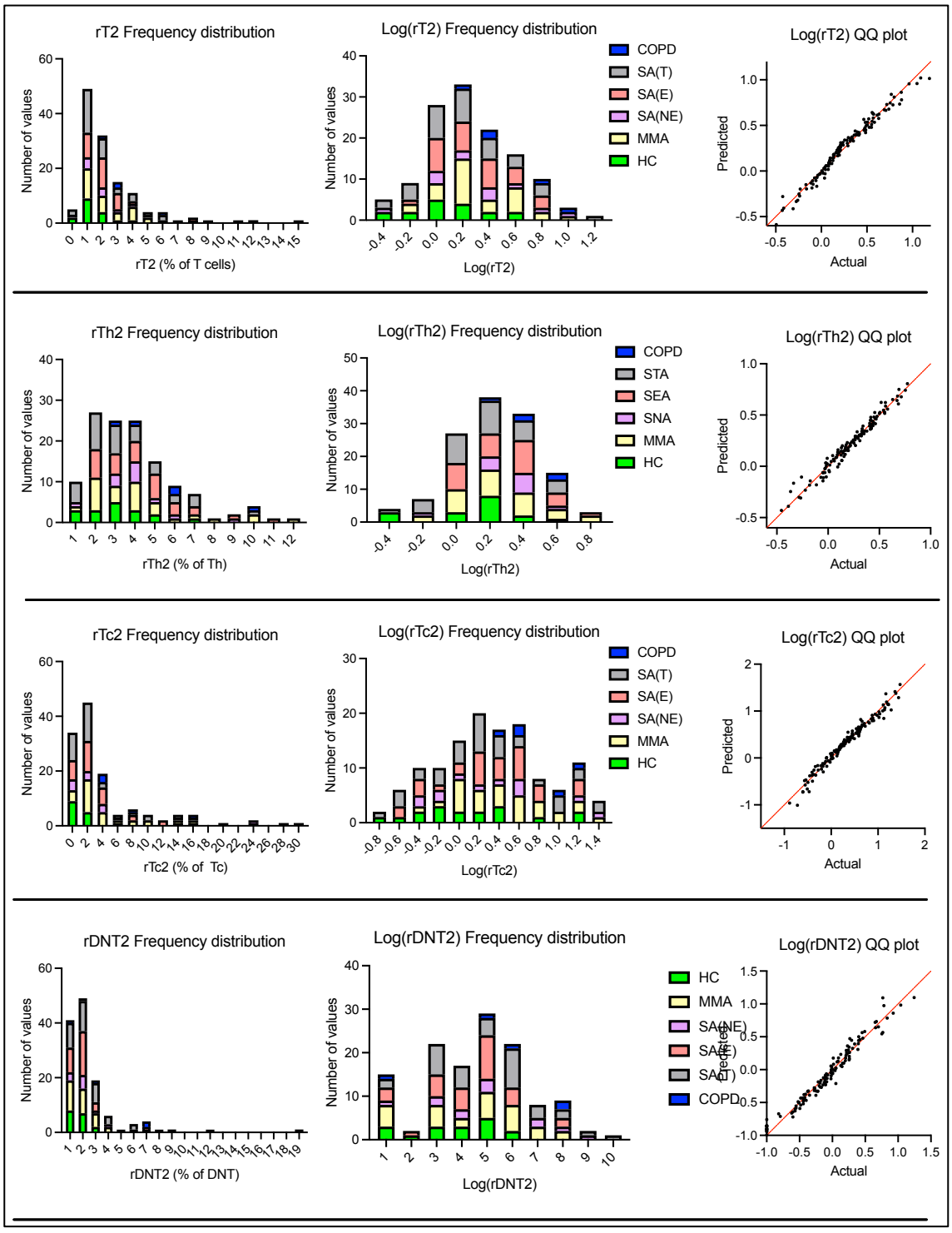
2.6.1 Distributions of data

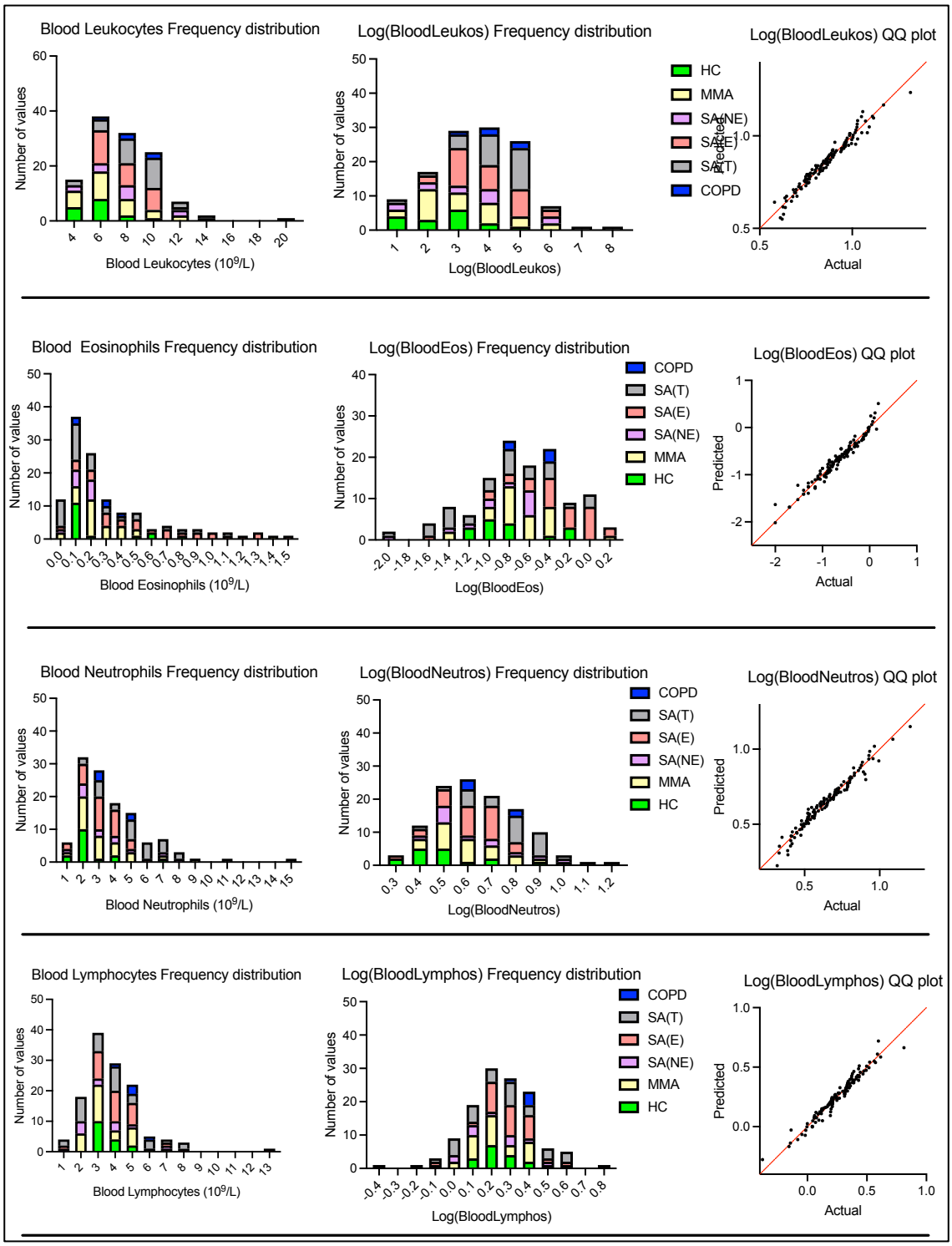


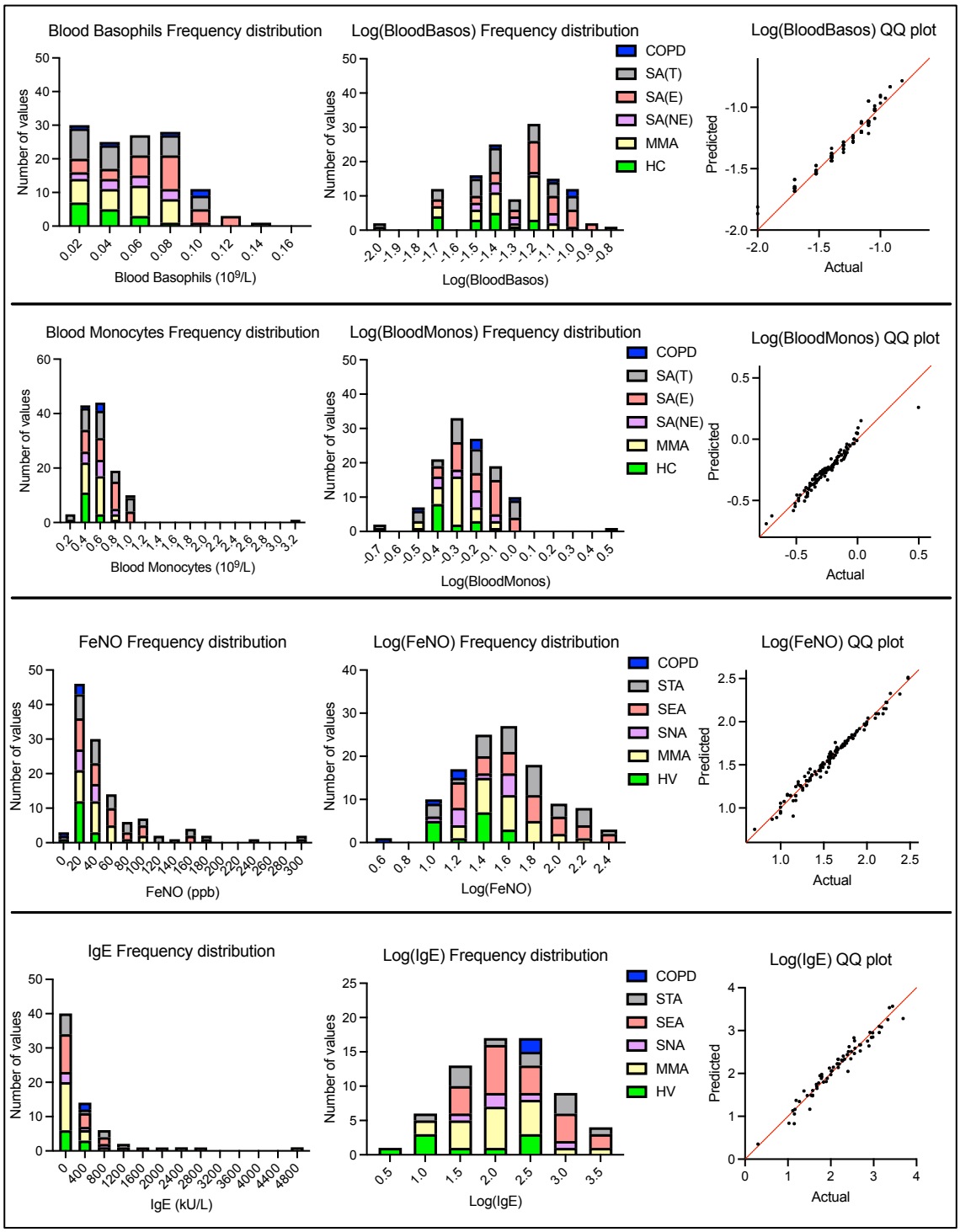
Supplementary Figure 1 Histograms and QQ-plots for normally distributed variables.

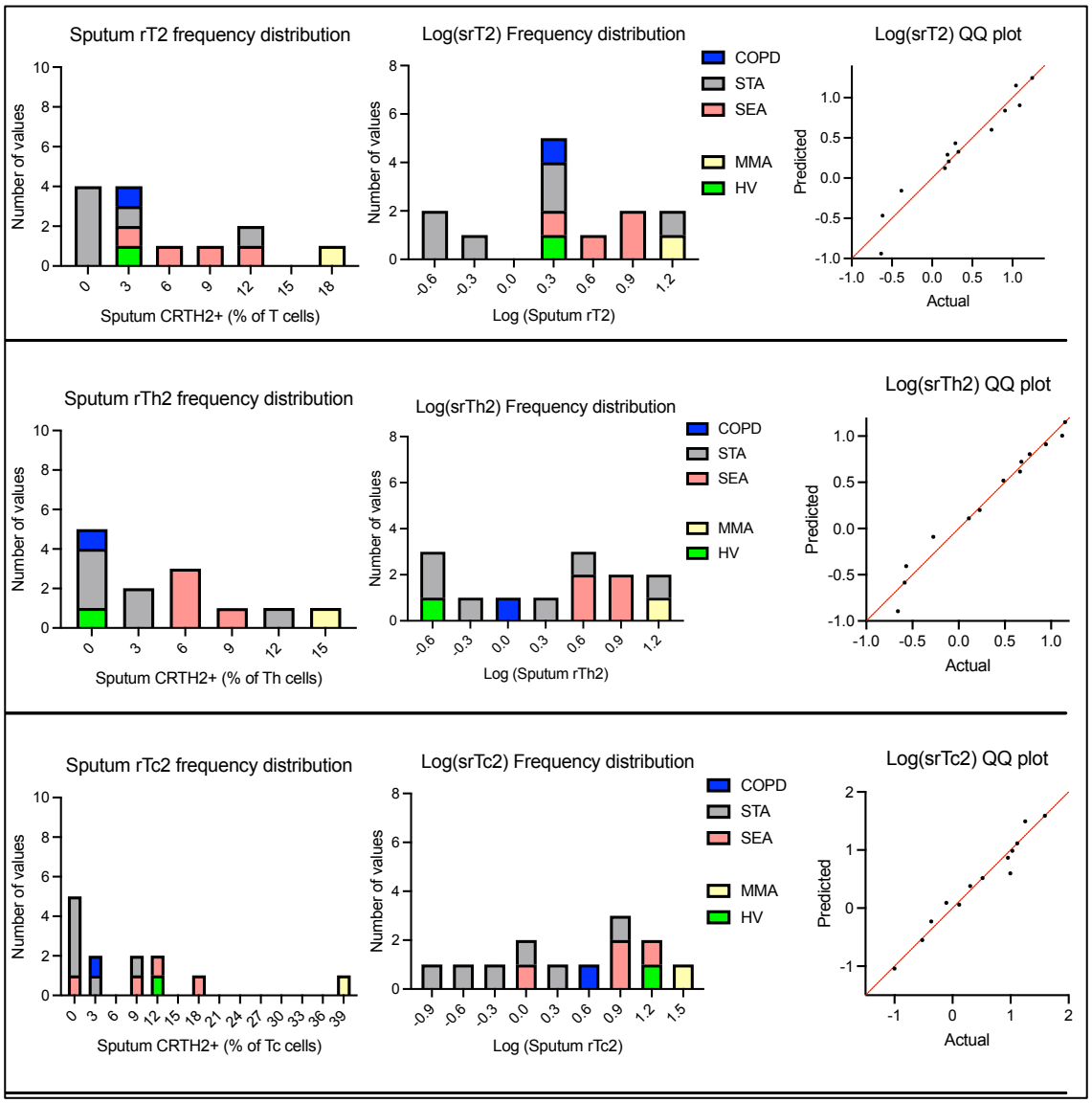
All QQ-plots compare to theoretical normally distributed data. Patient subcategories are shown by colour coding.


T2 Frequency distribution
Number of values
Cell count/mL
Log(T2) Frequency distribution
Log(T2)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(T2) QQ plot
Predicted
Actual
Th2 Frequency distribution
Log(Th2) Frequency distribution
Log(Th2)
Log(Th2) QQ plot
Tc2 Frequency distribution
Log(Tc2) Frequency distribution
Log(Tc2)
Log(Tc2) QQ plot
DNT2 Frequency distribution
Log(DNT2) Frequency distribution
Log(DNT2)
Log(DNT2) QQ plot


rT2 Frequency distribution
Number of values
rT2 (% of T cells)
Log(rT2) Frequency distribution
Log(rT2)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(rT2) QQ plot
Predicted
Actual
rTh2 Frequency distribution
rTh2 (% of Th)
Log(rTh2) Frequency distribution
Log(rTh2)
COPD
STA
SEA
SNA
MMA
HC
Log(rTh2) QQ plot
rTc2 Frequency distribution
rTc2 (% of Tc)
Log(rTc2) Frequency distribution
Log(rTc2)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(rTc2) QQ plot
rDNT2 Frequency distribution
rDNT2 (% of DNT)
Log(rDNT2) Frequency distribution
Log(rDNT2)
HC
MMA
SA(NE)
SA(E)
SA(T)
COPD
Log(rDNT2) QQ plot

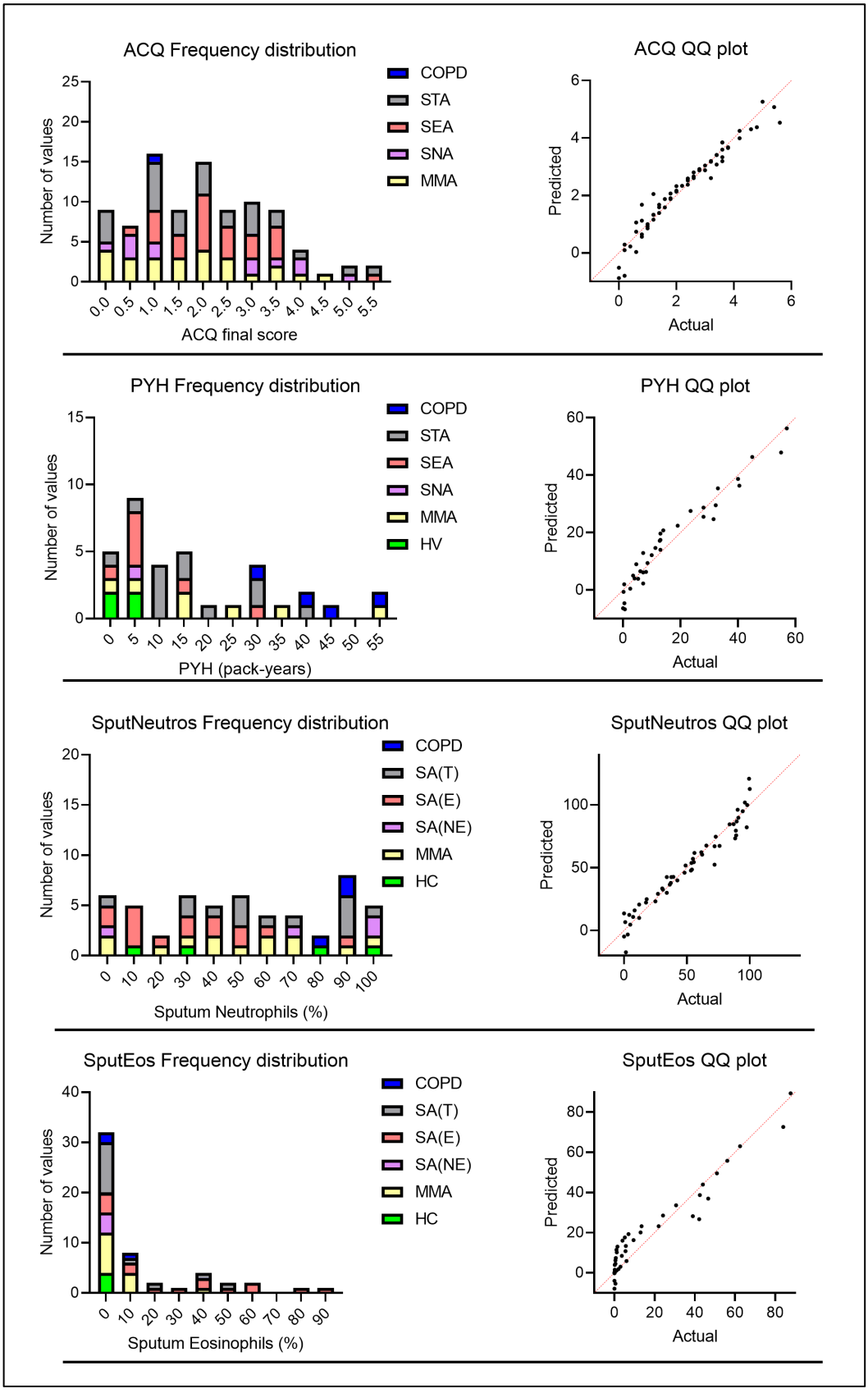

Blood Leukocytes Frequency distribution
Number of values
Blood Leukocytes (10^9/L)
Log(BloodLeukos) Frequency distribution
Number of values
Log(BloodLeukos)
Log(BloodLeukos) QQ plot
HC
MMA
SA(NE)
SA(E)
SA(T)
COPD
Predicted
Actual
Blood Eosinophils Frequency distribution
Number of values
Blood Eosinophils (10^9/L)
Log(BloodEos) Frequency distribution
Number of values
Log(BloodEos)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(BloodEos) QQ plot
Predicted
Actual
Blood Neutrophils Frequency distribution
Number of values
Blood Neutrophils (10^9/L)
Log(BloodNeutros) Frequency distribution
Number of values
Log(BloodNeutros)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(BloodNeutros) QQ plot
Predicted
Actual
Blood Lymphocytes Frequency distribution
Number of values
Blood Lymphocytes (10^9/L)
Log(BloodLymphos) Frequency distribution
Number of values
Log(BloodLymphos)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(BloodLymphos) QQ plot
Predicted
Actual

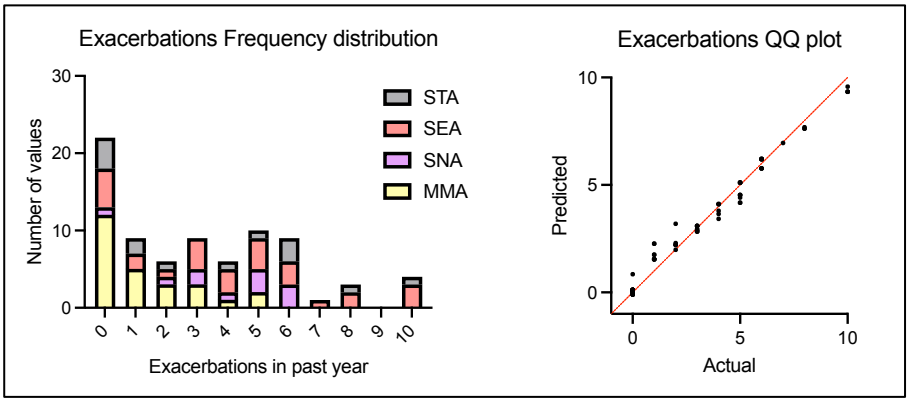

Blood Basophils Frequency distribution
Number of values
Blood Basophils (10^9/L)
Log(BloodBasos) Frequency distribution
Log(BloodBasos)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
Log(BloodBasos) QQ plot
Predicted
Actual
Blood Monocytes Frequency distribution
Blood Monocytes (10^9/L)
Log(BloodMonos) Frequency distribution
Log(BloodMonos)
Log(BloodMonos) QQ plot
FeNO Frequency distribution
FeNO (ppb)
Log(FeNO) Frequency distribution
Log(FeNO)
STA
SEA
SNA
HV
Log(FeNO) QQ plot
IgE Frequency distribution
IgE (kU/L)
Log(IgE) Frequency distribution
Log(IgE)
Log(IgE) QQ plot



Supplementary Figure 2 Histograms and QQ-plots for lognormally distributed variables.

For all variables I show the original distribution on the left, the log-transformed distribution in the centre, and the QQ-plot of the log-transformed distribution on the right. All QQ-plots compare to theoretical normally distributed data. Different patient groups are indicated by colour coding.

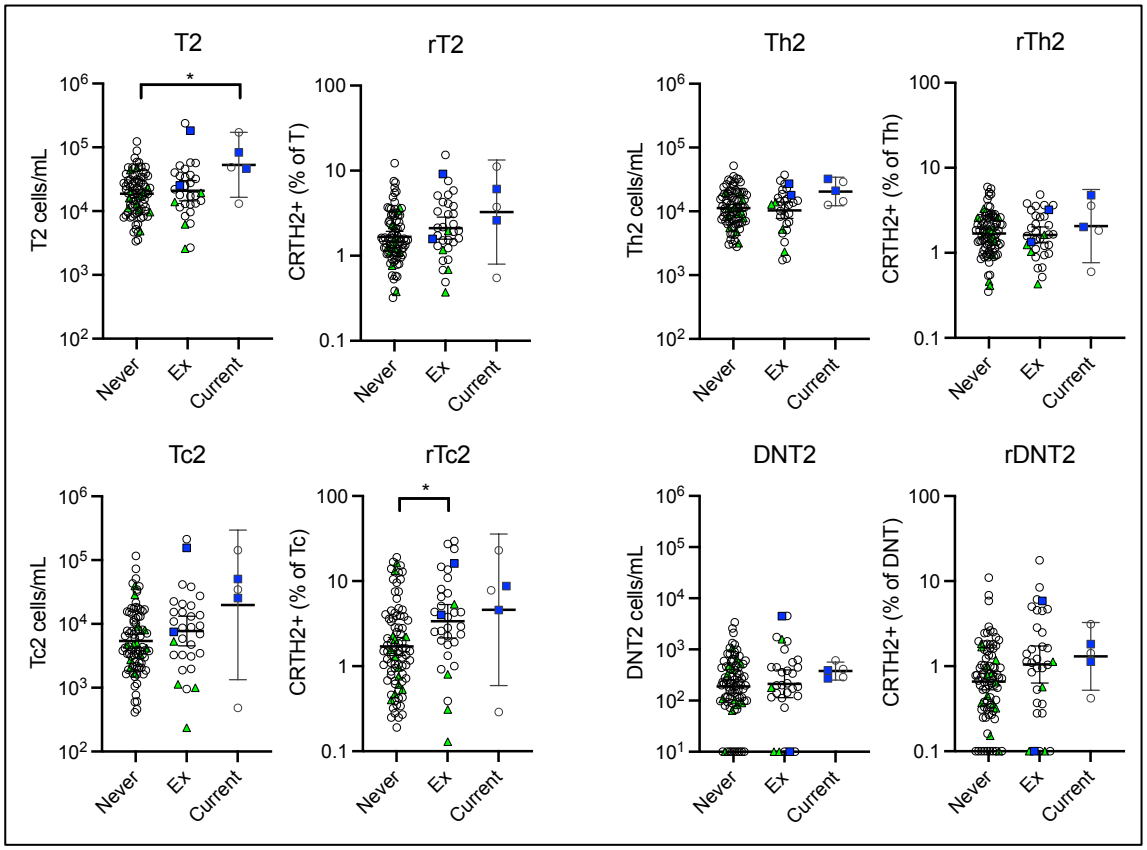

ACQ Frequency distribution
Number of values
ACQ final score
COPD
STA
SEA
SNA
MMA
ACQ QQ plot
Predicted
Actual
PYH Frequency distribution
Number of values
PYH (pack-years)
COPD
STA
SEA
SNA
MMA
HV
PYH QQ plot
Predicted
Actual
SputNeutros Frequency distribution
Number of values
Sputum Neutrophils (%)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
SputNeutros QQ plot
Predicted
Actual
SputEos Frequency distribution
Number of values
Sputum Eosinophils (%)
COPD
SA(T)
SA(E)
SA(NE)
MMA
HC
SputEos QQ plot
Predicted
Actual



Supplementary Figure 3 Histograms and QQ-plots for nonparametric variables

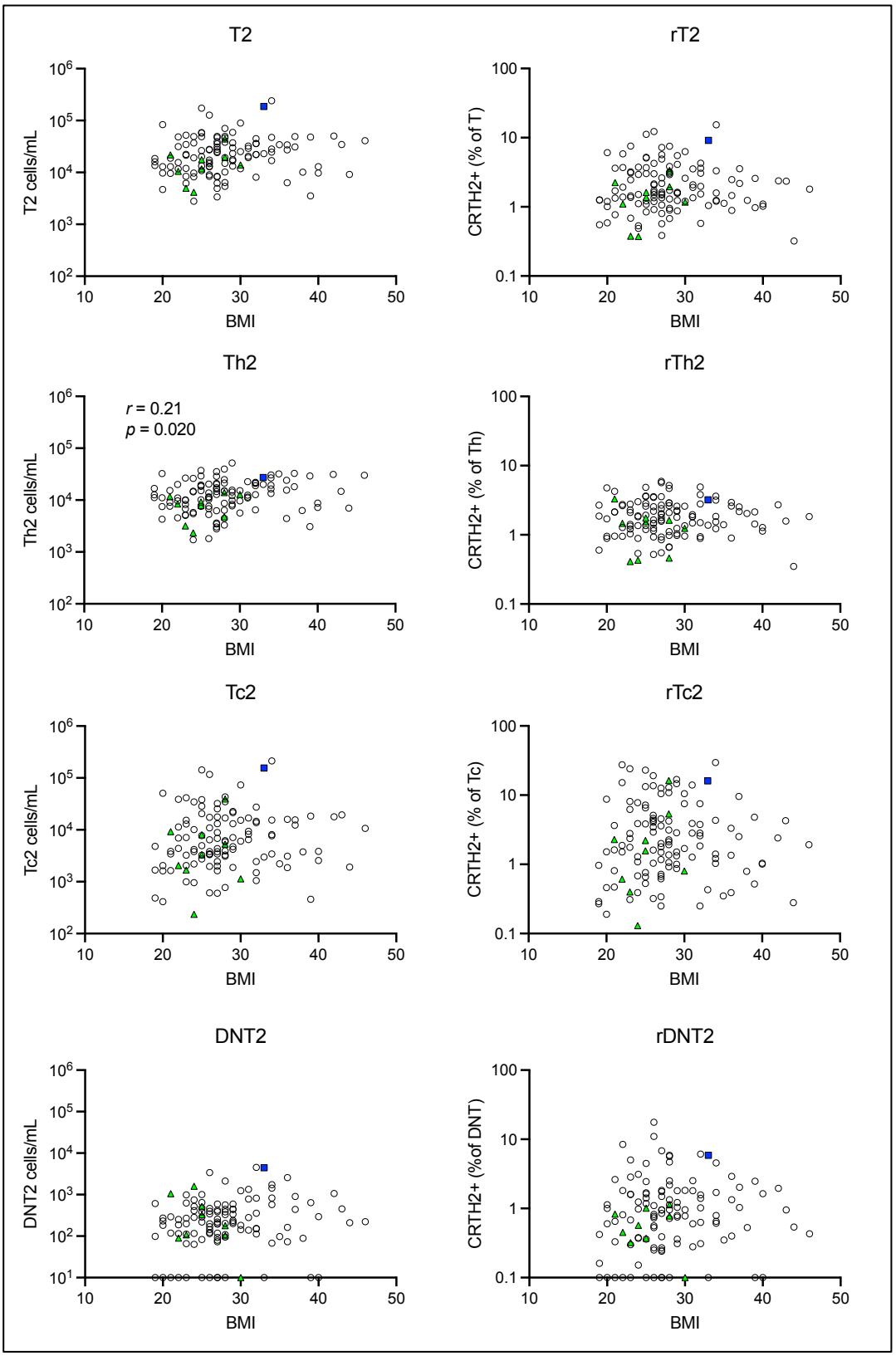
All QQ-plots compare to theoretical normally distributed data. Patient subcategories are shown by colour coding.

2.6.2 Associations between CRTH2⁺ T cells and other variables



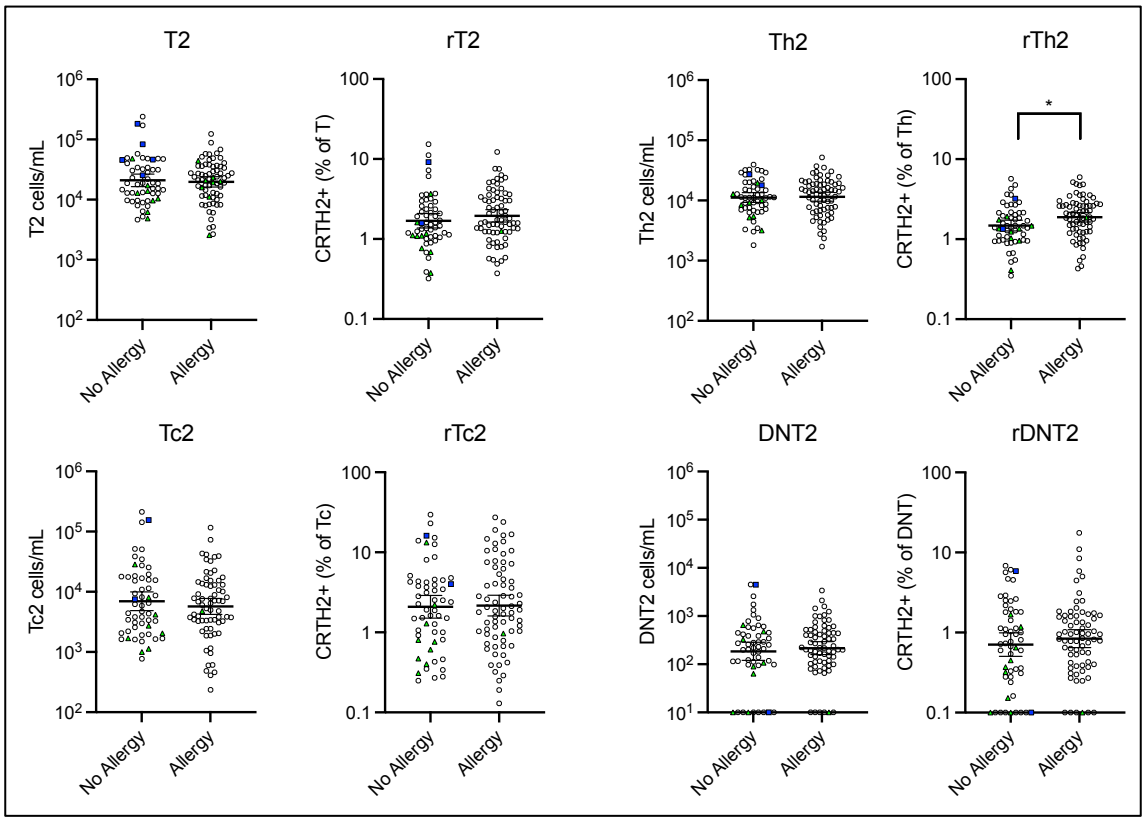
Supplementary Figure 4 Current smokers had higher T2 cell count than never smokers

Log-transformed data were analysed by one-way ANOVA. Specific comparisons between all groups were made by Tukey-Kramer test. Bars show geometric mean and confidence interval. Sample sizes never = 87, ex = 31, current = 5. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares.



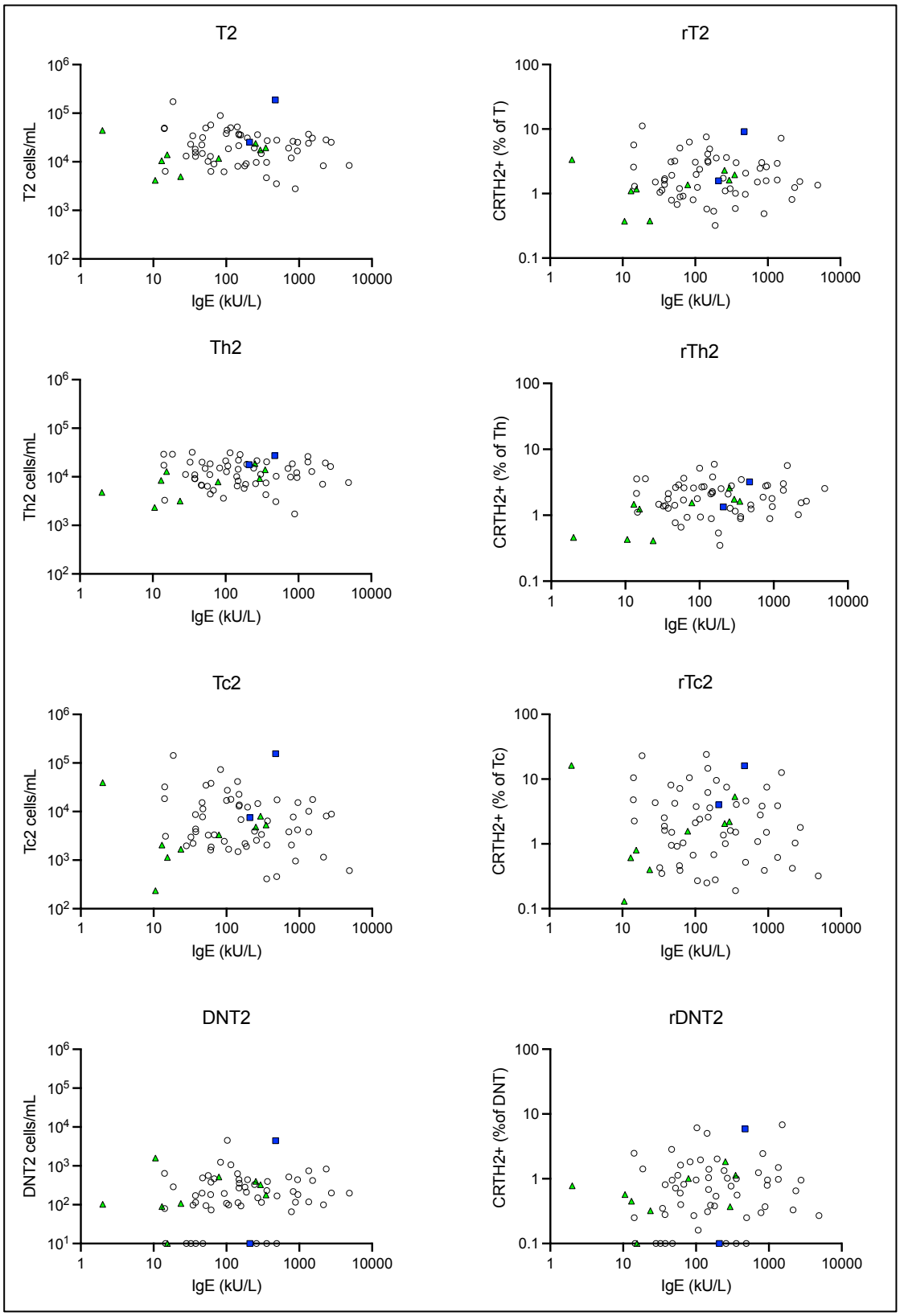
Supplementary Figure 5 No relationship observed between BMI and T2 cells

Variables were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 121.



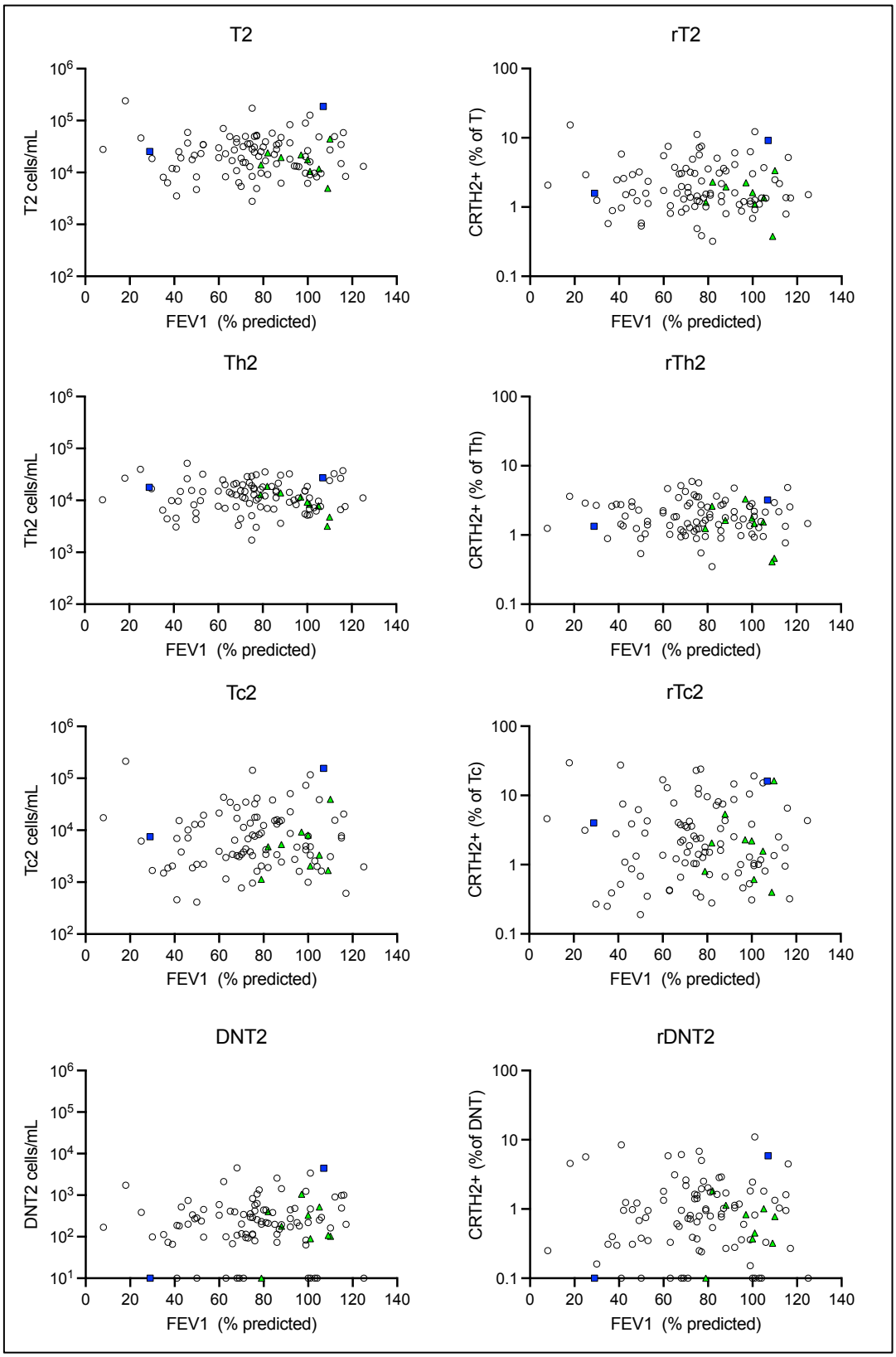
Supplementary Figure 6 Participants with allergies had increased proportion of Th2 cells

Log-transformed data were analysed by two-tailed *t*-test. Bars show geometric mean and confidence interval. Sample sizes no allergy = 55, allergy = 69. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares.



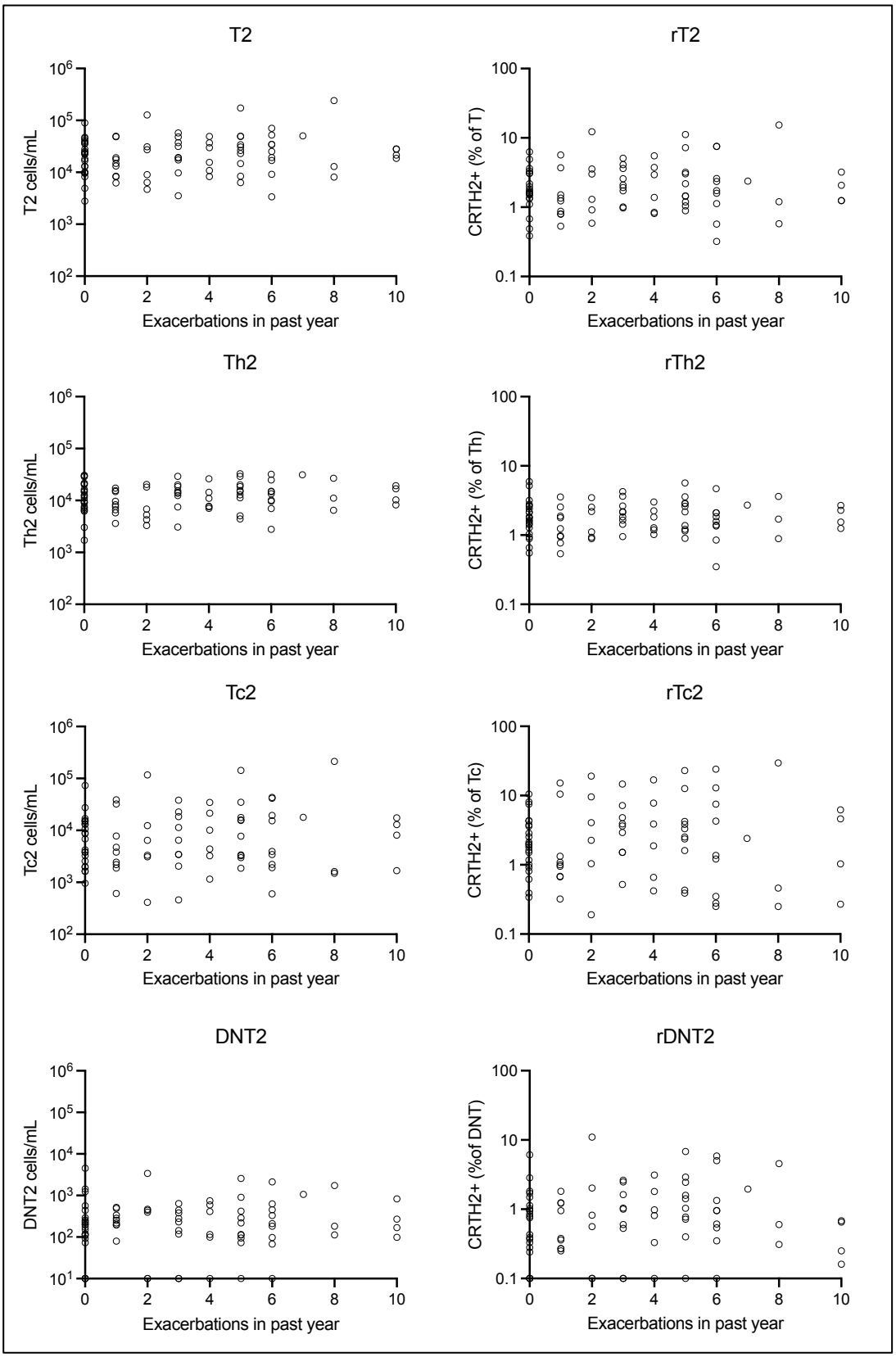
Supplementary Figure 7 IgE was not associated with T2 cells

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 67.



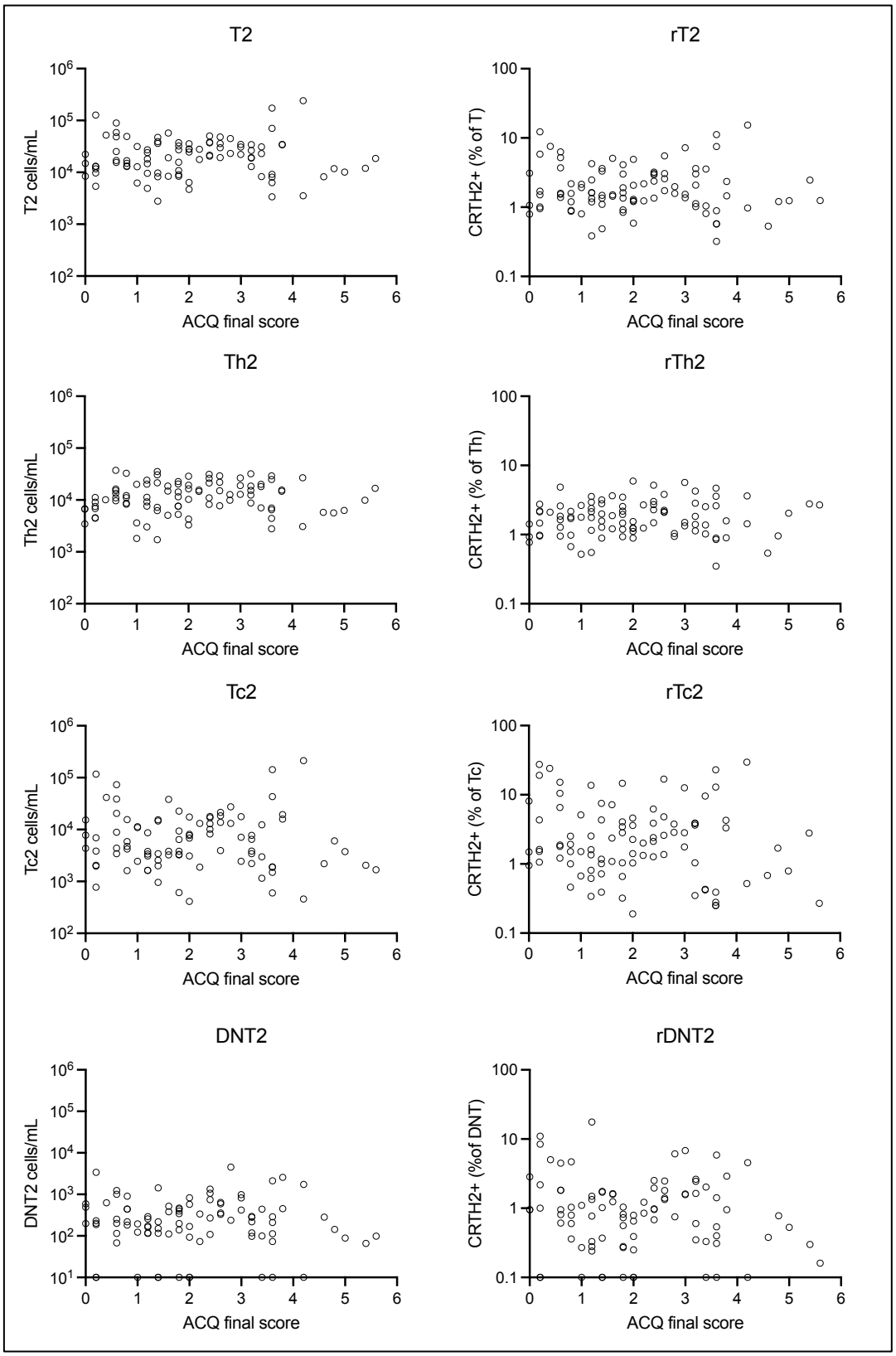
Supplementary Figure 8 No relationship between lung function and blood T2 cells

Variables were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 105



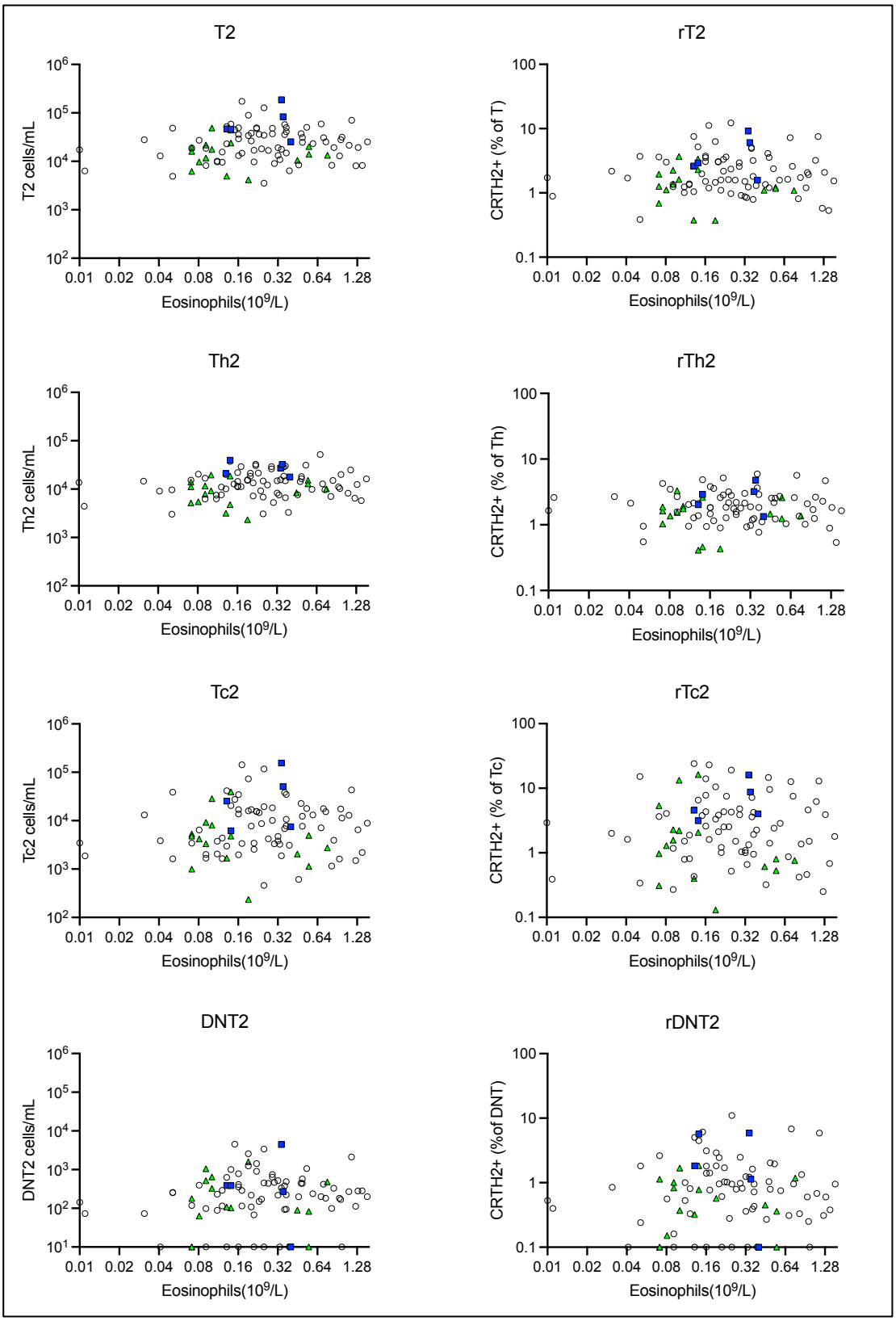
Supplementary Figure 9 No association between asthma exacerbations in the past year and blood T2 cells

Variables were compared by Spearman correlation. N = 58.



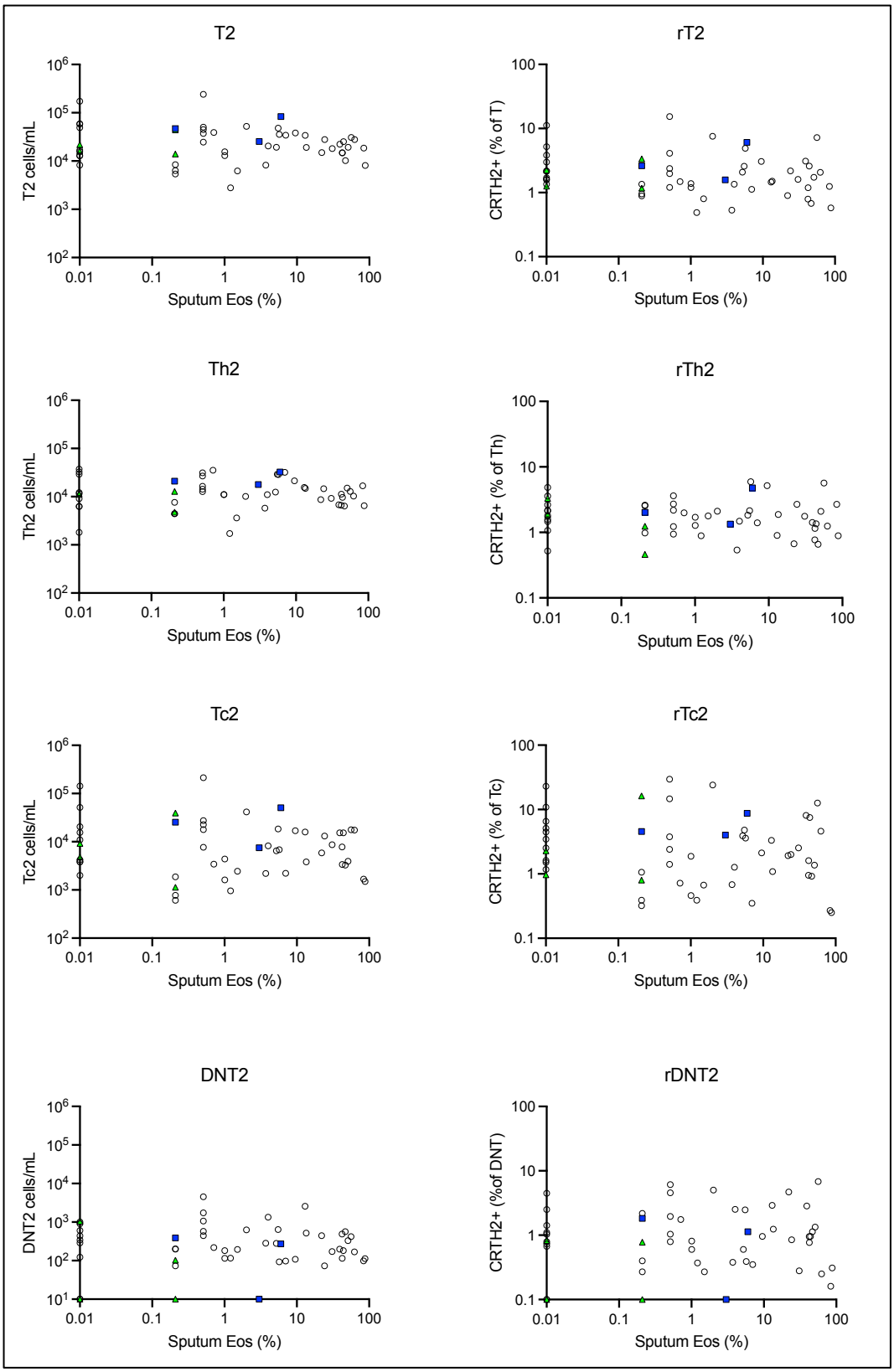
Supplementary Figure 10 No relationship between asthma control and blood T2 cells

Variables were compared by Spearman correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 91.



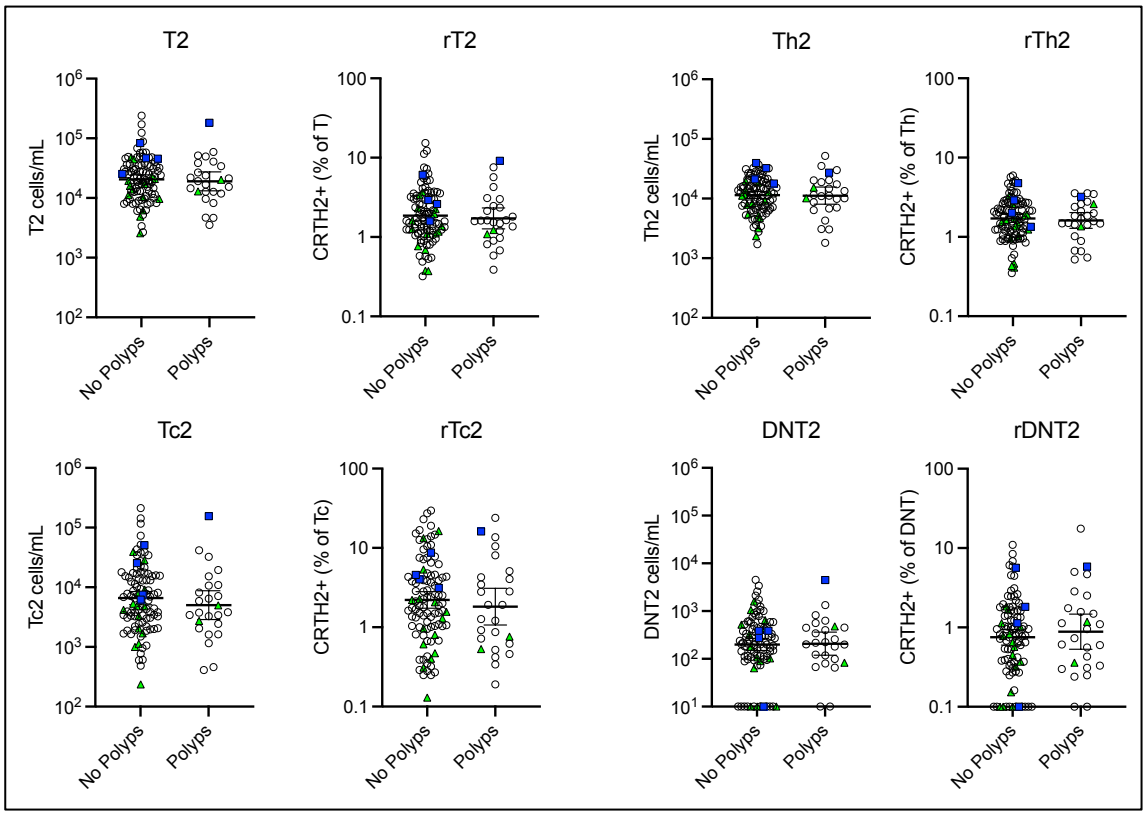
Supplementary Figure 11 No relationship between blood eosinophil and T2 cell counts

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. STA group was excluded from the analysis. N = 92.



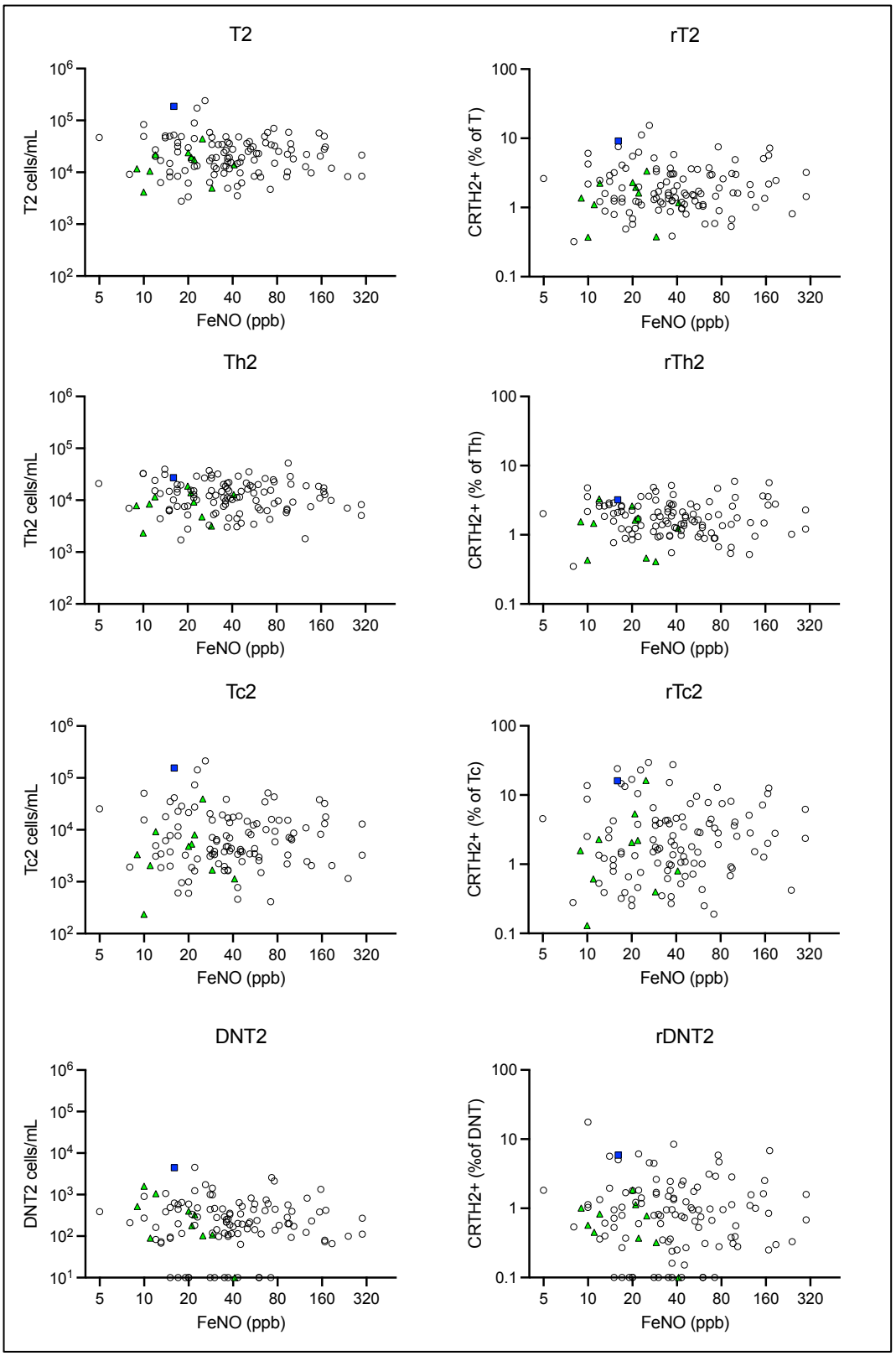
Supplementary Figure 12 No association between sputum eosinophils and blood T2 cells

Variables were compared by Spearman correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 53.



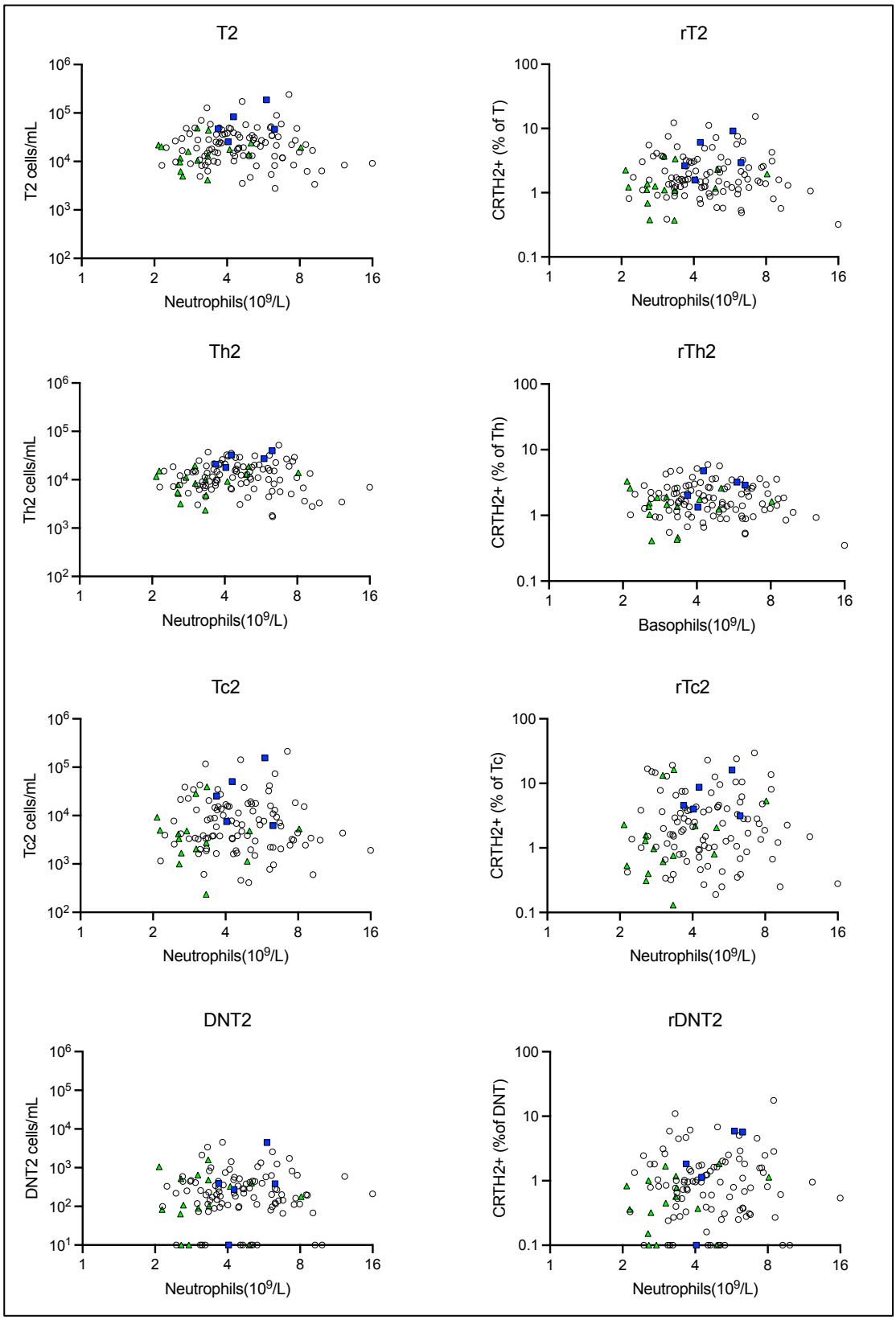
Supplementary Figure 13 No relationship between nasal polyposis and T2 cell counts.

Log-transformed data were analysed by two-tailed *t*-test. Bars show geometric mean and confidence interval. Sample sizes no polyps = 99, polyps = 25. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares.



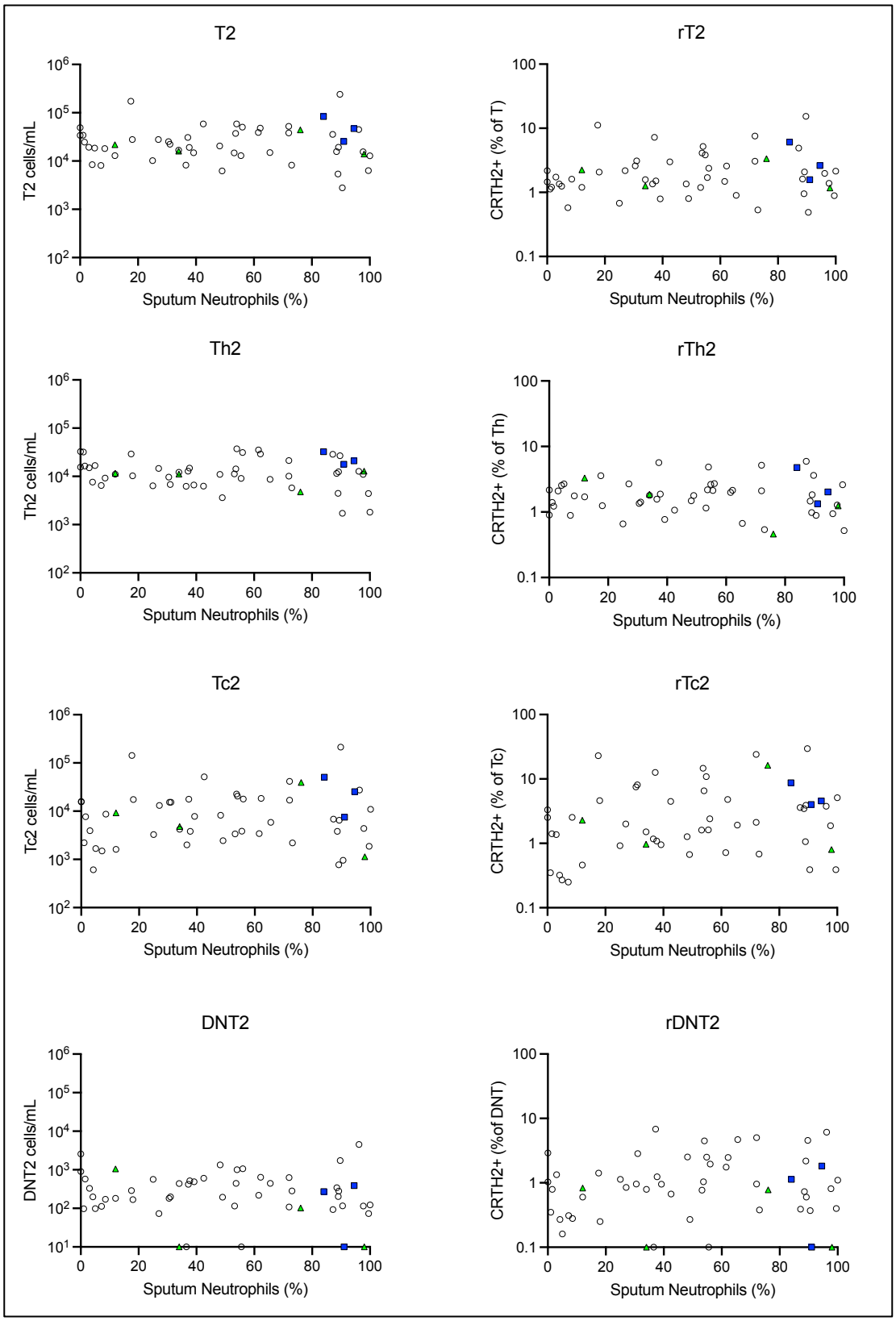
Supplementary Figure 14 No relationship between FeNO and T2 cell counts

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 118.



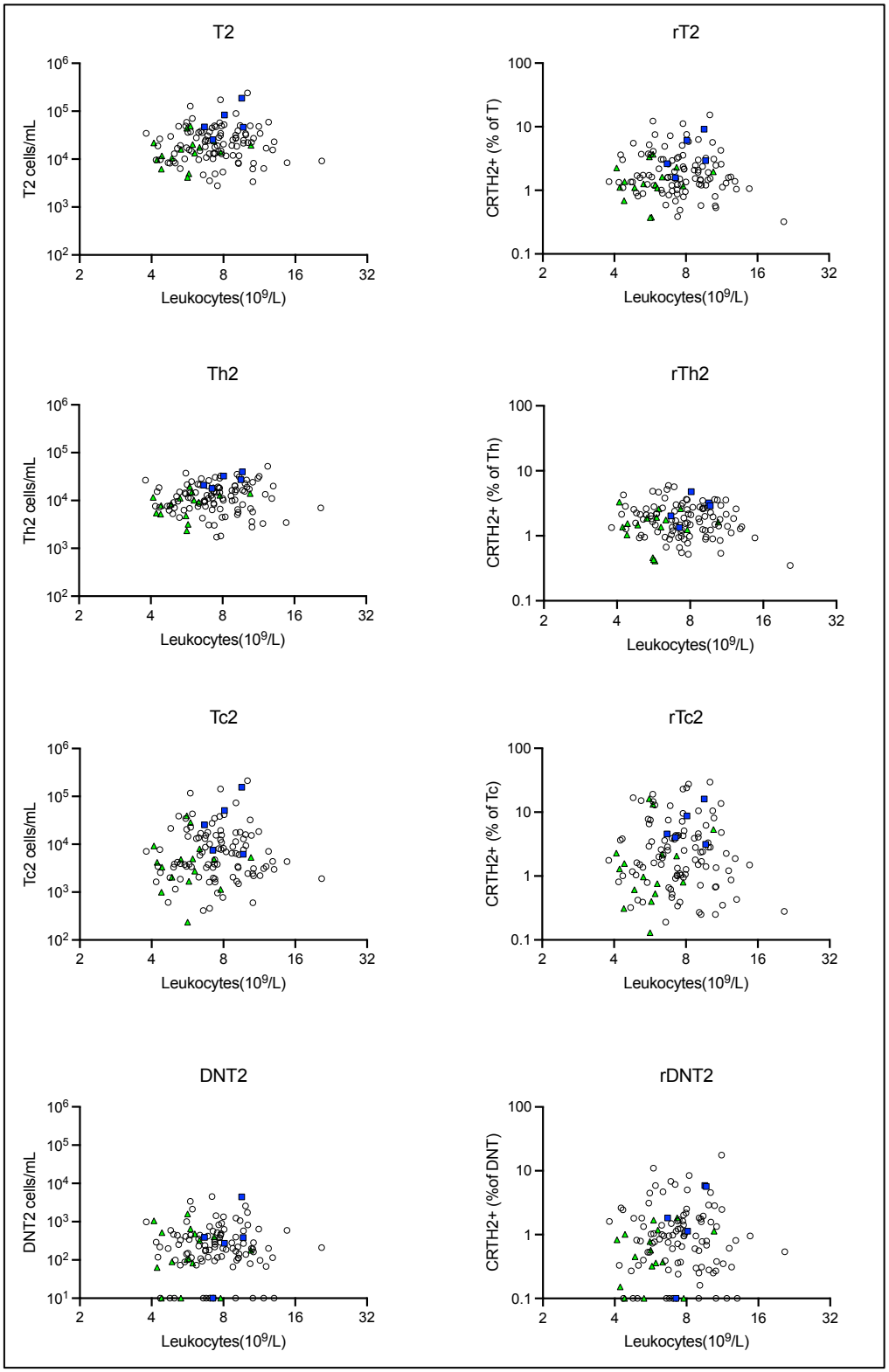
Supplementary Figure 15 No relationship between neutrophil count and T2 cells

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. STA group was excluded from the analysis. N = 92.



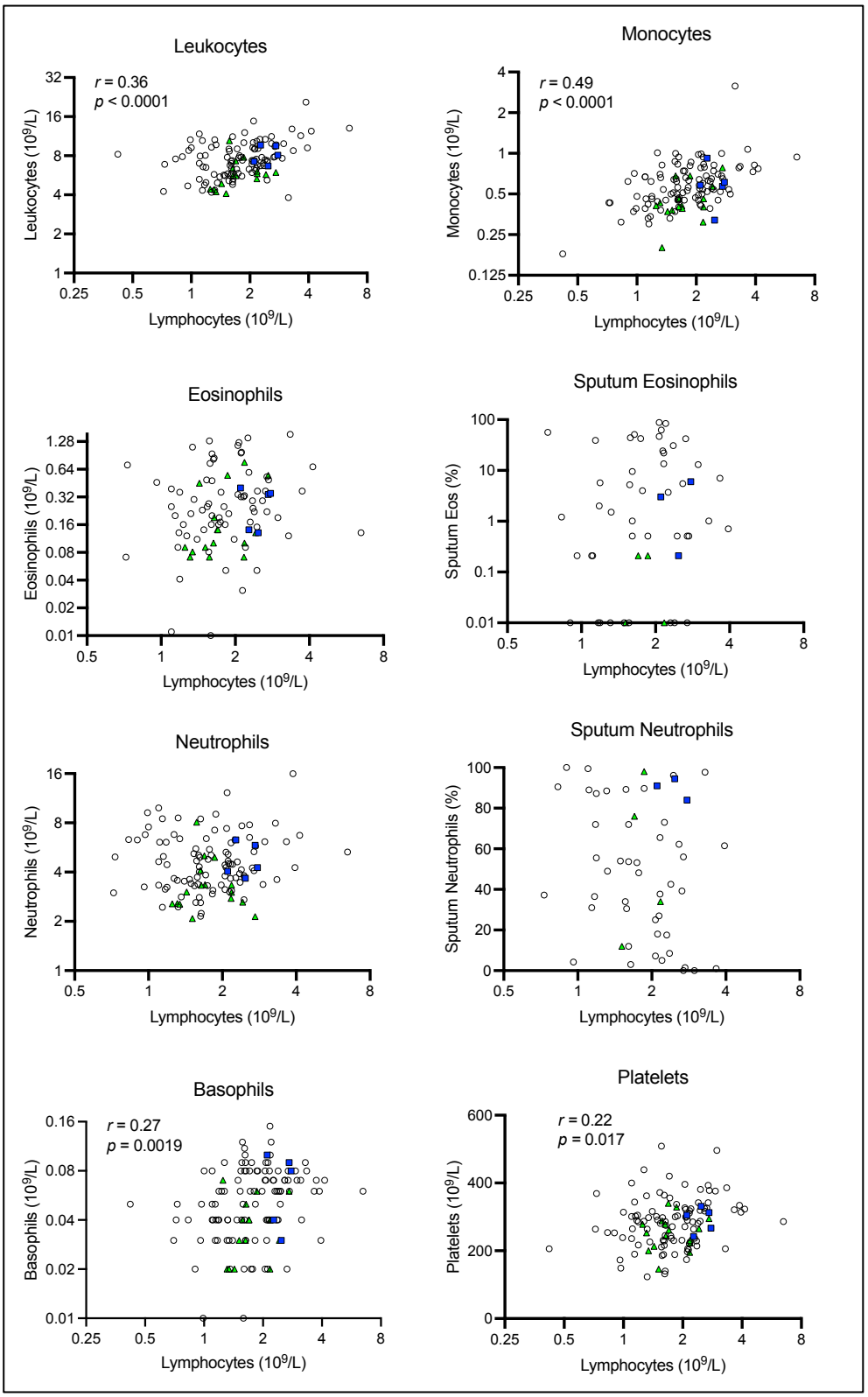
Supplementary Figure 16 No associations between sputum neutrophils and T2 cells

Variables were compared by Spearman correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 53.



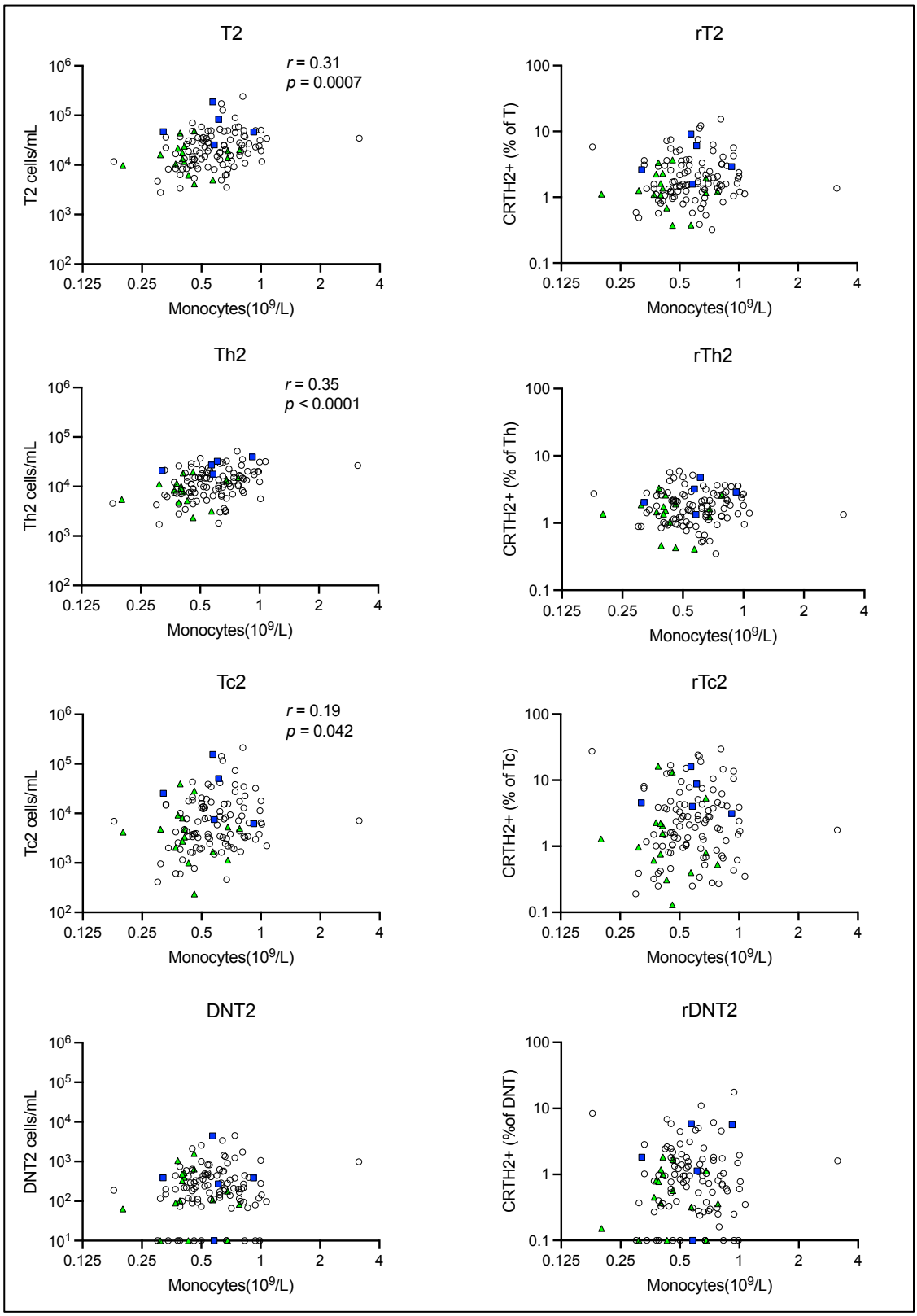
Supplementary Figure 17 Total leukocytes were not associated with T2 cells

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 120.



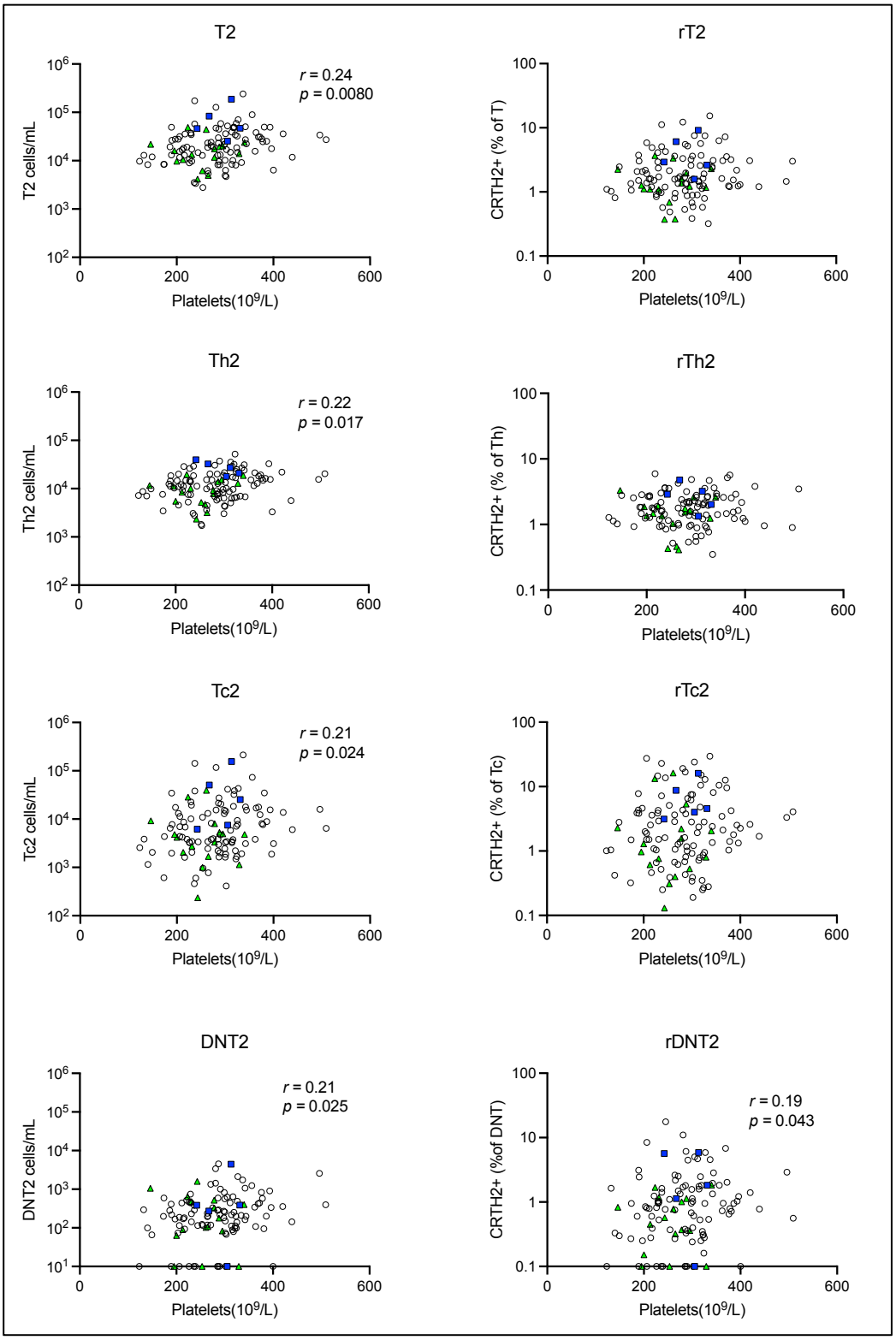
Supplementary Figure 18 Lymphocyte count was related to leukocytes, monocytes, platelets, and basophils

Log-transformed (except platelets) data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 121.



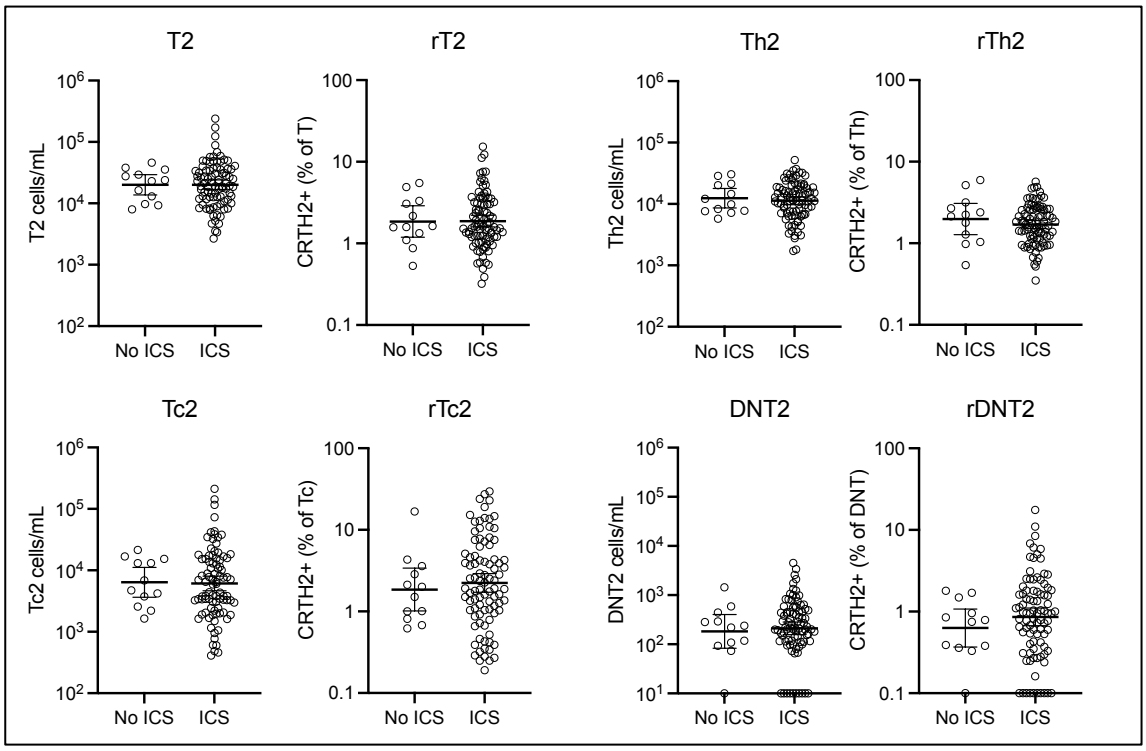
Supplementary Figure 19 Monocytes correlated with absolute T2 cell counts

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 121.



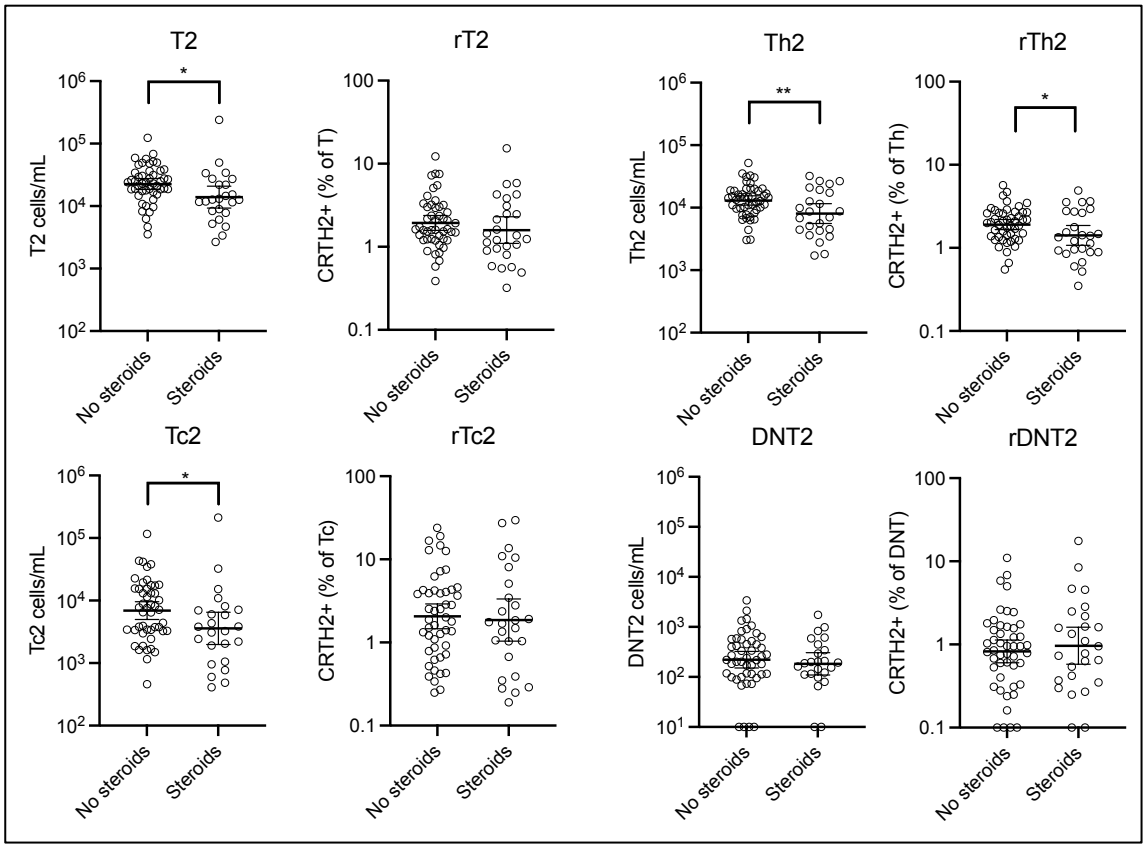
Supplementary Figure 20 Platelet count correlated with absolute T2 cell counts

Log-transformed data were compared by Pearson correlation. Data points from healthy volunteers are indicated with green triangles, asthma patients with white circles, and COPD patients with blue squares. N = 121.



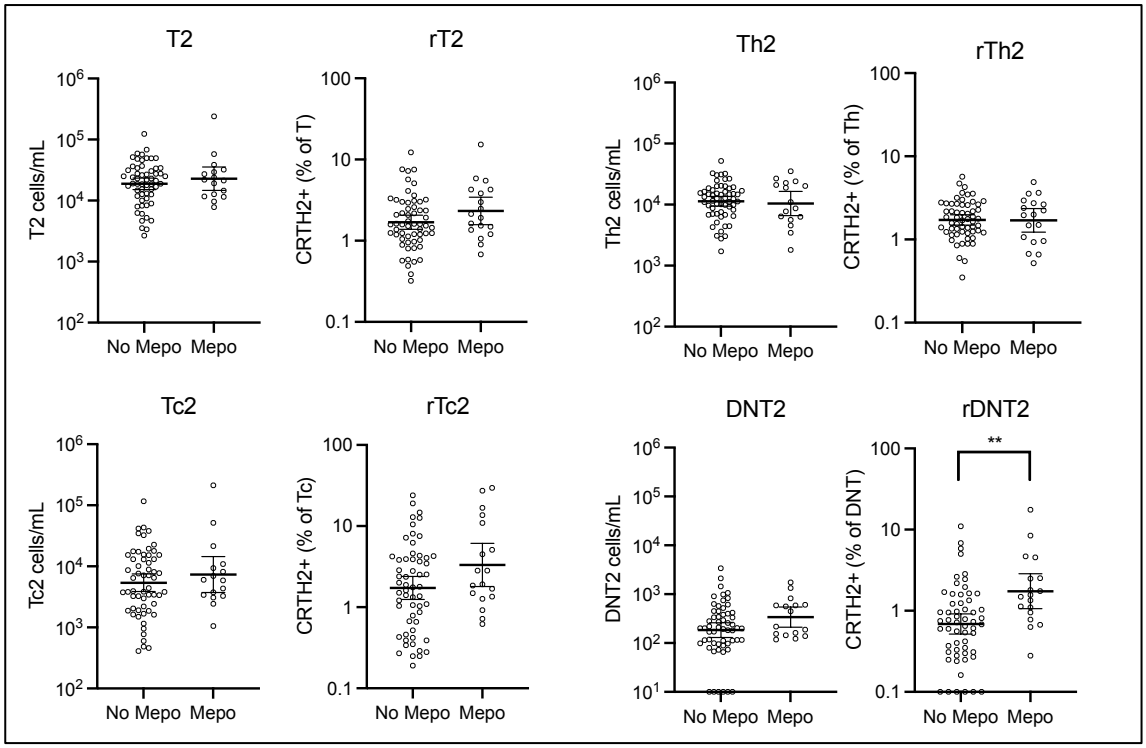
Supplementary Figure 21 No relationship between ICS use and T2 cells.

Log-transformed data were analysed by two-tailed t-test. Bars show geometric mean and confidence interval. Only patients with asthma considered. Sample sizes no ICS = 12, ICS = 89.



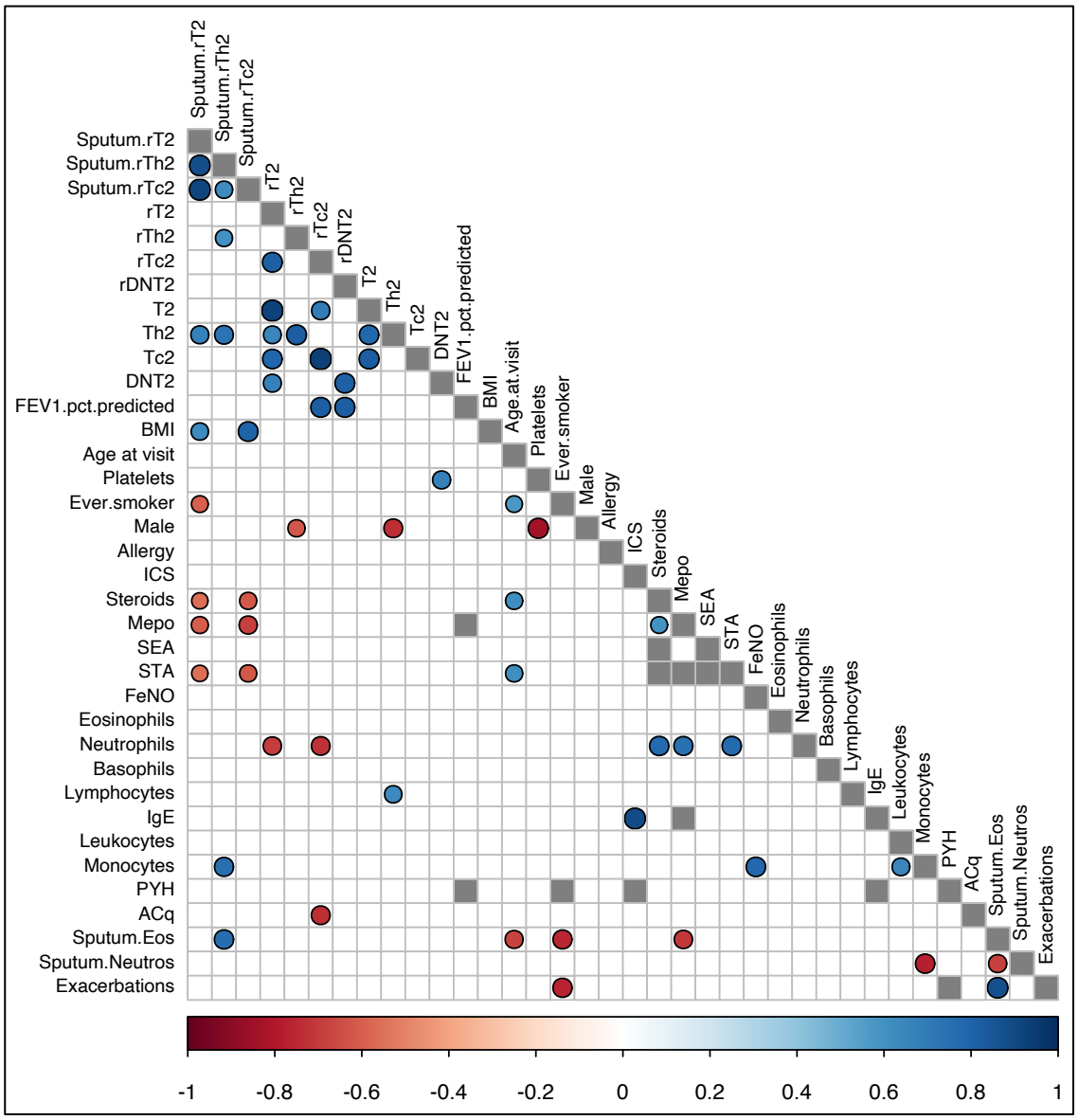
Supplementary Figure 22 Systemic steroid use was associated with reduced T2 cells

Log-transformed data were analysed by two-tailed t-test. Bars show geometric mean and confidence interval. Sample sizes no steroids = 48, steroids = 26. Only participants with severe asthma were considered in this analysis.



Supplementary Figure 23 Mepolizumab use was associated with CRTH2 expression on DNT cells

Log-transformed data were analysed by two-tailed t-test. Bars show geometric mean and confidence interval. Sample sizes no mepo = 58, mepo = 16. Only participants with severe asthma were considered in this analysis.



Supplementary Figure 24 Correlogram of associations between sputum CRTH2⁺ T cells and other variables

Only associations below threshold p=0.05 are shown, nonsignificant associations are whited out. The size of each circle, and its colour, are proportional to the strength of the association between two variables as measured by *r* value. Mutually exclusive (e.g., SEA vs STA) or artificial (e.g., STA vs steroid use) comparisons, or comparisons for which there was insufficient data to do any test, were excluded and are greyed out. Data from all participants (n=13) for all variables was included unless missing or deemed irrelevant. The statistical tests are as follows: two parametric variables compared by Pearson's correlation; one parametric and one dichotomous by point biserial correlation; two dichotomous by phi coefficient; one nonparametric and one parametric, or two nonparametric, by Spearman's rank correlation; and one nonparametric and one dichotomous by rank biserial correlation. Cell counts refer to blood except for where indicated as referring to sputum. Exacerbations refers to number of asthma exacerbations in the past 12 months.

Chapter 3

CRTH2 as a marker of type 2 T cells

3.1 Introduction

Type 2 T cells are defined by their ability to secrete cytokines IL-4, IL-5 and IL-13 (T. Nakayama et al., 2017; J. A. Walker & McKenzie, 2018). This definition carries with it some practical difficulties. In order to identify a type 2 T cell, the cell must first be activated to induce cytokine production. However, not all type 2 T cells necessarily respond to a single stimulus. Type 2 cytokines can then be detected internally by intracellular cytokine staining (ICS), a method that kills the cells, or externally by a cytokine secretion assay, a challenge to perform on three markers simultaneously.

It is therefore desirable to identify type 2 T cells by surface marker expression if possible, and ideally at resting state. CRTH2 remains the best candidate for doing so, because most $CRTH2^+$ T cells produce detectable levels of type 2 cytokines after stimulation (Cosmi et al., 2000). However, it remains unclear what proportion of type 2 T cells are $CRTH2^+$, or whether CRTH2 expression is associated with a particular type 2 cytokine. Considering this, the first aim of this chapter is to quantify the relationship between surface CRTH2 expression, and production of each of the three type 2 cytokines after T cell stimulation.

The rarity of $CRTH2^+$ T cells in the bulk T cell population makes it challenging to study them. Standard biological methods to quantify ribonucleic acid (RNA) or proteins can require a pure population of thousands or millions of cells to generate clear data. Of course, single cell techniques (e.g., flow cytometry, single cell RNA sequencing (scRNA-seq)) can be used but these come with their own limitations in terms of sensitivity and financial cost.

Several approaches have been explored to isolate large numbers of $CRTH2^+$ T cells for study. Firstly, type 2 T cells can be differentiated from bulk T cells *in vitro* using

recombinant IL-2 and IL-4, and these cells express CRTH2 (Nagata, Tanaka, et al., 1999; Xue et al., 2005). However, questions remain about how closely *in vitro* differentiation models the *in vivo* process (J. Zhu et al., 2010). Secondly, $CRTH2^+$ T cells can be sorted directly (using magnetic beads or FACS) from very large samples containing hundreds of millions of T cells, such as leukocyte cones (Hilvering et al., 2018). The disadvantages of this method are high costs (bead sorting an entire leukocyte cone consumes up to an entire magnetic bead kit per sample), and the low numbers of $CRTH2^+$ T cells isolated when smaller samples of blood or tissue are used.

For researchers interested in $CRTH2^+$ T cell biology, it may be helpful to expand $CRTH2^+$ T cells after they have been isolated from a sample of interest. Clonal expansion describes the process by which a lymphocyte recognises cognate antigen and, if costimulatory requirements are met, undergoes massive proliferation (N. M. Adams et al., 2020). This process can be synthetically induced in the laboratory by providing required activation signals and nutrients (Riddell & Greenberg, 1990). However, it has not been determined whether $CRTH2^+$ $CD8^+$ cells can be expanded this way whilst retaining their identity. The second aim of this chapter is therefore to assess whether $CRTH2^+$ $CD8^+$ cells can be expanded from a small number of clones to produce a massive population of daughter cells, whilst retaining the cells' immunological properties as described in the chapter's first aim.

3.2 Methods

3.2.1 PBMC preparation from leukocyte cones

Leukocyte cones containing residual products of platelet donation were ordered from NHS Blood and Transplant (NHSBT). PBMCs were isolated from the cone by density gradient

centrifugation with Lymphoprep (Stemcell, 07851) following the manufacturer's protocol (StemCell Technologies, 2017). The Lymphoprep reagent has a density between low density PBMCs and high density erythrocytes/granulocytes due to a specific concentration of high density sodium diatrizoate. The presence of polysaccharide promotes erythrocyte agglutination, increasing their density and sedimentation. After centrifugation, the PBMCs layer between the Lymphoprep and plasma.

Live cells were counted by haemocytometer (Biosigma, BVS100) using Trypan blue as a viability dye (Thermo Fisher, 15250061) and following the manufacturer's protocol (Biosigma, 2014). Cells were cryopreserved in a freezing medium of 90 % foetal calf serum (FCS) (Sigma, F9665) and 10 % cryoprotectant dimethyl sulphoxide DMSO (Sigma, 276855-1), which protects cells from damage by ice crystals and hypertonicity (Wowk, 2007). PBMCs were stored at 10^7/mL in 2 mL cryotubes (Thermo, 377267). Cryotubes were transferred to a Mr Frosty freezing container (Thermo Fisher, 5100-0001) and stored as per instructions (Thermo Fisher Scientific, n.d.-c). The freezing device was stored at -80 °C overnight and then cryotubes were transferred to liquid nitrogen for long term storage. To thaw frozen PBMCs, I incubated them in a water bath at 37 °C until almost melted, and then transferred the contents of each cryotube to 10 mL RPMI (Sigma, R8758-500ML) + 10 % FCS at 37 °C (Hønge et al., 2017).

3.2.2 *In vitro* expansion of CRTH2$^+$ Tc cells

The protocol I used for T cell expansion follows instructions shared with me by Amina Ismaeel (Professor Graham Ogg group, Radcliffe Department of Medicine, Oxford). The protocol is derived from a rapid expansion protocol first published in 1990 (Riddell & Greenberg, 1990) to generate cytomegalovirus-specific $CD8^+$ T cells for adoptive transfer (Riddell et al., 1992). Those authors achieved massive monoclonal T cell expansion by a

combination of anti-CD3 antibody, allogenic feeder PBMCs, and high-dose IL-2. It was adapted by the National Institutes of Health (M. E. Dudley, 2002; Mark E. Dudley et al., 2003) to a single round of rapid expansion of many clones.

PBMCs containing target cells for expansion were stained by extracellular staining as per manufacturer protocol (BioLegend, n.d.-a) with the antibodies listed in Supplementary Table 1. The samples were resuspended in a sort buffer of Dulbecco's PBS with 2% FCS and 20 mM HEPES buffer for pH stability (Cossarizza et al., 2017; Shipman, 1969), and DAPI DNA-binding fluorescent dye (BioLegend, 422801, reconstituted in 2 mL deionised H20) for exclusion of dead cells (Thermo Fisher Scientific, n.d.-b). Target cells were sorted by FACS using the BD FACS Aria III at the NDM flow cytometry facility (John Radcliffe Hospital, Oxford) operated by the facility staff. The sort conditions were 85 μm nozzle diameter and purity sort mode. The gating strategy for the four target cell types (Th vs Tc, $CRTH2^+$ vs $CRTH2^-$) is shown in Supplementary Figure 25.

Target cells were sorted into 96-well round-bottom plates (Corning, 3799) at 1 cell per well for monoclonal T cell cultures, or 2-50 cells per well for polyclonal T cell cultures. Each well contained a collection buffer of Rapid Expansion Medium (REM) (Supplementary Table 2) supplemented with anti-CD3 and irradiated feeder PBMCs. The anti-CD3 antibody was clone OKT3 (BioLegend, 317302) at 50 ng/mL, sufficient to induce clonal expansion by TCR activation (Y. Li & Kurlander, 2010; Ochoa et al., 1989). Irradiated allogenic feeder PBMC cells from three donors were present at 1 million/mL, similar to previous protocols (Mark E. Dudley et al., 2003), to promote T cell activation (Riddell & Greenberg, 1990). To irradiate feeder cells, I exposed PBMCs to 25 Gy of radiation to ablate PBMC proliferation (Y.-Q. Wei et al., 2017) by DNA damage.

The culture was incubated at 37 °C and 5 % CO_2 (Mark E. Dudley et al., 2003). After three days, I replaced half the collection buffer with REM. Wells containing expanding T cells were identified by colour change towards yellow, indicating pH change due to CO_2 and lactic acid production. A pellet could also be seen building on the bottom of the well. Wells containing expanding populations were transferred to a 48-well plate. Half of the culture medium was replaced by fresh REM twice per week, and confluent wells were split as required. After 21 days the cells were tested or cryopreserved. Prior study indicates that expanded T cells retain function when thawed and rested (Sadeghi et al., 2013).

3.2.3 Magnetic bead sorting

Cells can be fractionated by surface marker expression using magnetic bead sorting. I sorted $CRTH2^+$ and $CRTH2^-$ T cells using the CD294 (CRTH2) Microbead kit (Miltenyi, 130-091-274) following the manufacturer's protocol (Miltenyi Biotec, 2019). To summarise, the cells are first stained with phycoerythrin (PE)-conjugated anti-CRTH2. They are incubated with magnetic beads coated with anti-PE antibodies, such that $CRTH2^+$ cells bind to the magnetic beads. The sample is transferred to an LS column (Miltenyi, 130-042-401) containing a matrix of spheres that can amplify a magnetic field (Miltenyi Biotec, 2020), which was pre-placed in a strong magnet (Miltenyi, 130-090-976). The column retains bead-bound cells whilst allowing unbound (i.e., $CRTH2^-$) cells to wash through. The column is removed from the magnet before flushing out bead-bound (i.e., $CRTH2^+$) cells.

3.2.4 Flow cytometry

3.2.4.1 Determination of viability and staining of surface markers

Surface marker expression was assessed by flow cytometry. Cells were incubated with the Zombie Aqua fixable viability dye (BioLegend, 423102) at 1:400 dilution following the manufacturer's instructions (BioLegend, n.d.-d). The dye is excluded by intact cell

membrane but enters through the permeable membrane of dead/dying cells to react with free amines (Perfetto et al., 2010). Extracellular staining was performed as per the manufacturer's instructions (BioLegend, n.d.-a) using antibodies listed in Supplementary Table 3. Flow cytometry was performed as described in section 2.2.3. A CRTH2-fluorescence minus one (FMO) control was used to set gate position. Cultured lymphocytes had elevated forward and side scatter properties, which is consistent with changes observed during cell activation (H. R. MacDonald & Zaech, 1982).

3.2.4.2 Detection of type 2 cytokines by stimulation and intracellular cytokine staining (ICS)

ICS was performed to detect production of type 2 cytokines after stimulation. I followed the manufacturer's protocol (BioLegend, n.d.-b) using recommended stimulation conditions for detection of type 2 cytokines (BioLegend, n.d.-c). The stimulation consisted of a six hour incubation with: phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) (Cayman, CAY400145-1), a phorbol ester which mimics diacylglycerol (DAG) to activate protein kinase C (PKC) (Smith-Garvin et al., 2009); ionomycin (1 μg/mL) (Cayman, CAY10004974-1), a calcium ionophore (it transports $Ca2^{+}$ ions across membranes); and Brefeldin A (5 μg/mL) (BioLegend, 420601) added after one hour to block cytokine secretion by interference with the Golgi apparatus (Klausner et al., 1992). After stimulation, cells were stained for viability and surface markers (Supplementary Table 4). The sample was fixed with a formaldehyde based fixative (eBioscience, 00-8222-49) and permeabilised with a buffer containing 0.1 % saponin (eBioscience, 00-8333-56), a detergent which binds and removes cholesterol to leave behind a porous membrane (Hosokawa & Morikawa, 2010). Intracellular cytokines were stained (Supplementary Table 4) and samples analysed with flow cytometry (see 2.2.3).

3.2.4.3 Detection of GATA3 transcription factor by intranuclear staining

To detect the intranuclear transcription factor GATA3, I used the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00), following the manufacturer's protocol (Thermo Fisher Scientific, n.d.-a). Cells were first stained for viability and extracellular surface markers (Supplementary Table 5). Then, they were resuspended in 1 mL of 1:3 fixation/permeabilization concentrate to fixation/permeabilization diluent for 45 minutes at room temperature in the dark. This reagent contains formaldehyde as a fixative and other unknown (due to commercial confidentiality) ingredients for permeabilization. Cells were washed in permeabilization buffer as with ICS, then stained for GATA3 at room temperature in the dark for 45 minutes. Finally, cells were washed in staining buffer (PBS + 2 % FCS) and analysed with flow cytometry (see 2.2.3).

3.2.5 Chemotaxis assay

The chemotaxis assay was performed using a ChemoTx Disposable Chemotaxis System (Neuro Probe, SKU:101-5, 5 μm pore size, 8 mm^2 cell site area, 30 μL well capacity) following the manufacturer's instructions (Neuro Probe, 2020). In this system, there are two chambers separated by a porous membrane (Boyden, 1962). Cells are loaded in the upper chamber and migrate through the porous membrane towards chemotactants in the lower chamber. In my experiments, I loaded 20 000 cells per test, the concentration of PGD_2 in the lower chamber was 20 nM, the medium was RPMI + 10 % FCS, and the incubation time was one hour. These conditions are similar to previous studies (Hirai et al., 2001). All tests were carried out in triplicate and the median value was taken for analysis.

To quantify the cell migration to the lower chamber I used CellTiter-Glo! Solution (Promega, G7570) following the manufacturer's protocol (Promega, 2015). Cells from each lower chamber were mixed with the same volume of the reagent in a white 96-well

microplate (Greiner, 655075). The reagent lyses cells and ATP released powers a luciferase reaction, converting luciferin to luminescent oxyluciferin (Stanley & Williams, 1969) detected by a FLUOstar Optima microplate reader. Cell migration count was approximated by comparing luminescence values to a standard curve of known cell numbers.

3.2.6 Statistics

For sensitivity, specificity, positive predictor value (PPV) and negative predictor value (NPV), I used definitions listed elsewhere (Trevethan, 2017). These values were used to assess the use of CRTH2 surface staining as test for type 2 cell identity as defined by the capacity to produce type 2 cytokines after stimulation. In this case, CRTH2 surface marker staining is considered as the *screening* test and cytokine production detected by ICS as the *gold standard* test for type 2 T cell identification. With that in mind, values *a, b, c,* and *d* were calculated as described in Table 6. Instead of absolute numbers of cells, I used proportions in the calculations (e.g., if 5 % of cells were $CRTH2^+$ and $cytokine^+$, then $a = 0.05$).

Table 6 Calculations used for sensitivity, specificity, PPV and NPV values

Value	CRTH2 expression	Cytokine production
***a* (true positive)**	Positive	Positive
***b* (false positive)**	Positive	Negative
***c* (false negative)**	Negative	Positive
***d* (true negative)**	Negative	Negative
	Calculation	
Sensitivity	[a/(a + c)] X 100	
Specificity	[d/(b + d)] X 100	
PPV	[a/(a + b)] X 100	
NPV	[d/(c + d)] X 100	

3.3 Results

3.3.1 CRTH2 as a marker of type 2 T cells

The first aim of this chapter is to quantify the link between surface CRTH2 expression and production of the canonical type 2 cytokines IL-4, IL-5, and IL-13 after T cell activation.

3.3.1.1 CRTH2 is downregulated after stimulation with PMA and ionomycin

Previous studies showed that CRTH2 is downregulated after T cell activation (MacLean Scott et al., 2018; Nagata, Tanaka, et al., 1999). I asked whether this downregulation inhibits identification of activated CRTH2$^+$ T cells by flow cytometry. Stimulation of PBMCs with PMA and ionomycin (see 3.2.4.2) visibly decreased CRTH2 expression on T cells (Supplementary Figure 26). The proportion of cells staining CRTH2$^+$ was lower after stimulation for both Tc (*MR* = 0.47, *CI* = 0.36 to 0.62, $p = 0.0003$) and Th (*MR* = 0.58, *CI* = 0.45 to 0.75, $p = 0.0015$) cells compared to the stimulated condition, and in both cases the median fluorescence intensity of positively stained cells fell after stimulation (Figure 30). Therefore, in a mixed population of positive and negative cells, the identification of CRTH2$^+$ T cells becomes compromised after stimulation with PMA and ionomycin.

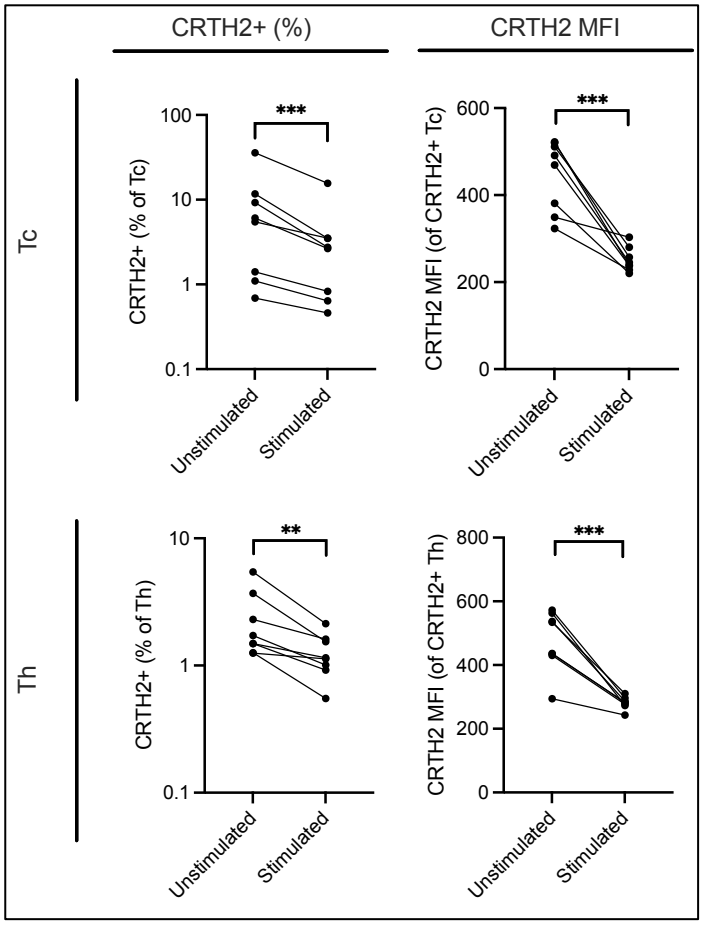


Figure 30 CRTH2 is downregulated after stimulation with PMA and ionomycin

PBMCs were stimulated with PMA and ionomycin as described in 3.2.4.2, then stained and analysed as described in 3.2.4.1. Data regarding CRTH2+ (%) follow a lognormal distribution so were log-transformed before statistical analysis. A paired t-test was used to compare unstimulated and stimulated conditions. N = 8 biological replicates.

3.3.1.2 Sorting CRTH2+ cells prior to stimulation restores accuracy of identification

Because CRTH2$^+$ T cells could not be accurately identified in whole PBMCs after stimulation, I separated them prior to stimulation using magnetic bead isolation (Cosmi et al., 2000). PBMCs were sorted into CRTH2$^+$ and CRTH2$^-$ fractions. The two populations were stimulated with PMA and ionomycin and cytokine production was detected by ICS (3.2.4.2). In this way, both the detection of surface CRTH2 and internal type 2 cytokines could be resolved by flow cytometry despite the downregulation of CRTH2 after stimulation (Figure 31). Cells that were sorted into the CRTH2$^+$ fraction before stimulation and stained positively for CRTH2 after stimulation were treated as true CRTH2$^+$ cells. Conversely, cells which sorted into the CRTH2 negative fraction before stimulation and remained negative for CRTH2 after stimulation were treated as CRTH2$^-$ cells. This partially accounts for imperfect sorting efficiency and background staining effects.

3.3.1.3 Assessment of CRTH2 as a marker of type 2 T cells

Using the gating strategy in Figure 31, I compared the production of type 2 cytokines by T cells in the CRTH2$^+$ vs CRTH2$^-$ fractions. In all cases the proportion of type 2 cytokine-producing cells was higher in CRTH2$^+$ fraction (Table 7 and Figure 32A). A second observation was that within cytokine producing cells, CRTH2$^+$ T cells generally produced greater quantities of cytokine than CRTH2$^-$ T cells (Figure 32B).

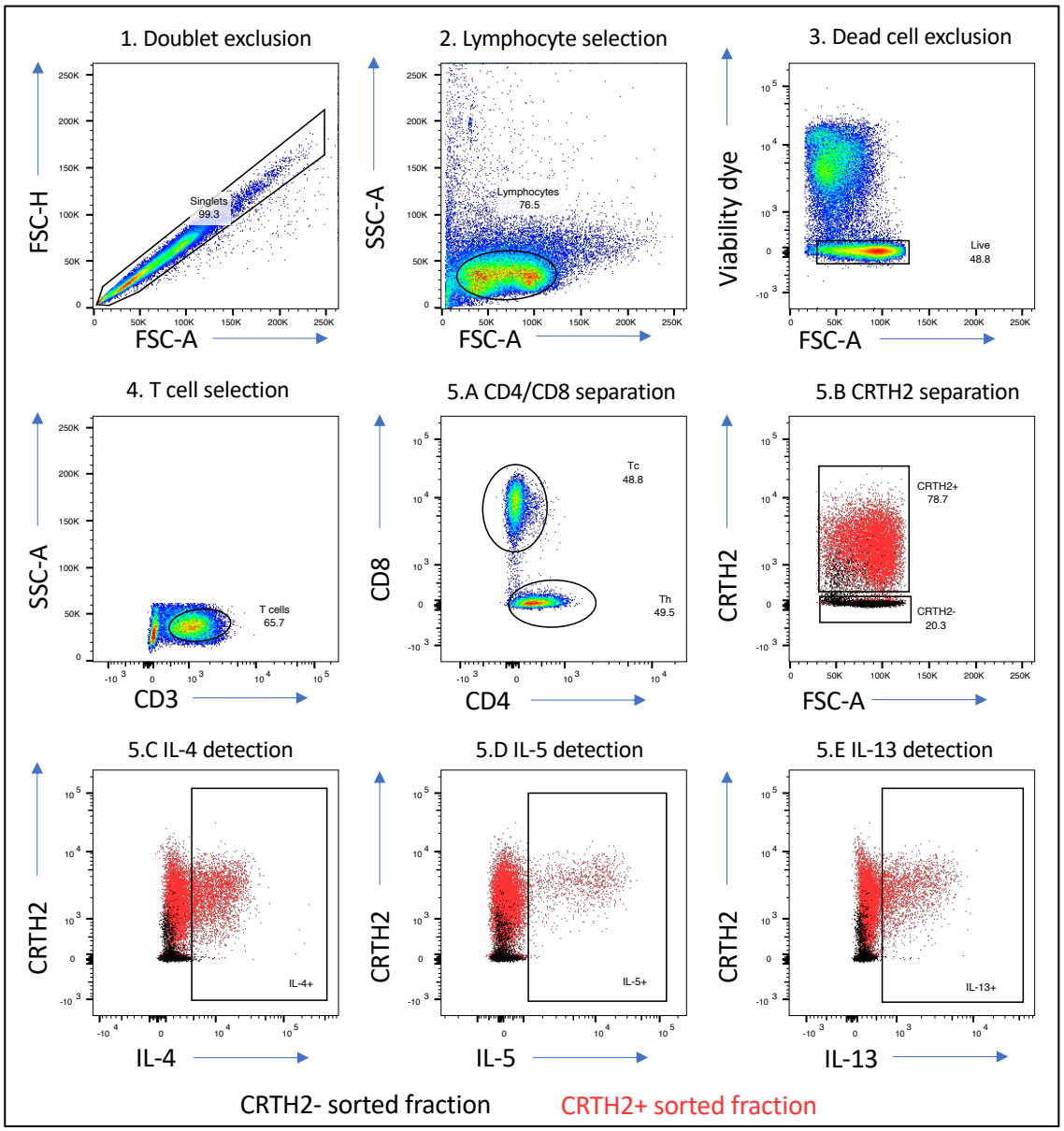


Figure 31 Gating strategy for simultaneous detection of CRTH2 and type 2 cytokines

PBMCs were first fractionated by CRTH2 expression by magnetic beads sorting (3.2.3). Sorted populations were stimulated, surface markers were stained, and cytokine production was detected by ICS (3.2.4.2). The order of gating for analysis is indicated numerically in the figure above each scatter plot. Gates 5A to 5E are implemented on the same populations and can be combined as required by Boolean gating. Although both CRTH2^{+} and CRTH2^{-} fractions are shown in this gating figure, they were ran as separate samples on the cytometer.

Table 7 The proportion of CRTH2$^+$ or CRTH2$^-$ T cells that produced type 2 cytokines

Values in the table are derived from the data presented in Figure 32. They show the proportion of CRTH2$^+$ or CRTH2$^-$ T cells (indicated in columns) that produced type 2 cytokines (indicated in rows) after stimulation. For example, 22.9 % of CRTH2+ Tc cells produced IL-4. *p*-value refers to the *p*-value of a two-tailed paired *t*-test between the CRTH2$^+$ and CRTH2$^-$ groups, for each cytokine tested (IL-4, IL-5, or IL-13) per cell type (Tc or Th).

Cell	Cytokine Profile	CRTH2$^+$		CRTH2$^-$		
		Mean	SD	Mean	SD	*p*-value
Tc	IL-4$^+$	22.9	9.1	0.27	0.30	0.0002
	IL-5$^+$	7.3	3.5	0.091	0.082	0.0007
	IL-13$^+$	19.3	8.0	0.31	0.36	0.0003
Th	IL-4$^+$	33.7	10.5	0.42	0.37	<0.0001
	IL-5$^+$	10.2	5.6	0.052	0.025	0.0015
	IL-13$^+$	29.0	8.9	0.46	0.24	<0.0001

The proportions of different cytokine profiles (e.g., IL-4$^+$IL-5$^+$) within the CRTH2$^+$ T cell population were calculated (Supplementary Table 6) and visualised as a Venn diagram (Figure 33) to illustrate the overlap between production of different type 2 cytokines within stimulated CRTH2$^+$ T cells. Generally, a greater proportion of cells stained positive for a type 2 cytokine in the CRTH2$^+$ Th population (M = 43.7, SD = 12.4) than the CRTH2$^+$ Tc (M = 30.7, SD = 12.0, p = 0.0014) after stimulation with PMA and ionomycin. All possible combinations of type 2 cytokine production were observed, but it was clear that the populations largely overlapped i.e., production of IL-4, IL-5 and IL-13 are closely linked.

Next, I performed an analysis which considered type 2 cytokine production across all Th or Tc cells, including both the CRTH2$^+$ and CRTH2$^-$ populations. To calculate the proportions of CRTH2 expression and type 2 cytokine production for each cytokine within Th or Tc cells, and all possible intersections (e.g., IL-4$^+$CRTH2$^+$, IL-5$^+$IL-13$^+$CRTH2$^-$, etc.), I first weighted the proportion of type 2 cytokine producing T cells in the stimulated CRTH2$^+$ and CRTH2$^-$ fractions, by the proportion of CRTH2$^+$ and CRTH2$^-$ cells in the T cell compartment of unstimulated PBMCs taken from the same samples

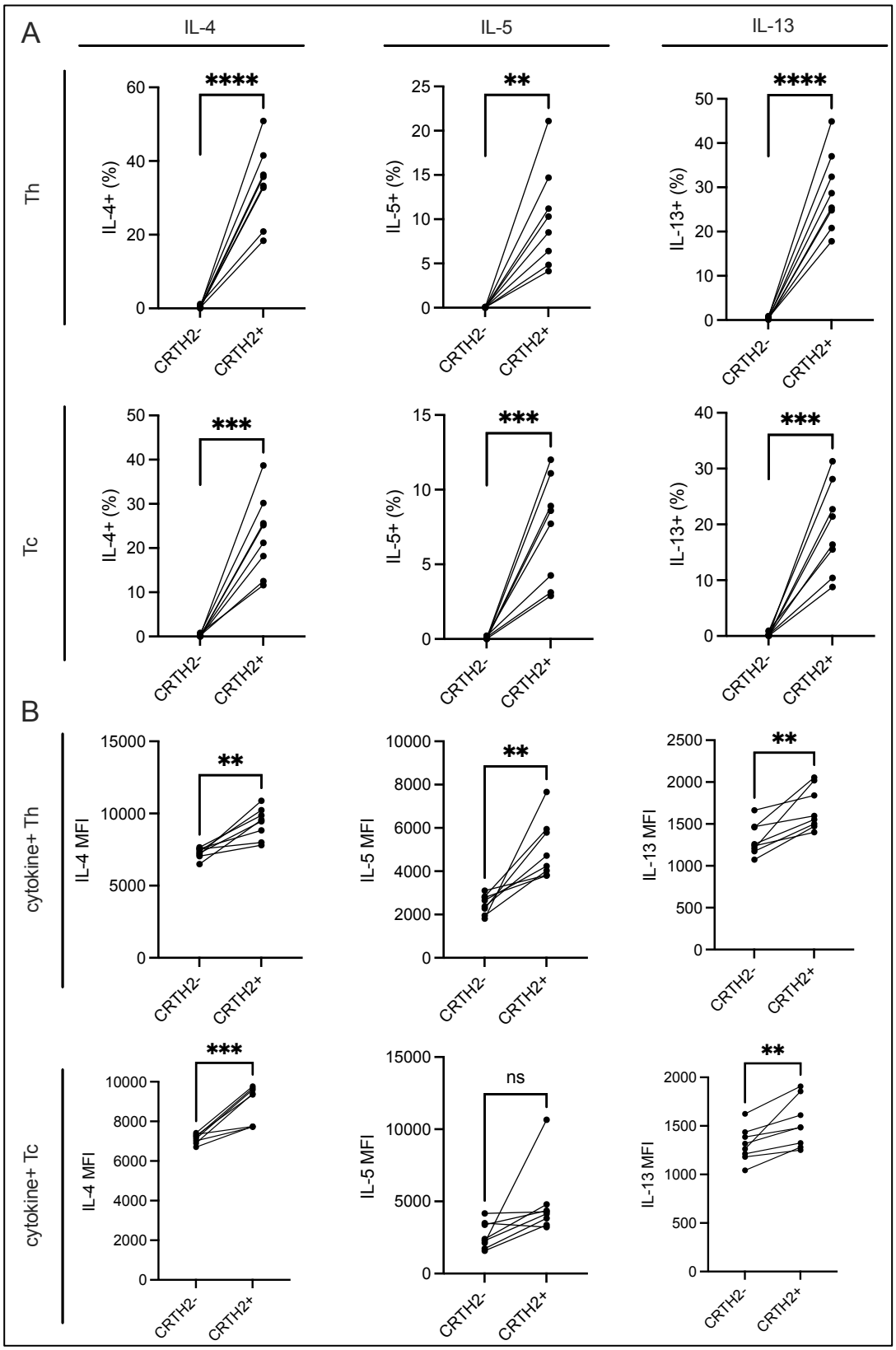


Figure 32 *Ex vivo* CRTH2⁺ T cells are enriched for type 2 cytokine production

Data were acquired as described in Figure 31. Groups were compared by two-tailed paired t-test. N = 8 biological replicates. (A) Percentage of cytokine+ Th or Tc cells. (B) mean fluorescence intensity (MFI) of cytokine production within positively staining Th or Tc cells.

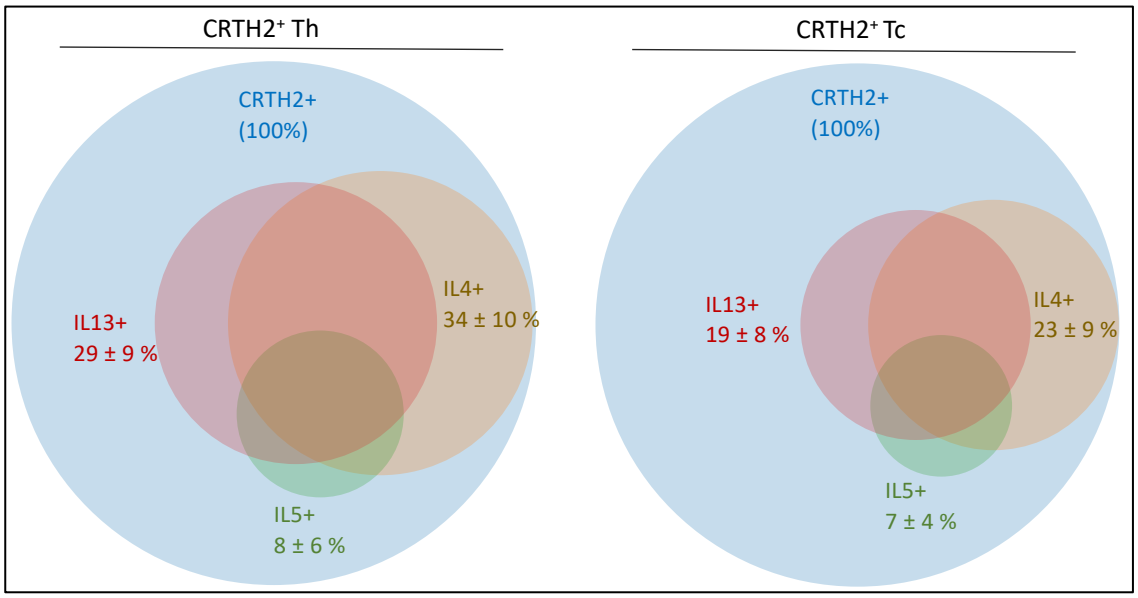


Figure 33 Type 2 cytokine profiles within the CRTH2⁺ Th and Tc cell population after stimulation with PMA and ionomycin

Numbers represent the percentage of sorted CRTH2⁺ Th or Tc cells from PBMCs that expressed cytokine after stimulation. Data were acquired as described in Figure 31. Logical gating was used to quantify type 2 cytokine production (Supplementary Table 6). Venn diagrams were constructed on meta-chart.com using mean values, and annotated with mean ± SD % cytokine production. N = 8 donors. The area of each circle is scaled to represent the proportion of that group within the CRTH2⁺ T cell population.

(Supplementary Table 7 to Supplementary Table 10). This was necessary because, as described in 3.3.1.1, the proportion of CRTH2⁺ T cells could not be accurately ascertained in stimulated samples alone, so it was instead measured in the unstimulated samples. From this data I derived the averages of all possible combinations of CRTH2 and type 2 cytokine production in Th and Tc cells for each donor (Supplementary Table 11 and Supplementary Table 12). These values were used to construct two sets of Venn diagrams. The first set shows an area-proportional diagram of the overlap between CRTH2 expression and the production of any type 2 cytokine (IL-4, IL-5, or IL-13) (Figure 34A). In the second set of diagrams, the type 2 cytokines are considered individually, but the number of mathematical sets is too high to retain area proportionality (Figure 34B).

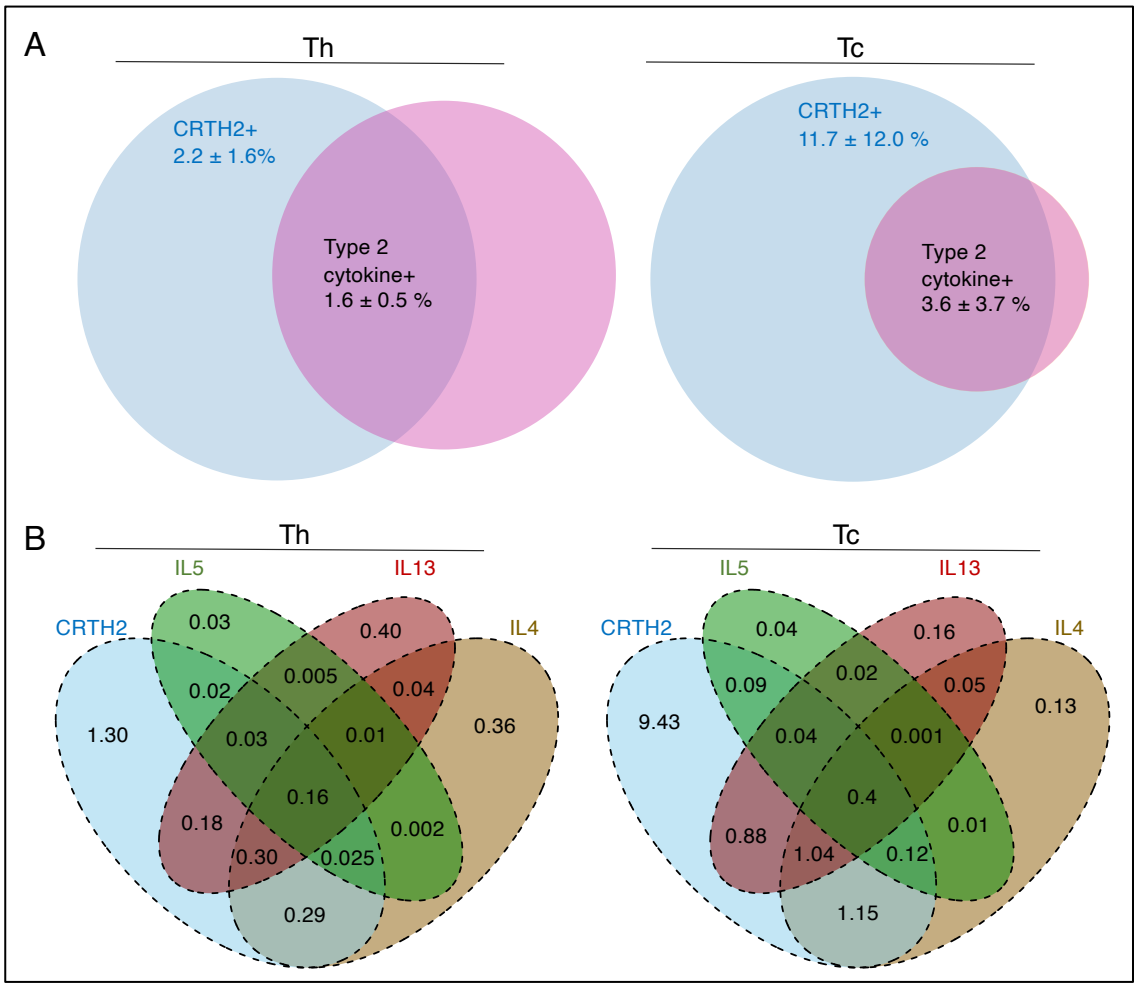


Figure 34 Venn diagrams of CRTH2 expression and type 2 cytokine production by T cells

Numbers represent the percentage of Th or Tc cells from PBMCs that express the indicated surface marker before stimulation and/or cytokine after stimulation. Type 2 cytokine production for CRTH2$^+$ and CRTH2$^-$ T cells was measured as described in the legend for Figure 31. CRTH2 expression data were taken from the unstimulated group described in Figure 30. (A) in this case IL-4, IL-5 or IL-13 are considered together as type 2 cytokines (in purple) and compared to CRTH2 expression (in blue) as an area-proportional Venn diagram, drawn on meta-chart.com. Tc and Th are considered separately and are not proportionate to each other. Values indicate mean ± SD. (B) Percentages of CRTH2 expression and production of individual type 2 cytokines. Diagrams were calculated with the VennDiagram package (H. Chen, 2018).

The final part of this first chapter aim is to quantify the ability of surface CRTH2 staining to act as a screening test for type 2 T cells, where production of type 2 cytokines is considered as the 'gold standard'. Using the same data (Supplementary Table 11 and Supplementary Table 12), I derived proportions of true positives (e.g., CRTH2$^+$ IL-5$^+$), true negatives (e.g., CRTH2$^-$ IL-5$^-$), false positives (e.g., CRTH2$^+$ IL-5$^-$) and false negatives (CRTH2$^-$ IL-5$^+$) for each cytokine individually, and for expression of any cytokine (IL-4,

IL-5, or Il-13) or a combination of all of them (IL-4, IL-5 and IL-13). These parameters were used to derive values for four test indicators: sensitivity, specificity, PPV, and NPV (see 3.2.6 for method) and the results are shown in Table 8. The test indicator values were compared between Th and Tc cells (Table 8). In general, CRTH2 was a more sensitive marker of Tc2 cells than Th2 cells but it was also less specific and had a lower PPV in the Tc group. The NPV was very high in all cases. Secondly, the test indicator values were compared between the different cytokine profiles (e.g., IL-4 vs IL-5), within Th or Tc cells (Figure 35). This showed that CRTH2 was most sensitive as a marker of triple positive type 2 T cells. For Th2 cells, CRTH2 was a more sensitive as a marker of IL-5 than IL-13, and the PPV was always lowest (and NPV highest) for IL-5 production.

Table 8 Summary table of sensitivity, specificity, PPV, and NPV for surface CRTH2 as a test for capacity to produce type 2 cytokines, compared between Th and Tc

For definitions of sensitivity, specificity, PPV and NPV, see section 3.2.6. Values shown are the mean ± SD of eight biological replicates. Groups were analysed by 2-way ANOVA in which the row factor was the cytokine profile and the column factor was the cell type (Th or Tc). Stars after the test name indicate significance of the difference between Th and Tc for that test overall. Specific comparisons for each combination of test indicator and cytokine profile (e.g., sensitivity of CRTH2 for IL-4 detection), between Th and Tc, were made by Šidák's multiple comparisons test. Significant results are indicated by stars after the value. Comparisons within one cell type (e.g., Th) between cytokine profiles, for each individual test, are shown in Figure 35.

		Test indicator			
	Cytokine profile	**Sensitivity******	**Specificity******	**PPV******	**NPV**
Tc	**IL-4+**	88.8 ± 8.0**	90.1 ± 10.9	22.9 ± 9.1	99.7 ± 0.3
	IL-5+	87.2 ± 9.0	88.8 ± 11.8	7.3 ± 3.5	99.9 ±0.1
	IL-13+	84.8 ± 12.0**	89.9 ± 10.9	19.3 ± 8.0*	99.7 ± 0.4
	Any T2C (4, 5, OR 13+)	83.2 ± 11.0***	90.9 ± 10.3	30.7 ± 12.0	99.4 ± 0.5
	All T2C (4, 5, AND 13+)	95.5 ± 3.5	88.6 ± 11.9	4.1 ± 2.5	100.0 ± 0.0
Th	**IL-4+**	62.7 ± 23.2**	98.4 ± 1.3	33.7 ± 10.5	99.6 ± 0.4
	IL-5+	74.3 ± 19.4	98.0 ± 1.5	10.1 ± 5.6	99.9 ± 0.0
	IL-13+	56.4 ± 19.9**	98.3 ± 1.3	29.0 ± 8.9*	99.5 ± 0.2
	Any T2C (4, 5, OR 13+)	53.6 ± 21.3***	98.6 ± 1.2	43.7 ± 12.4	99.2 ± 0.4
	All T2C (4, 5, AND 13+)	89.6 ± 6.7	97.9 ± 1.5	6.8 ± 4.3	100.0 ± 0.0

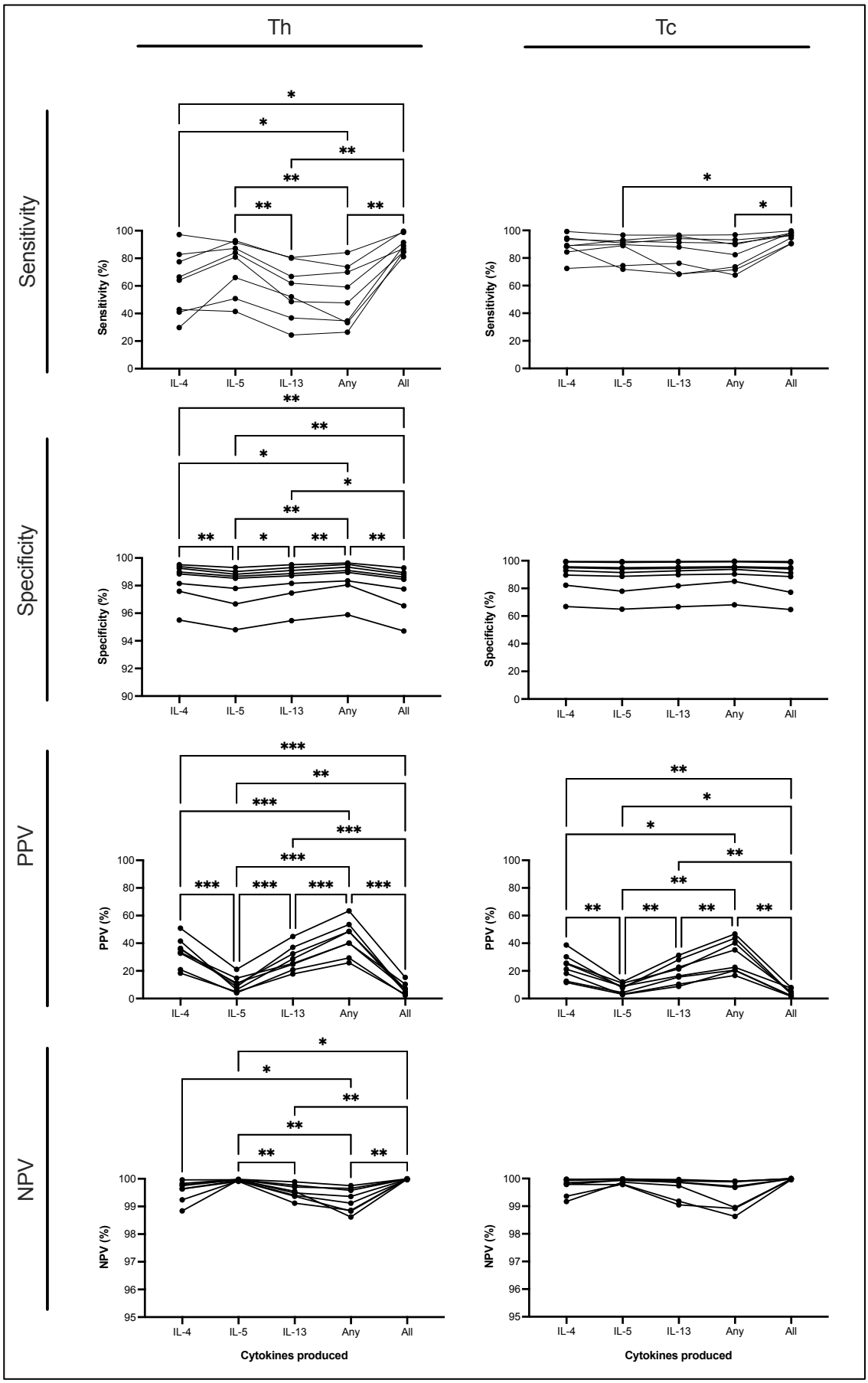

Th
Tc
Sensitivity
Specificity
PPV
NPV
Sensitivity (%)
Specificity (%)
PPV (%)
NPV (%)
IL-4
IL-5
IL-13
Any
All
Cytokines produced

Figure 35 Comparisons between cytokine profiles, within Th or Tc, of sensitivity, specificity, PPV, and NPV values for CRTH2 as a marker of type 2 T cells

Within each graph, the cytokine profiles on the x-axis were compared overall by one-way ANOVA and individual comparisons made by Tukey's multiple comparisons test. On the x-axis, the cytokine profile is indicated, for example 'IL-5' indicates the population of IL-5$^+$ cells as detected by ICS. 'Any' refers to IL-4, IL-5 OR IL-13 production i.e., any type 2 T cell. 'All' refers to the IL-4, IL-5, AND IL-13, i.e., the triple-positive cell population. Data points from the same donor are linked by lines and treated as paired data.

3.3.2 CRTH2$^+$ T cells can be expanded *in vitro* and retain their functional identity

The second aim of this chapter was to determine if a pure population of CRTH2$^+$ Tc cells can be cultured *in vitro* by the rapid expansion method outlined in 3.2.2. This would allow for downstream experiments that require a larger number of cells than can be easily obtained *ex vivo*.

3.3.2.1 Expanded CRTH2$^+$ T cells retain expression of surface markers

Bulk CRTH2$^+$ Tc or Th cell populations were sorted from PBMCs and expanded as described in 3.2.2. Purity of the resulting cultures was assessed by flow cytometry (3.2.4.1). Expanded CRTH2$^+$ Th or Tc cells generally retained stable expression of their surface markers, although there was variation between biological donors (Figure 36). Addition of polarising cytokines such as IL-4 or alarmin cytokines to the medium for the duration of culture did not reliably affect CRTH2 expression (Supplementary Figure 27). The level of surface CRTH2 expression did not appear to be affected by the culturing process itself (Supplementary Figure 28). There was no difference in CRTH2 expression level between cells cultured from healthy controls and patients with severe T2-high asthma (Supplementary Figure 28).

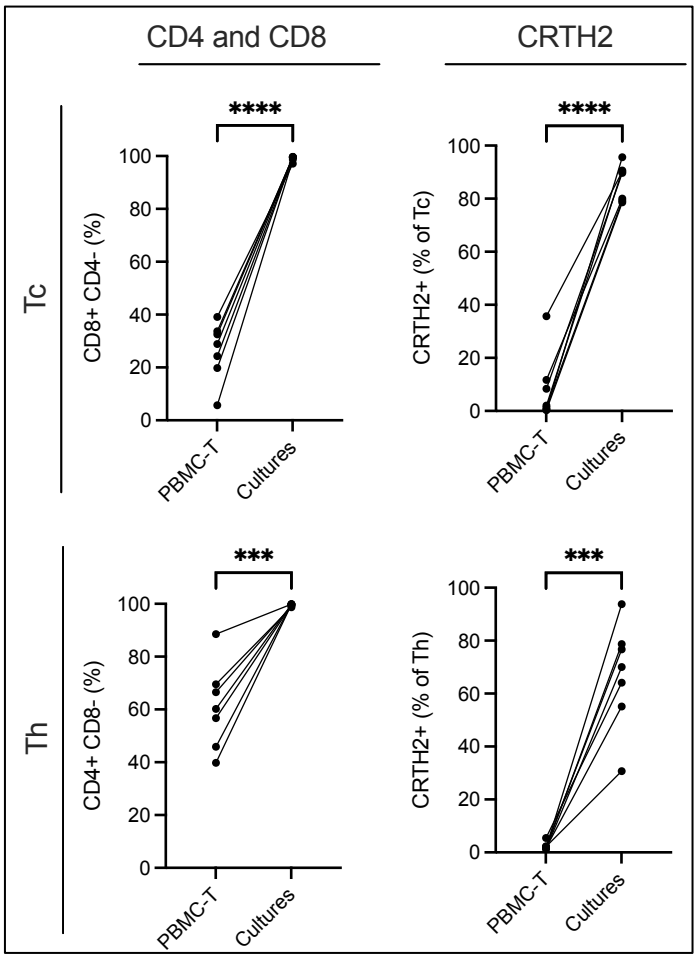


Figure 36 Expanded CRTH2$^+$ T cells retain their defining surface markers

CRTH2$^+$ T cells were sorted from PBMCs and expanded as described in 3.2.2. CD4, CD8, and CRTH2 expression were measured by flow cytometry (3.2.4.1). Enrichment of the target population was compared to T cells from PBMCs ('PBMC-T'). The two groups were compared by two-tailed paired t-test. N = 7 biological replicates.

It was important to rule out the possibility that CRTH2 expression was being induced by the culture method itself. Therefore, I expanded CRTH2$^-$ CD8$^+$ T cells using the same method. Initial attempts showed that cultured CRTH2$^-$ CD8$^+$ T cells contained a proportion of contaminating CRTH2$^+$ T cells, although expression was lower than for CRTH2$^+$ Tc cell cultures (Supplementary Figure 29). The culture method was improved by my colleague Dr Jian Luo, who hypothesised that FACS was not sensitive enough to detect low level CRTH2 expression and this could be the reason for CRTH2^{low} cell contamination in the CRTH2$^-$ cultures, rather than cells becoming CRTH2$^+$ during the culture. Instead, he

generated CRTH2$^-$ CD8$^+$ T cell cultures by first depleting CRTH2$^+$ cells from the sample by magnetic bead sorting (3.2.3) before proceeding in the same way (3.2.2). I analysed the resulting CRTH2$^-$ Tc cultures by flow cytometry and found the purity to be improved with this double-depletion technique for isolating initial CRTH2$^-$ Tc cells (Figure 37).

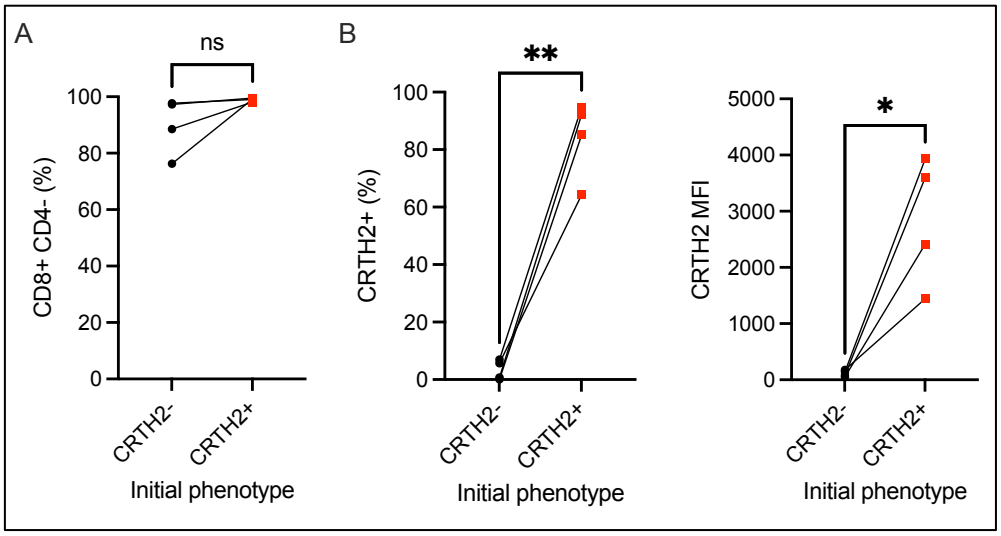


Figure 37 CRTH2$^-$ Tc cells can be cultured if CRTH2$^+$ T cells are depleted first

CRTH2$^+$ T cells were depleted from PBMCs by magnetic bead sorting (3.2.3) and then CRTH2$^-$ Tc cells were sorted and expanded (3.2.2). The surface protein profiles of the expanded CRTH2$^-$ Tc cells are compared to cells from CRTH2$^+$ Tc cultures. (A) Comparison of Tc purity within T cells (B) comparison of CRTH2 expression (gating and MFI) between cultured CRTH2$^-$ or CRTH2$^+$ Tc cells. Paired *t*-tests were used to compare means. N = 4 biological replicates.

3.3.2.2 Expanded CRTH2$^+$ Tc cells are potent type 2 T cells particularly associated with production of IL-5

In addition to retention of surface markers, I asked if cultured CRTH2$^+$ T cells retain their type 2 identity compared to CRTH2$^-$ Tc cells. The most important aspect of this is the capacity to produce type 2 cytokines after stimulation. This was assessed by stimulation with PMA and ionomycin, followed by simultaneous ICS for IL-4, IL-5, and IL-13 (3.2.4.2). The proportion of cells that produced detectable cytokine, and the average fluorescence reading for each cytokine, were greater in the CRTH2$^+$ CD8$^+$ cultures for all three cytokines (Figure 38).

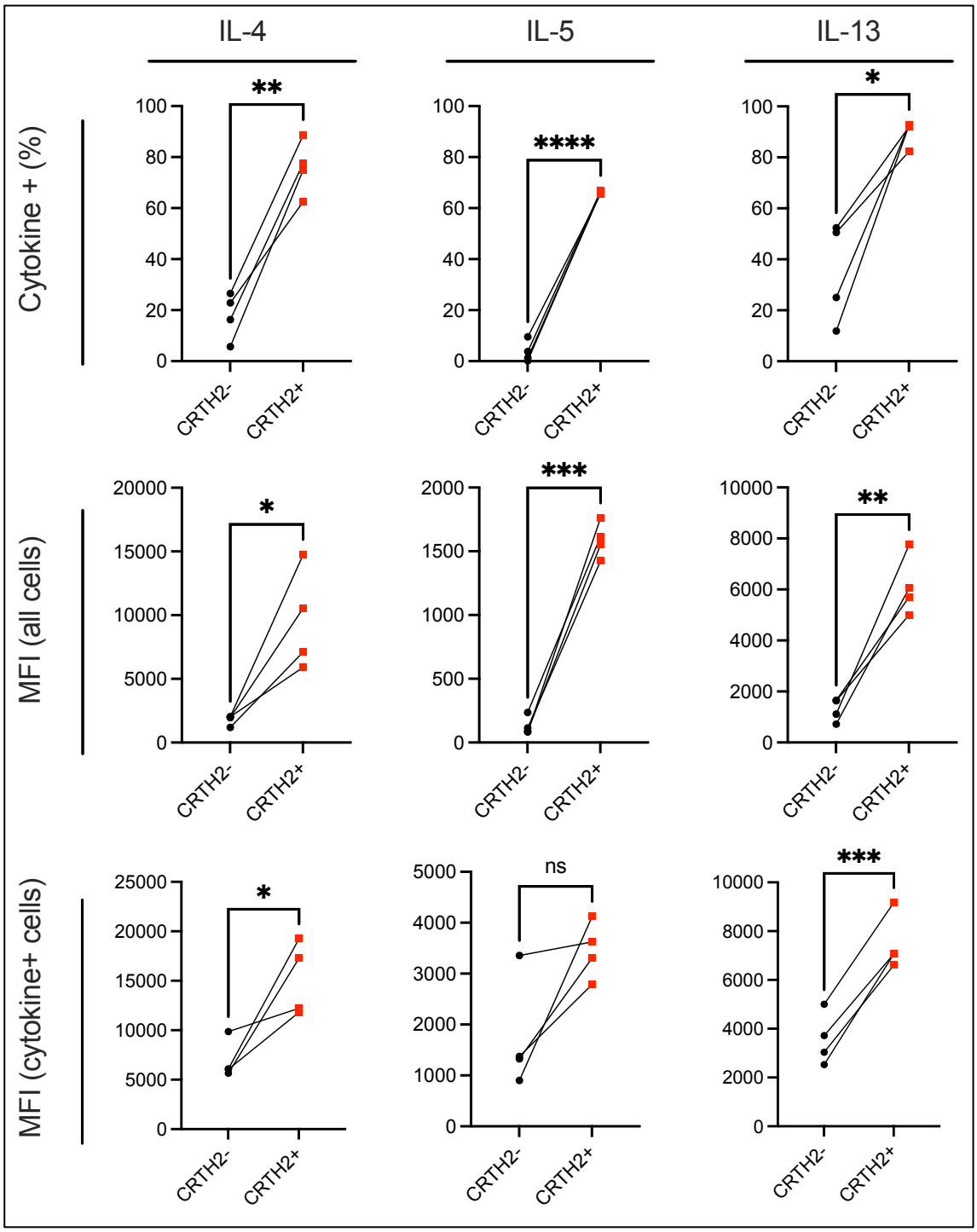


Figure 38 Type 2 cytokines produced by activated expanded CRTH2⁺ Tc cells

Cultured Tc cells were generated as described in 3.2.2. For CRTH2⁻ Tc cultures, depletion of CRTH2⁺ T cells was performed first as discussed in 3.3.2.1. Stimulation and cytokine detection were performed as described in 3.2.4.2. Groups were compared by paired *t*-test. N = 4 biological replicates.

CRTH2⁺ Tc cells were particularly dominant producers of IL-5, which was almost undetectable in CRTH2⁻ CD8⁺ cultures (Figure 38). For IL-4 and IL-13, a significant

minority of CRTH2$^-$ CD8$^+$ T cells could produce detectable cytokine (Figure 38). However, within positively staining cells, CRTH2$^+$ Tc cells were more potent producers of IL-4 and IL-13 than CRTH2$^-$ equivalents (Figure 38). In summary, CRTH2$^+$ Tc cells can produce high quantities of IL-4, IL-5, and IL-13, whereas most CRTH2$^-$ CD8$^+$ T cells cannot produce any type 2 cytokines. A subpopulation of CRTH2$^-$ CD8$^+$ T cells can produce IL-4 and IL-13 to a lesser degree than CRTH2$^+$ equivalents. This is in agreement with the *ex vivo* data (Figure 34B).

3.3.2.3 Expanded CRTH2$^+$ Tc cells migrate towards PGD_2

The most well-established function of CRTH2 is as a chemotactic receptor that facilitates migration along gradients of PGD_2 (Hirai et al., 2001). Cultured CRTH2$^+$ Tc cells retained the ability to migrated towards PGD_2, whereas CRTH2$^-$ controls did not (Figure 39).

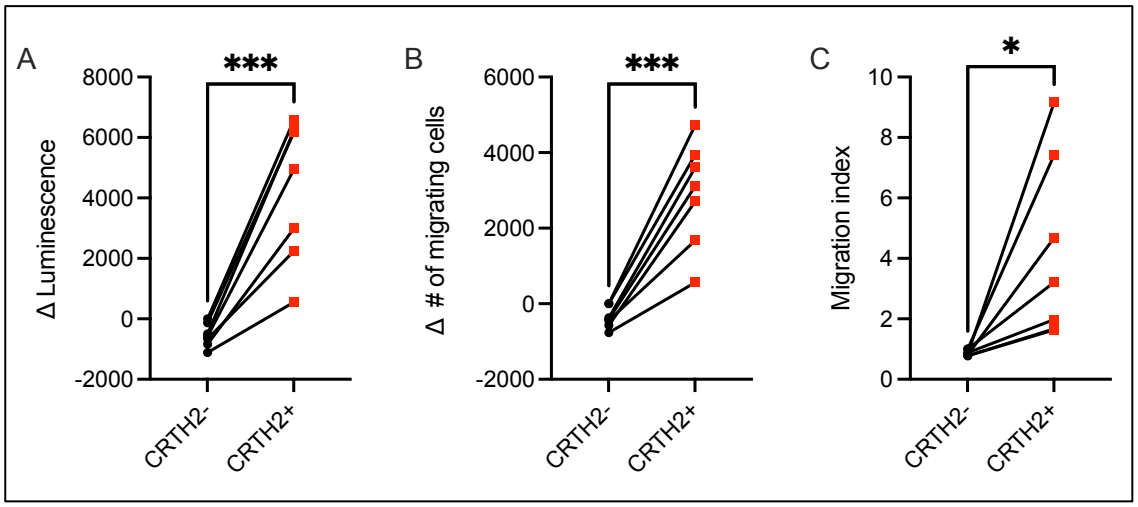


Figure 39 Expanded CRTH2$^+$ Tc cells migrate towards PGD_2

Expanded CRTH2$^+$ or CRTH2$^-$ Tc cells were assayed for migration towards PGD_2 using a chemotaxis assay (3.2.5). (A) ΔLuminescence refers to the difference between luminescence values measured in 'test' lower chambers containing 20nM PGD_2 and 'control' chambers containing only medium. (B) 'Δ # of migrating cells' is equivalent to 'ΔLuminescence' except that luminescence values have been converted to cell counts based on a standard curve (Supplementary Figure 30). Migration index is the ratio of number of cells migrated towards the test medium (with PGD_2) vs control medium. All data points are the median of three technical replicates. Groups were compared by a paired *t*-test. N = 7 biological replicates.

3.3.2.4 Expanded CRTH2+ Tc cells express elevated GATA3 protein

Type 2 T cell function is associated with expression of the transcription factor GATA3 (T. Nakayama et al., 2017). Therefore, I asked if cultured CRTH2+ Tc cells were enriched for GATA3 compared to CRTH2- equivalents. CRTH2+ Tc cells were very highly enriched for GATA3 expression on flow cytometry plots (Figure 40A), with an increased proportion staining positive (Figure 40B) and higher mean fluorescence intensity (MFI) values both in the overall population (Figure 40C) and within GATA3+ staining cells (Figure 40D).

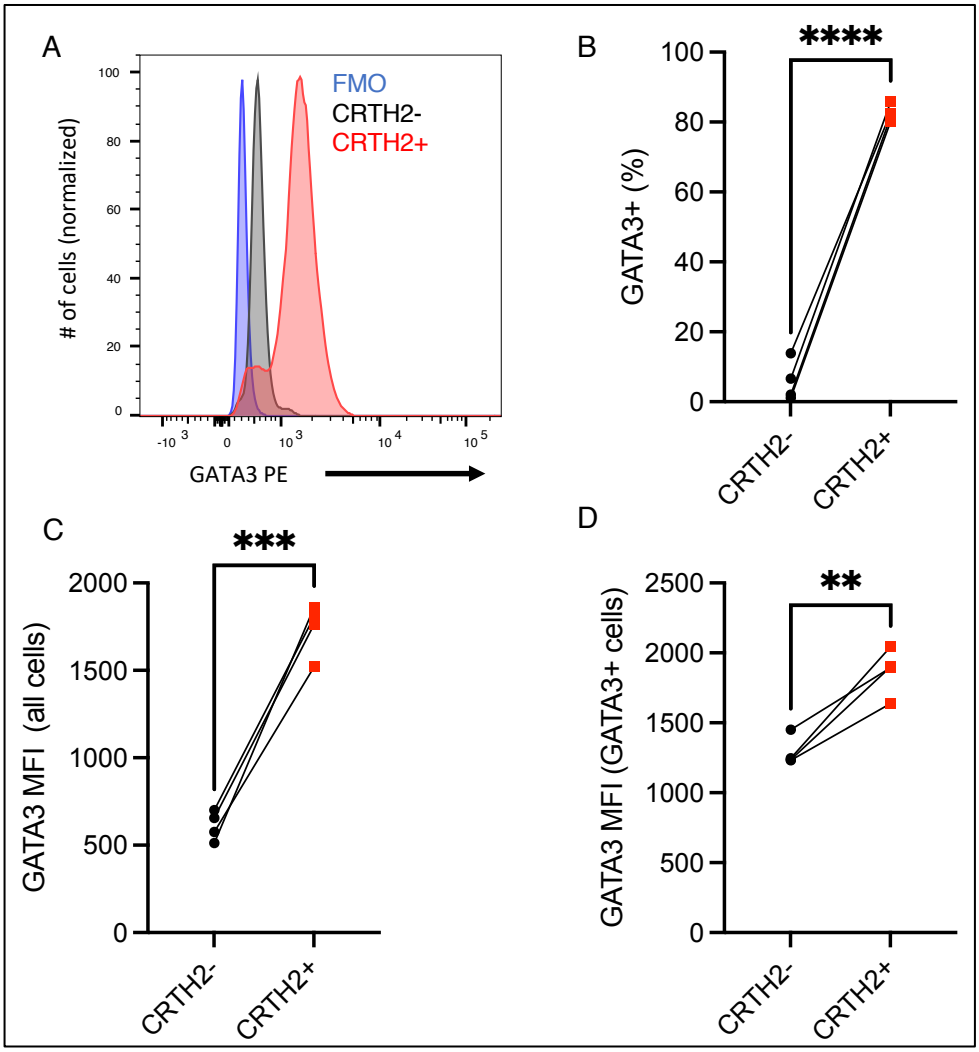


Figure 40 Expanded CRTH2+ Tc cells are enriched for GATA3 transcription factor

Expanded CRTH2+ or CRTH2- Tc cells were stained for surface markers and intranuclear GATA3 protein as described in 3.2.4.3. N = 4 biological replicates. FMO = fluorescence minus one (GATA3-PE) control carried out on a CRTH2+ population to ensure GATA3 detection was unaffected by the level of other surface markers.

3.4 Discussion

3.4.1 CRTH2 is a good, but not universal, marker of type 2 T cells

This chapter explored the relationship between surface CRTH2 expression and production of the type 2 cytokines IL-4, IL-5, and IL13. I treated CRTH2 as a 'test' marker and production of type 2 cytokines after stimulation as a 'gold standard' of identification. The data supported the use of CRTH2 as a marker of type 2 T cells in both Th and Tc subsets. In general, sensitivity was greater than 75 % for Tc2 cells and greater than 50 % for Th2 cells, and specificity was approximately 90 % for Tc2 and 98 % for Th2, depending on which cytokine profile was used to define a type 2 T cell. Naturally, these values raise the questions: what are the biological roles of the large populations of $CRTH2^-$ Th2 cells? And why is there a large proportion of $CRTH2^+$ Tc cells which did not appear to be Tc2? The NPV value of CRTH2 was generally extremely high, but the PPV value was quite low. These values can all be considered by researchers who are using CRTH2 as a surrogate for type 2 T cells, as different test indicator values will be considered acceptable for different applications.

Improvements in fluorescence chemistry (Chattopadhyay et al., 2012) since previous similar research (Cosmi et al., 2000) has allowed quantification of all three type 2 cytokines simultaneously rather than separately, which is an advantage of this study. However, a technical limitation of this work is the sensitivity of detection of CRTH2 and type 2 cytokines by fluorochrome-conjugated antibodies. Weakly-positive cells could have been missed because they were not resolved from negative populations on the flow plots. The expansion experiments suggested that FACS alone is not sufficient to identify the full $CRTH2^+$ population, because we required an extra sorting step with magnetic beads in order to isolate and culture a truly CRTH2-negative T cell population. In this study I

compensated for CRTH2 downregulation after stimulation by fractionating the cells before stimulation, but prestaining for CRTH2 may be a more economical and equally effective strategy. Type 2 cytokines may be even harder to stain than CRTH2, being located inside the cell. Furthermore, a single stimulation time point was used, ignoring the kinetics of cytokine production (North et al., 1996) which are likely to differ between individual cells and cytokines. It is therefore likely that the specificity and PPV of CRTH2 has been underestimated in this study. In future, antibody clones may be refined to have higher specificity or affinity, or perhaps nucleotide-conjugated antibodies (Shahi et al., 2017) will enhance assay sensitivity.

The data presented here suggest heterogeneity of type 2 T cells, with a population of $CRTH2^+$ T cells capable of producing high levels of IL-4, IL-5, and IL-13, and subpopulations of $CRTH2^-$ T cells capable of producing lower levels of IL-4 or IL-13. This is in line with the CRTH2 discovery paper which found that $CRTH2^+$ T cells make more type 2 cytokines than $CRTH2^-$ Th2 cells (Nagata, Tanaka, et al., 1999). It is also coherent with single cell transcriptomic data that identified a cluster of $CRTH2^+$ T cells termed the Th2A subset which were the sole producers of IL-5, but several clusters of IL-13 producing T cells (Bangert et al., 2021). The $CRTH2^+$ T cells in this study may therefore represent a *Tc2A* phenotype. Alternatively, a Tpath2 phenotype has also been described to involve IL-5 and CRTH2 (T. Nakayama et al., 2017). It is not clear to what extent Tpath2 and Th2A describe the same cells. I did not measure cytokines associated with other T cell phenotypes (e.g., Th1-associated IFNγ) and cannot comment on whether $CRTH2^+$ Tc cells are capable of more than a type 2 response, known as a hybrid population (J. A. Walker & McKenzie, 2018). The question of heterogeneity within type 2 T cell populations will be further explored as more single cell multi-omics data are made publicly available.

3.4.2 CRTH2$^+$ Tc cells are a stable population that retain their identity after massive expansion

Secondly, CRTH2$^+$ Tc cells were isolated and massively amplified. Their identity as type 2 cells remained stable after expansion and culture. Although CRTH2 expression was linked to all three type 2 cytokines, there was a particularly strong relationship between CRTH2 and IL-5, implying a role in eosinophilic inflammation (Rosenberg et al., 2013). The proportion of cytokine producing cells was higher in cultured cells than *ex vivo,* and this is in agreement with previous data (Nagata, Tanaka, et al., 1999).

The differentiation of type 2 T cells has been investigated by many immunologists including the eminent William E. Paul (W. E. Paul, 2010), who wrote about the importance of the distinction between *in vitro* and *in vivo* type 2 differentiation of T cells. The key difference between this study and other studies is that the CRTH2$^+$ T cells were differentiated *in vivo* and merely expanded *in vitro*, rather than being wholly or partially differentiated *in vitro.* This idea is supported by the observation that CRTH2$^-$ controls remained negative after culture. It is notable that the identity of both CRTH2$^+$ and CRTH2$^-$ Tc cells remained stable despite strong TCR triggering and high dose IL-2 in the culture medium.

This data contrasts with a previous claim that Tc2 identity was unstable after *in vitro* differentiation with type 2 cytokines (A. Fox et al., 2018). To me, this suggests that there are fundamental differences between the *in vitro* and *in vivo* differentiation processes, with the latter apparently resulting in a more stable phenotype. It may be informative to compare the identities of type 2 T cells which have been differentiated *in vitro* with these *in vivo* differentiated CRTH2$^+$ T cells. The stability of CRTH2$^+$ T cell identity after expansion and culture, even in the presence of a variety of cytokines, also suggests an underlying

epigenetic mechanism. Modern techniques for assessing the epigenome, such as ATAC-sequencing, are effective using small numbers of cells (Corces et al., 2017), or even single cells (Buenrostro et al., 2015), and would therefore be appropriate for the study of *ex vivo* $CRTH2^+$ T cells from blood and/or lung tissue samples.

3.4.3 Future applications of this method

Future research could use the massive expansion technique applied here to study type 2 T cell subsets of interest. We have informally tried expanding $CRTH2^+$ Tc cells from lung tissue and the results were similar to PBMC (Supplementary Figure 31). If tissue identities are stable after culture, it will be possible to compare $CRTH2^+$ T cells from different sites. Instead of relying on leukocyte cones, $CRTH2^+$ T cells can be isolated and expanded from blood or tissue samples of patients of specific clinical interest, to determine if type 2 T cells are fundamentally altered in disease contexts. Of course, this applies to any T cell subset depending on the availability of a specific surface marker like CRTH2 and its stability after the expansion process. In this way, rare subsets identified by large screening experiments could be amplified to facilitate downstream assays that explore their behaviour. It should also be possible to study $CRTH2^-$ type 2 T cell subsets, although these could be harder to isolate. One possible strategy being explored by our group is to use a cytokine secretion assay (Miltenyi Biotec, 2018) in conjunction with CRTH2 staining: the $CRTH2^-$ type 2 T cells could be isolated and expanded in the same way as described here.

A second potential application of this kind of work is in the field of therapeutic T cells. The rapid expansion protocol was originally developed to generate massive populations of antigen-specific T cells for therapeutic use (Riddell & Greenberg, 1990). Current protocols for manufacturing therapeutic T cells (Vormittag et al., 2018) do not necessarily consider the nature of the starting material beyond antigen specificity and some basic parameters

such as the CD4/CD8 ratio. Sometimes gamma chain cytokines such as IL-7 and IL-15 are included to promote memory cell formation, but I could not find examples of cytokines included to promote a specific 'flavour' of T cell response. As this therapeutic strategy develops, it may become clear that specific T cell subsets are desirable in different contexts, and these subsets may be identified prior to expansion or differentiated by adding different cytokines to the expansion medium.

3.5 Conclusion

CRTH2 can be used as a marker of type 2 T cells in both the Tc and Th subsets, although its test indicator values (e.g., sensitivity and specificity) are variable between donors and depending on what cytokine profile is used to define a type 2 T cell. Generally, CRTH2 is a more sensitive marker of Tc2 than Th2, due to a larger population of $CRTH2^-$ type 2 T cells in the Th compartment than Tc. These $CRTH2^-$ type 2 T cells produce IL-4 and/or IL-13, but to a lesser extent than $CRTH2^+$ type 2 T cells. CRTH2 is a more specific marker of Th2 than Tc2, because many $CRTH2^+$ Tc cells did not produce measurable quantities of cytokine. This could mean those $CRTH2^+$ Tc cells are not type 2 T cells, or it could be due to the stimulation and type 2 cytokine detection assay parameters.

$CRTH2^+$ Tc cells can be expanded *in vitro* by a rapid expansion protocol, to be studied as a bulk population. They retain key elements of their identity: production of type 2 cytokines after stimulation, chemotaxis towards PGD_2, and enrichment of the GATA3 transcription factor. This method permits the study of amplified populations of $CRTH2^+$ Tc cells originating from small samples.

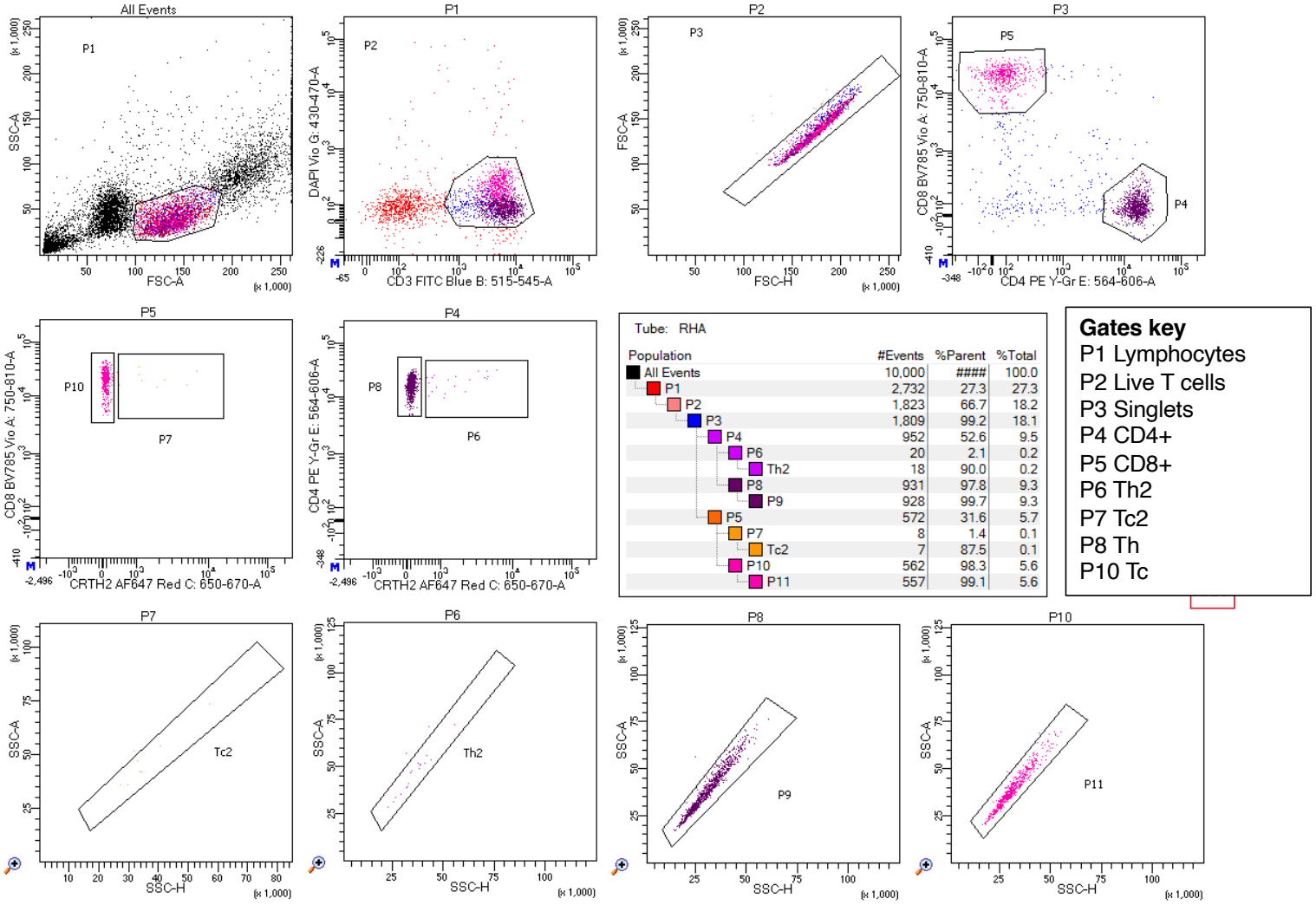
3.6 Appendix

Supplementary Table 1 Antibody panel for cell sorting

Cultured T cell panel					
Antigen	**Clone**	**Fluorochrome**	**Supplier**	**Number**	**Dilution**
CD3	OKT3	FitC	BioLegend	317306	100
CD4	RPA-T4	PE	BioLegend	300508	800
CD8	RPA-T8	BV785	BioLegend	344740	100
CRTH2	BM16	AF647	BioLegend	350104	50
Viability dye		DAPI	BioLegend	422801	50 000

Supplementary Table 2 Contents of Rapid Expansion Medium (REM)

Reagent (concentration)	**Supplier (code)**	**Purpose**
RPMI (90 %)	Sigma, (R8758-500ML)	Suitable cell culture medium for T cells (Riddell & Greenberg, 1990).
Human serum (10 %)	Sigma (H4522-100mL)	Contains a range of nutrients and growth factors (Master & O'Connor, 2018).
Interleukin 2 (250 U/mL)	BioLegend (589106)	Cytokine that controls T cell expansion (Boyman & Sprent, 2012) and is often added to T cell cultures (Master & O'Connor, 2018). Previous studies have used concentrations ranging from 50 U/mL (Riddell & Greenberg, 1990) to 6000 U/mL (Mark E. Dudley et al., 2003).
MEM NEAA (1 X)	Gibco (11140-050)	Amino acid supplement to support growth and proliferation (Master & O'Connor, 2018). Essential amino acids are already included in the culture medium.
Sodium Pyruvate (1 mM)	Gibco (11360-070)	Acts as a source of carbon and energy for cultured cells and may support clonal expansion (Freshney, 2016).
HEPES (1 mM)	Gibco (15630-056	A buffer to stabilise culture pH at physiological level (Riddell & Greenberg, 1990; Shipman, 1969)
2-Mercaptoethanol (1 X)	Gibco (31350-010)	Antioxidant which prevents oxidative stress by build-up of free radicals (Master & O'Connor, 2018; Riddell & Greenberg, 1990)



Supplementary Figure 25 Gating strategy for FACS of CRTH2⁺ T cells (courtesy of the NDM Experimental Medicine FACS facility)

Supplementary Table 3 Antibody panel for identification of type 2 T cells

Cultured T cell panel					
Antigen	**Clone**	**Fluorochrome**	**Supplier**	**Number**	**Dilution**
CD3	OKT3	FitC	BioLegend	317306	100
CD4	RPA-T4	PE	BioLegend	300508	800
CD8	RPA-T8	PB	BioLegend	301026	1000
CRTH2	BM16	AF647	BioLegend	350104	50
Viability dye		Zombie Aqua	BioLegend	423102	400

Supplementary Table 4 Antibodies for ICS of type 2 cytokines

ICS panel					
Antigen	**Clone**	**Fluorochrome**	**Supplier**	**Number**	**Dilution**
Viability dye		Zombie near-IR	BioLegend	423105	400
Extracellular Stain					
CD3	HIT8a	PB	BioLegend	317314	200
CD4	OKT4	AF700	BioLegend	317426	200
CD8	SK1	BV785	BioLegend	344740	100
CRTH2	BM16	PE	Miltenyi	130-113-600	100
Intracellular Stain					
IL-4	MP4-25D2	BV605	BioLegend	500827	50
IL-5	JES1-39D10	APC	BioLegend	500904	50
IL-13	85BRD	FitC	eBioscience	11-7136-41	50

Supplementary Table 5 Antibodies for intranuclear staining of GATA3

ICS panel					
Antigen	**Clone**	**Fluorochrome**	**Supplier**	**Number**	**Dilution**
Viability dye		Zombie Aqua	BioLegend	423102	400
Extracellular Stain					
CD3	OKT3	FitC	BioLegend	317306	100
CD4	OKT4	AF700	BioLegend	317426	200
CD8	RPA-T8	PB	BioLegend	301026	1000
CRTH2	BM16	AF647	BioLegend	350104	50
Intranuclear Stain					
GATA3	L50-823	PE	BD	560074	50

Supplementary Table 6 type 2 cytokine profiles of purified CRTH2$^+$ T cells from PBMCs

PBMCs were fractionated into CRTH2$^+$ and CRTH2- populations (3.2.3). CRTH2$^+$ cells were stimulated, and stained for type 2 cytokine production (3.2.4.2). In FlowJo, the 'Boolean gating' function was used to calculate each permutation of type 2 cytokine production. Any T2C$^+$ refers to production of IL-4, IL-5 OR IL-13.

Cell	Population	Donor							
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Tc	**IL-4$^+$ (%)**	21.20	11.60	12.50	25.20	18.20	38.70	25.60	30.20
	IL-5$^+$	8.90	3.12	4.25	8.59	2.89	12.00	11.10	7.72
	IL-13$^+$	16.40	10.40	15.50	22.70	8.79	31.30	21.40	28.10
	IL4/5$^+$	8.22	2.00	2.41	6.17	2.29	9.24	5.98	5.21
	IL4/13$^+$	15.10	5.79	8.17	13.70	7.04	24.30	10.30	15.70
	IL5/13$^+$	8.22	2.25	3.28	6.25	1.99	9.64	5.13	5.08
	IL4/5/13$^+$	7.53	1.66	2.09	4.88	1.82	7.95	3.42	3.56
	Any T2C$^+$	22.6	16.7	20.5	35.2	20.4	46.7	40.2	43.6
Th	**IL-4$^+$**	32.80	20.90	18.40	35.70	33.30	41.50	50.90	36.30
	IL-5$^+$	11.20	4.13	4.85	10.30	14.70	6.40	21.10	8.51
	IL-13$^+$	24.80	20.80	17.80	32.40	25.40	28.70	44.90	37.00
	IL4/5$^+$	8.40	2.73	3.38	7.60	11.50	5.18	18.00	6.26
	IL4/13$^+$	19.60	12.70	10.70	20.40	20.10	22.20	33.50	20.50
	IL5/13$^+$	7.84	3.32	4.12	8.60	12.20	5.45	17.10	6.21
	IL4/5/13$^+$	7.09	2.38	3.03	6.76	10.40	4.70	15.30	4.71
	Any T2C$^+$	40.1	29.4	25.9	48.5	40	48.5	63.4	53.5

Supplementary Table 7 Calculations to estimate the proportion of type 2 cytokine$^+$ and CRTH2$^+$ Tc cells within all Tc cells

Any T2C refers to IL-4, IL-5 OR IL-13 production. IL-4/5/13$^+$ indicates triple positivity for all three cytokines. The unweighted values indicate the percentage of cells in the CRTH2$^+$ Tc population which express each combination of cytokines. These are then weighted by the proportion of CRTH2$^+$ Tc cells in unstimulated samples of the same PBMCs, to give a final weighted value estimate for each population in the total Tc population. Abbreviations: T2C = type 2 cytokine.

		Donor							
	Population	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Unweighted (percentage of T2C$^+$ in CRTH2$^+$ Tc)	**IL-4$^+$ (%)**	21.20	11.60	12.50	25.20	18.20	38.70	25.60	30.20
	IL-5$^+$	8.90	3.12	4.25	8.59	2.89	12.00	11.10	7.72
	IL-13$^+$	16.40	10.40	15.50	22.70	8.79	31.30	21.40	28.10
	IL4/5$^+$	8.22	2.00	2.41	6.17	2.29	9.24	5.98	5.21
	IL4/13$^+$	15.10	5.79	8.17	13.70	7.04	24.30	10.30	15.70
	IL5/13$^+$	8.22	2.25	3.28	6.25	1.99	9.64	5.13	5.08
	IL4/5/13$^+$	7.53	1.66	2.09	4.88	1.82	7.95	3.42	3.56
	Any T2C$^+$	22.6	16.7	20.5	35.2	20.4	46.7	40.2	43.6
Weighting (i.e., proportion of CRTH2$^+$ in Tc)		0.055	0.357	0.117	0.093	0.061	0.014	0.007	0.357
Weighted (percentage of T2C$^+$ CRTH2$^+$ in Tc)	**IL-4$^+$**	1.16	4.14	1.46	2.33	1.10	0.55	0.18	10.78
	IL-5$^+$	0.49	1.11	0.50	0.80	0.18	0.17	0.08	2.76
	IL-13$^+$	0.90	3.71	1.81	2.10	0.53	0.44	0.15	10.03
	IL4/5$^+$	0.45	0.71	0.28	0.57	0.14	0.13	0.04	1.86
	IL4/13$^+$	0.83	2.07	0.96	1.27	0.43	0.34	0.07	5.60
	IL5/13+	0.45	0.80	0.38	0.58	0.12	0.14	0.04	1.81
	IL4/5/13$^+$	0.41	0.59	0.24	0.45	0.11	0.11	0.02	1.27
	Any T2C$^+$	1.24	5.96	2.40	3.26	1.24	0.66	0.28	15.57

Supplementary Table 8 Calculations to estimate the proportion of type 2 cytokine$^+$ and CRTH2$^-$ Tc cells within all Tc cells

Any T2C refers to IL-4, IL-5 OR IL-13 production. IL-4/5/13$^+$ indicates triple positivity for all three cytokines. The unweighted values indicate the percentage of cells in the CRTH2$^-$ Tc population which express each combination of cytokines. These are then weighted by the proportion of CRTH2$^-$ Tc cells in unstimulated samples of the same PBMCs, to give a final weighted value estimate for each population in the total Tc population. Abbreviations: T2C = type 2 cytokine.

		Donor							
	Population	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Unweighted (percentage of T2C$^+$ in CRTH2$^-$ Tc)	**IL-4$^+$**	0.150	0.830	0.210	0.021	0.070	0.210	0.033	0.640
	IL-5$^+$	0.059	0.130	0.220	0.031	0.019	0.059	0.010	0.200
	IL-13$^+$	0.130	0.260	0.950	0.084	0.036	0.140	0.069	0.820
	IL4/5$^+$	0.008	0.030	0.034	0.006	0.004	0.015	0.003	0.070
	IL4/13$^+$	0.047	0.110	0.130	0.006	0.020	0.066	0.007	0.180
	IL5/13$^+$	0.016	0.053	0.160	0.008	0.007	0.016	0.001	0.079
	IL4/5/13$^+$	0.008	0.018	0.029	0.002	0.004	0.012	0.001	0.042
	Any T2C$^+$	0.280	1.050	1.080	0.120	0.097	0.320	0.100	1.370
Weighting (i.e., proportion of CRTH2$^-$ in Tc)		0.945	0.643	0.883	0.907	0.939	0.986	0.993	0.643
Weighted (percentage of T2C$^+$ CRTH2$^-$ in Tc)	**IL-4$^+$**	0.142	0.534	0.185	0.019	0.066	0.207	0.033	0.412
	IL-5$^+$	0.056	0.084	0.194	0.028	0.018	0.058	0.009	0.129
	IL-13$^+$	0.123	0.167	0.839	0.076	0.034	0.138	0.069	0.527
	IL4/5$^+$	0.007	0.019	0.030	0.005	0.004	0.015	0.003	0.045
	IL4/13$^+$	0.044	0.071	0.115	0.005	0.019	0.065	0.007	0.116
	IL5/13$^+$	0.015	0.034	0.141	0.007	0.007	0.016	0.001	0.051
	IL4/5/13$^+$	0.007	0.012	0.026	0.002	0.004	0.012	0.001	0.027
	Any T2C$^+$	0.265	0.675	0.954	0.109	0.091	0.315	0.099	0.881

Supplementary Table 9 Calculations to estimate the proportion of type 2 cytokine$^+$ and CRTH2$^+$ Th cells within all Th cells

Any T2C refers to IL-4, IL-5 OR IL-13 production. IL-4/5/13$^+$ indicates triple positivity for all three cytokines. The unweighted values indicate the percentage of cells in the CRTH2$^+$ Th population which express each combination of cytokines. These are then weighted by the proportion of CRTH2$^+$ Th cells in unstimulated samples of the same PBMCs, to give a final weighted value estimate for each population in the total Th population. Abbreviations: T2C = type 2 cytokine.

		Donor							
	Population	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Unweighted (percentage of T2C$^+$ in CRTH2$^+$ Th)	IL-4$^+$	32.80	20.90	18.40	35.70	33.30	41.50	50.90	36.30
	IL-5$^+$	11.20	4.13	4.85	10.30	14.70	6.40	21.10	8.51
	IL-13$^+$	24.80	20.80	17.80	32.40	25.40	28.70	44.90	37.00
	IL4/5$^+$	8.40	2.73	3.38	7.60	11.50	5.18	18.00	6.26
	IL4/13$^+$	19.60	12.70	10.70	20.40	20.10	22.20	33.50	20.50
	IL5/13$^+$	7.84	3.32	4.12	8.60	12.20	5.45	17.10	6.21
	IL4/5/13$^+$	7.09	2.38	3.03	6.76	10.40	4.70	15.30	4.71
	Any T2C$^+$	40.1	29.4	25.9	48.5	40	48.5	63.4	53.5
Weighting (i.e., proportion of CRTH2$^+$ in Th)		0.015	0.023	0.054	0.037	0.017	0.013	0.013	0.013
Weighted (percentage of T2C$^+$ CRTH2$^+$ in Th)	IL-4$^+$	0.489	0.481	1.001	1.321	0.573	0.523	0.636	0.457
	IL-5$^+$	0.167	0.095	0.264	0.381	0.253	0.081	0.264	0.107
	IL-13$^+$	0.370	0.478	0.968	1.199	0.437	0.362	0.561	0.466
	IL4/5$^+$	0.125	0.063	0.184	0.281	0.198	0.065	0.225	0.079
	IL4/13$^+$	0.292	0.292	0.582	0.755	0.346	0.280	0.419	0.258
	IL5/13$^+$	0.117	0.076	0.224	0.318	0.210	0.069	0.214	0.078
	IL4/5/13$^+$	0.106	0.055	0.165	0.250	0.179	0.059	0.191	0.059
	Any T2C$^+$	0.597	0.676	1.409	1.795	0.688	0.611	0.793	0.674

Supplementary Table 10 Calculations to estimate the proportion of type 2 cytokine$^+$ and CRTH2$^-$ Th cells within all Th cells

Any T2C refers to IL-4, IL-5 OR IL-13 production. IL-4/5/13$^+$ indicates triple positivity for all three cytokines. The unweighted values indicate the percentage of cells in the CRTH2$^-$ Th population which express each combination of cytokines. These are then weighted by the proportion of CRTH2$^-$ Th cells in unstimulated samples of the same PBMCs, to give a final weighted value estimate for each population in the total Th population. Abbreviations: T2C = type 2 cytokine.

		Donor							
	Population	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Unweighted (percentage of T2C$^+$ in CRTH2$^-$ Th)	**IL-4$^+$**	0.250	1.160	0.220	0.039	0.170	0.760	0.360	0.370
	IL-5$^+$	0.032	0.050	0.041	0.037	0.020	0.079	0.063	0.092
	IL-13$^+$	0.230	0.450	0.510	0.300	0.110	0.630	0.600	0.880
	IL4/5$^+$	0.013	0.015	0.029	0.003	0.001	0.008	0.043	0.009
	IL4/13$^+$	0.041	0.064	0.085	0.004	0.014	0.065	0.110	0.027
	IL5/13$^+$	0.011	0.015	0.053	0.006	0.001	0.013	0.043	0.012
	IL4/5/13$^+$	0.010	0.013	0.026	0.003	0.001	0.007	0.035	0.006
	Any T2C$^+$	0.420	1.380	0.640	0.350	0.250	1.170	0.880	1.140
Weighting (i.e., proportion of CRTH2$^-$ in Th)		0.985	0.977	0.946	0.963	0.983	0.987	0.988	0.987
Weighted (percentage of T2C$^+$ CRTH2$^-$ in Th)	**IL-4$^+$**	0.246	1.133	0.208	0.038	0.167	0.750	0.356	0.365
	IL-5$^+$	0.032	0.049	0.039	0.036	0.020	0.078	0.062	0.091
	IL-13$^+$	0.227	0.440	0.482	0.289	0.108	0.622	0.593	0.869
	IL4/5$^+$	0.013	0.015	0.027	0.003	0.001	0.008	0.042	0.009
	IL4/13$^+$	0.040	0.063	0.080	0.004	0.014	0.064	0.109	0.027
	IL5/13$^+$	0.011	0.015	0.050	0.006	0.001	0.013	0.042	0.012
	IL4/5/13$^+$	0.010	0.013	0.025	0.003	0.001	0.007	0.035	0.006
	Any T2C$^+$	0.414	1.348	0.605	0.337	0.246	1.155	0.869	1.131

Supplementary Table 11 Derived proportions of each possible combination of CRTH2 expression and type 2 cytokine production within Tc cells

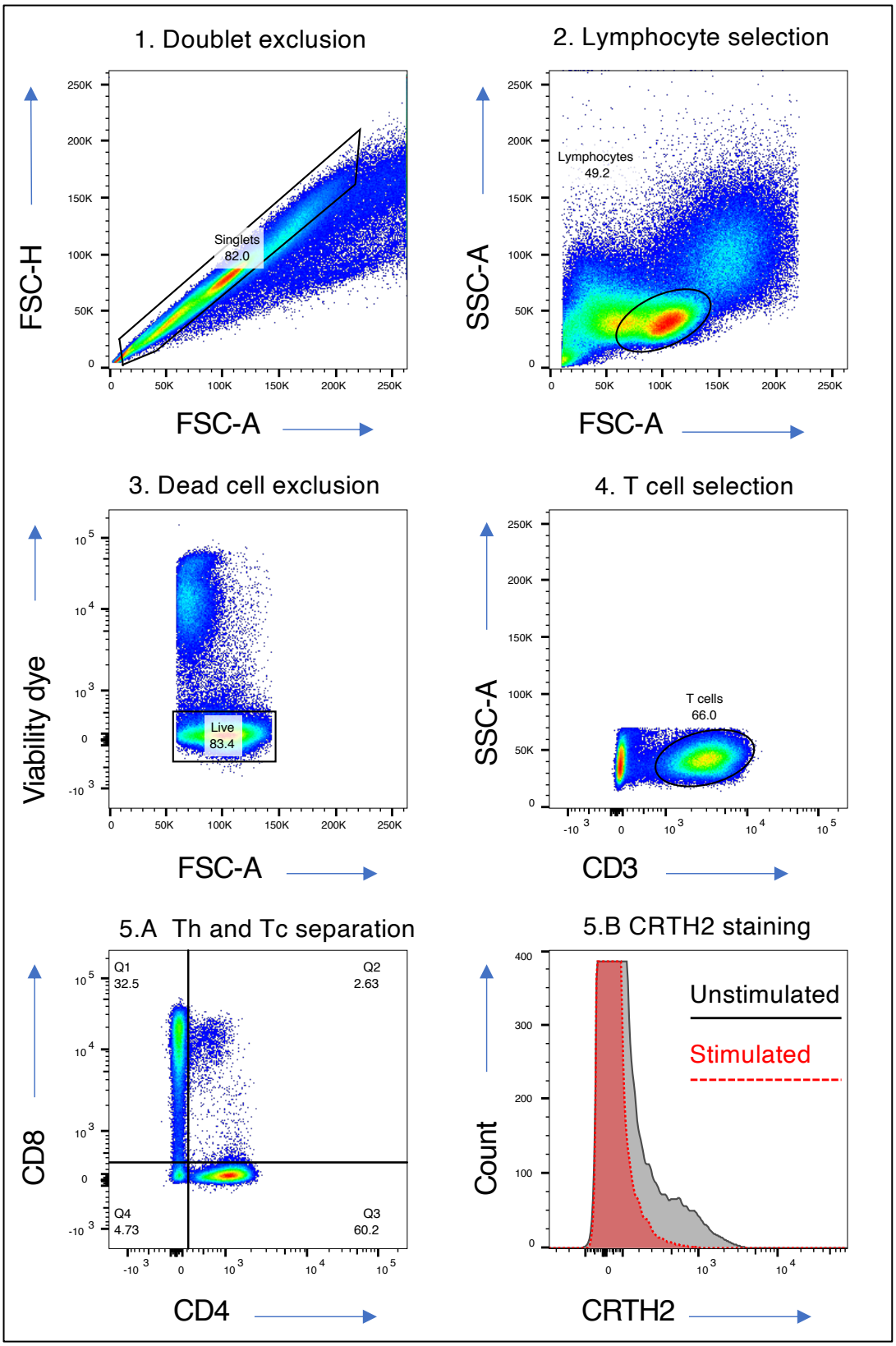
These data represent the estimated proportions of each permutation of CRTH2 and/or type 2 cytokine expression within the total Tc population. When necessary they are calculated by summing the weighted values found in Supplementary Table 7 and Supplementary Table 8 (e.g., IL-4$^+$ was calculated by summing the weighted value of CRTH2$^+$IL-4$^+$ and CRTH2$^-$IL-4$^+$). Phenotypes that are CRTH2$^+$ are equal to the weighted values in Supplementary Table 7.

	Donor							
Phenotype	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
CRTH2$^+$	5.470	35.700	11.700	9.260	6.070	1.410	0.690	23.400
IL4$^+$	1.301	4.675	1.648	2.353	1.170	0.753	0.209	7.557
IL5$^+$	0.543	1.197	0.692	0.824	0.193	0.227	0.086	1.960
IL13$^+$	1.020	3.880	2.652	2.178	0.567	0.579	0.216	7.204
CRTH2$^+$IL4$^+$	1.160	4.141	1.463	2.334	1.105	0.546	0.177	7.067
CRTH2$^+$IL5$^+$	0.487	1.114	0.497	0.795	0.175	0.169	0.077	1.806
CRTH2$^+$IL13$^+$	0.897	3.713	1.814	2.102	0.534	0.441	0.148	6.575
IL4$^+$IL5$^+$	0.457	0.733	0.312	0.577	0.143	0.145	0.044	1.273
IL4$^+$IL13$^+$	0.870	2.138	1.071	1.274	0.446	0.408	0.078	3.812
IL5$^+$IL13$^+$	0.465	0.837	0.525	0.586	0.128	0.152	0.037	1.249
CRTH2$^+$IL4$^+$IL5$^+$	0.450	0.714	0.282	0.571	0.139	0.130	0.041	1.219
CRTH2$^+$IL4$^+$IL13$^+$	0.826	2.067	0.956	1.269	0.427	0.343	0.071	3.674
CRTH2$^+$IL5$^+$IL13$^+$	0.450	0.803	0.384	0.579	0.121	0.136	0.035	1.189
IL4$^+$IL5$^+$IL13$^+$	0.419	0.604	0.270	0.454	0.115	0.124	0.025	0.865
CRTH2$^+$IL4$^+$IL5$^+$IL13$^+$	0.412	0.593	0.245	0.452	0.110	0.112	0.024	0.833
Any T2C$^+$	1.501	6.637	3.352	3.368	1.329	0.974	0.377	11.252
CRTH2$^+$ Any T2C$^+$	1.24	5.96	2.40	3.26	1.24	0.66	0.28	10.20

Supplementary Table 12 Derived proportions of each possible combination of CRTH2 expression and type 2 cytokine production within Th cells

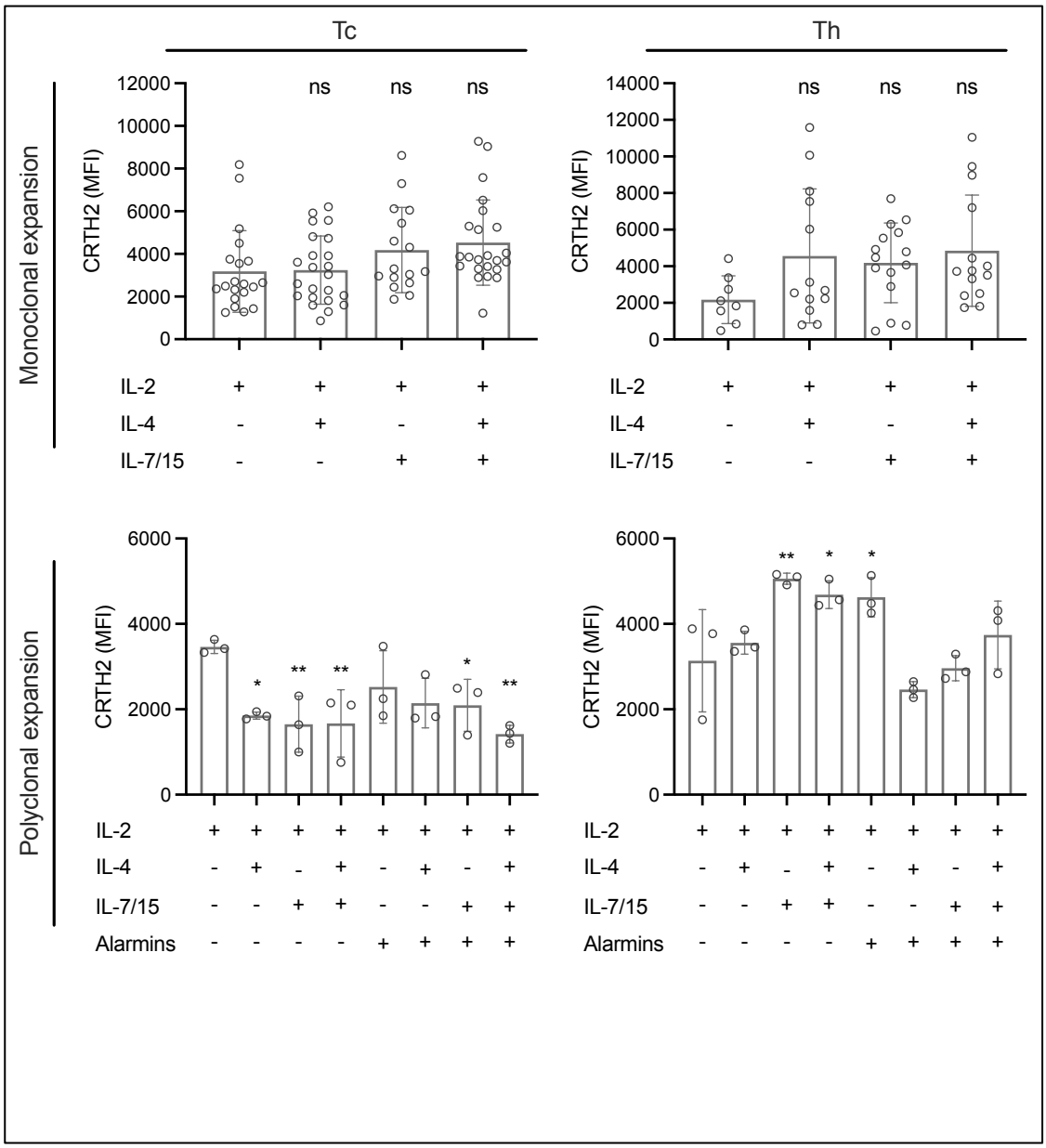
These data represent the estimated proportions of each permutation of CRTH2 and/or type 2 cytokine expression within the total Tc population. When necessary they are calculated by summing the weighted values found in Supplementary Table 9 and Supplementary Table 10 (e.g., IL-4$^+$ was calculated by summing the weighted value of CRTH2$^+$IL-4$^+$ and CRTH2$^-$IL-4$^+$). Phenotypes that are CRTH2$^+$ are equal to the weighted values in Supplementary Table 9.

	Donor							
Phenotype	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
CRTH2$^+$	1.490	2.300	5.440	3.700	1.720	1.260	1.250	0.760
IL4$^+$	0.735	1.614	1.209	1.358	0.740	1.273	0.992	0.643
IL5$^+$	0.198	0.144	0.303	0.417	0.272	0.159	0.326	0.156
IL13$^+$	0.596	0.918	1.451	1.488	0.545	0.984	1.154	1.155
CRTH2$^+$IL4$^+$	0.489	0.481	1.001	1.321	0.573	0.523	0.636	0.276
CRTH2$^+$IL5$^+$	0.167	0.095	0.264	0.381	0.253	0.081	0.264	0.065
CRTH2$^+$IL13$^+$	0.370	0.478	0.968	1.199	0.437	0.362	0.561	0.281
IL4$^+$IL5$^+$	0.138	0.077	0.211	0.284	0.198	0.073	0.267	0.056
IL4$^+$IL13$^+$	0.332	0.355	0.662	0.759	0.359	0.344	0.527	0.183
IL5$^+$IL13$^+$	0.128	0.091	0.274	0.324	0.211	0.082	0.256	0.059
CRTH2$^+$IL4$^+$IL5$^+$	0.125	0.063	0.184	0.281	0.198	0.065	0.225	0.048
CRTH2$^+$IL4$^+$IL13$^+$	0.292	0.292	0.582	0.755	0.346	0.280	0.419	0.156
CRTH2$^+$IL5$^+$IL13$^+$	0.117	0.076	0.224	0.318	0.210	0.069	0.214	0.047
IL4$^+$IL5$^+$IL13$^+$	0.115	0.067	0.189	0.253	0.180	0.066	0.226	0.042
CRTH2$^+$IL4$^+$IL5$^+$IL13$^+$	0.106	0.055	0.165	0.250	0.179	0.059	0.191	0.036
Any T2C$^+$	1.011	2.024	2.014	2.132	0.934	1.766	1.662	1.538
CRTH2$^+$ Any T2C+	0.597	0.676	1.409	1.795	0.688	0.611	0.793	0.407



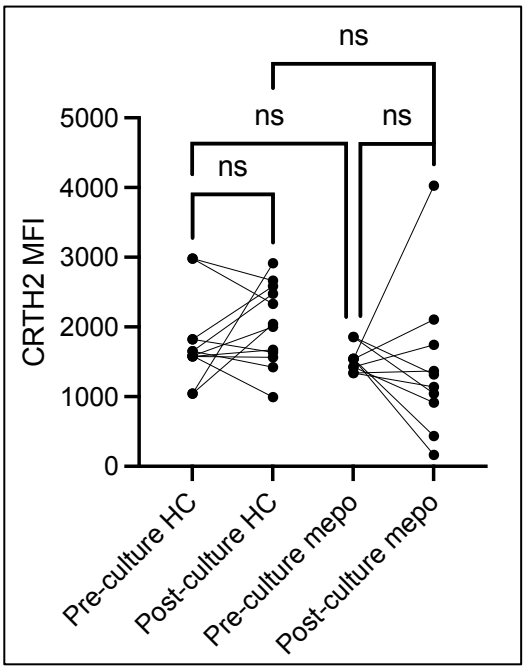
Supplementary Figure 26 CRTH2 downregulation after stimulation visible on flow plot

PBMCs were stimulated for six hours with PMA (50 ng/mL) and ionomycin (1 µg/mL) in the presence of Brefeldin A. Surface and viability staining was performed using the panel given in Supplementary Table 3. The order of gating is indicated numerically above the flow plots.



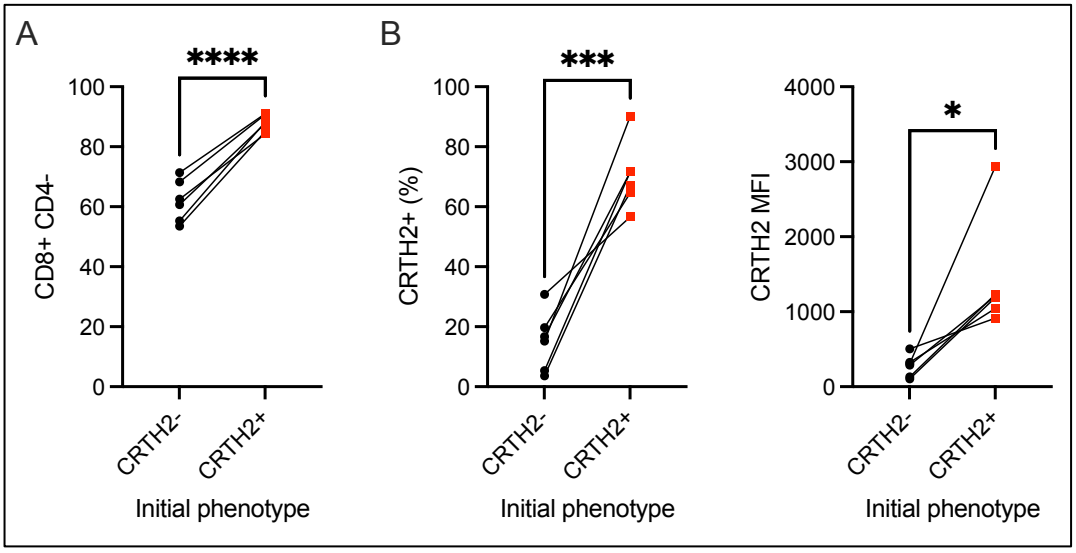
Supplementary Figure 27 Addition of polarising cytokines to the cell culture medium did not have reliable effects on CRTH2 expression

CRTH2+ Tc or Th cells were sorted and cultured as described in 3.2.2. Polarising cytokines were included in the culture medium as indicated in the figure. Concentrations of cytokine used were 10ng/mL except for IL-2 (250U/mL). Abbreviations: MFI, median fluorescence intensity. 'Alarmins' is a combination of IL-25, IL-33, and TSLP.



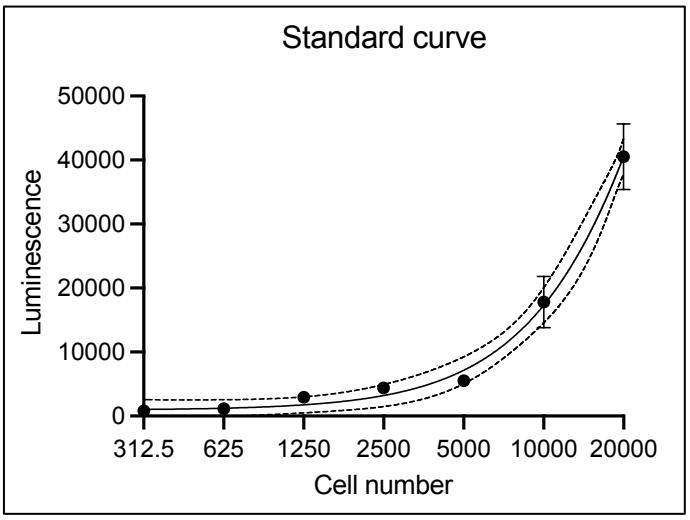
Supplementary Figure 28 No difference in CRTH2 expression on CRTH2⁺ Tc cells before vs after culture or in health vs type 2 high severe asthma

Data were collected from PBMCs ('pre-culture') or CRTH2⁺ Tc cells grown as described in 3.2.2 and analysed as described in 3.2.4.1 ('post-culture'). Groups were compared by one-way ANOVA with the Šidák correction for multiple comparisons. N = 5 donors for HC, 4 donors for mepolizumab. For each donor there is one value for the pre-culture MFI (the initial sample) and two random samples of post-culture cells were taken from the 48 well culture plate. Abbreviations: HC, healthy control; mepo, patients currently treated with mepolizumab for T2-high severe eosinophilic asthma.



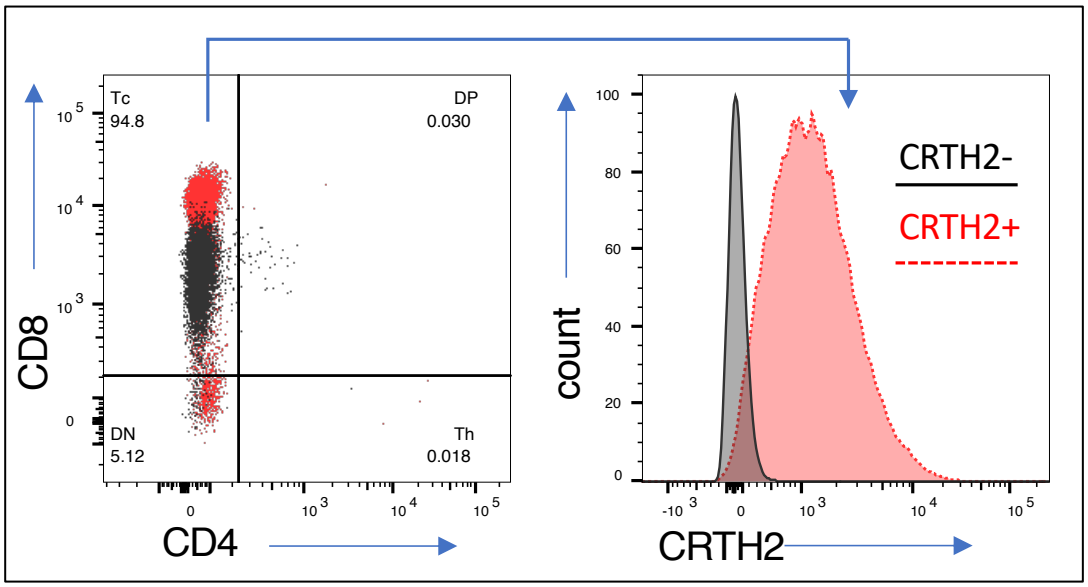
Supplementary Figure 29 CRTH2⁻ Tc cell cultures were partially positive for CRTH2 when starting CRTH2⁻ Tc cells were selected only by FACS

CRTH2⁺ Tc cells were cultured as described in 3.2.2 with the initial cells for culturing being selected only by FACS. Mean differences were assessed by paired *t*-test. (A) the proportion of cells at end of culture that were identifiable as Tc (CD8⁺ CD4⁻). (B) Expression of CRTH2 on cells at end of culture as measured by gating or MFI. N = 6 biological replicates.



Supplementary Figure 30 standard curve used for analysis of the chemotaxis assay

Cultured CRTH2+ Tc cells were counted by haemocytometer and plated in a 1:2 serial dilution.



Supplementary Figure 31 Purity of cultured CRTH2+ Tc cells derived from lung tissue

Cells from whole lung tissue were sorted and cultured as described in 3.2.2. After 3 weeks of expansion, cells were stained with antibodies outlined in Supplementary Table 1 and analysed by flow cytometry. Populations shown have previously been gated on live CD3+ T cells as outlined in Supplementary Figure 26 gates 1-4.

Chapter 4

Exploring the heterogeneity of CRTH2^{+} CD8^{+} T cell activation

4.1 Introduction

Asthma today is largely treated with beta agonists and corticosteroids (Pavord et al., 2018). Monoclonal therapies have been developed in recent years, mostly targeting elements of type 2 immunity, especially activators and products of type 2 lymphocytes (P. J. Barnes, 2018). One challenge with the application of these new drugs is to understand which patients will respond to which drugs. The benefit of a therapy for a heterogeneous disease can be missed if it is given to the wrong people through inadequate clinical phenotyping. This mistake was initially made in trials of mepolizumab, which missed a clinically relevant effect in the patient subgroup with a severe eosinophilic asthma phenotype (Pavord et al., 2018).

It is therefore important to understand type 2 lymphocyte activation, including Th2, Tc2, and ILC2 cell types. An unanswered question is whether type 2 lymphocytes display a uniform activation or if the response varies with the stimulus and cell identity. If the latter is true, then the airway inflammatory profiles of T2-high asthma (i.e., elevated eosinophils or FeNO) could exhibit a heterogeneity explained in part by the trigger of lymphocyte activation. A description of this complexity, if it exists, could contribute to rational design of monoclonal treatment plans for T2-high disease, and to further decipher the aetiology and pathogenesis of asthma.

The most well-described mode of T cell activation is through ligation of the TCR by cognate antigen presented on MHC molecules (Zinkernagel & Doherty, 1997). The downstream signalling that follows is complicated and incompletely understood (Hwang et al., 2020; Smith-Garvin et al., 2009). To summarise, a proximal signalling complex forms at the ligated TCR, containing activated phospholipase C-γ enzyme which catalyses the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers

inositol triphosphate (IP_3) and DAG (Figure 41). IP_3 triggers downstream calcium release and signalling including activation of nuclear factor of activated T-cells (NFAT) transcription factors (Trebak & Kinet, 2019). DAG goes on to activate the Ras proteins and PKC-θ. Activated Ras proteins induce signalling cascades including the MAPK/ERK pathway that promote cell growth, proliferation, differentiation, survival, and IL-2 production via the activator protein 1 (AP-1) transcriptional complex (Simanshu et al., 2017; Smith-Garvin et al., 2009). PKC-θ signalling has widespread effects such as activation of NF-κB, NFAT, and AP-1 (Jun/Fos) transcription factors which control aspects of T cell activation including effector cytokine production (Isakov & Altman, 2012; Pollizzi & Powell, 2014).

In addition to TCR triggering, ligation of CD28 on the T cell surface by CD80 or CD86 on the surface of activated APCs is part of the 'second signal' for complete cytotoxic T cell activation (van der Heide & Homann, 2016). Engagement of CD28 initiates complex signalling cascades from its cytoplasmic domain (reviewed in Esensten et al. 2016). One of the key downstream pathways involves recruitment of phosphoinositide 3-kinases (PI3Ks) (Smith-Garvin et al., 2009), which results in phospho-activation of PKC-θ through PDK1, and of Akt. Akt goes on to regulate the activity of mammalian target of rapamycin complex 1 (mTORC1), which has further complex effects including integration with the cell's metabolic status (Pollizzi & Powell, 2015). Other CD28 effects include augmented activation of Ras proteins and of NF-κB (via IκB kinase) (D. Bai et al., 2009).

There are mechanisms of type 2 T cell activation independent of antigen and the TCR. PGD_2 is a ligand for CRTH2 expressed on the surface of type 2 immune cells such as eosinophils and Th2 cells (Hirai et al., 2001). The PGD_2-CRTH2 axis plays a role in allergic and eosinophilic inflammation underlying some forms of asthma (Kupczyk & Kuna, 2017).

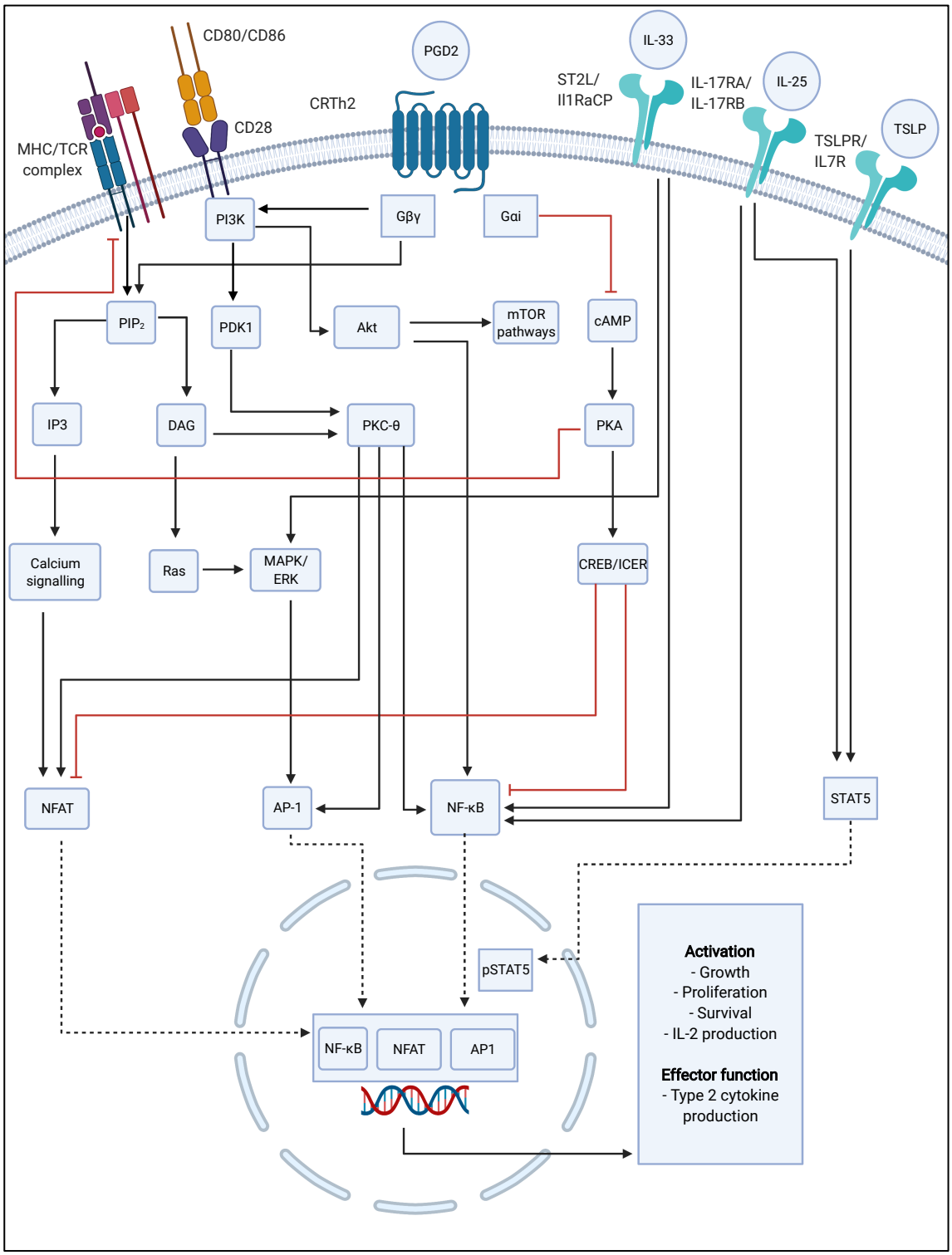


Figure 41 Schematic diagram of CRTH2+ T cell activation pathways

References to the steps of each pathway are in the main text. This diagram is a simplification of the full signalling pathways. Its design was influenced by other similar diagrams (Hwang et al., 2020; Pollizzi & Powell, 2014). Created with BioRender.com.

CRTH2 is a G-protein coupled receptor (Nagata, Tanaka, et al., 1999) which on ligation releases Gαi proteins, resulting in decreased adenylyl cyclase activity and cAMP levels (Kostenis & Ulven, 2006; Sawyer et al., 2002). The cAMP signalling pathway includes signalling through protein kinase A (PKA), which directly interferes with the proximal TCR complex (Wehbi & Taskén, 2016) and activates transcriptional repressors cAMP response element-binding protein (CREB) and inducible cAMP early repressor (ICER) that inhibit both NFAT (Bodor, 2017) and NF-κB (Wen et al., 2010). Thus, the PGD_2-CRTH2 signal releases a brake on NFAT and NF-κB activation. The Gβγ subunit is also released and goes on to catalyse formation of IP_3 and DAG (Kostenis & Ulven, 2006). Inhibition of calcium signalling downstream of IP_3 abrogates cytokine production (Xue et al., 2007). Finally, the CRTH2 receptor signals through PI3K, probably via its Gβγ subunit (Xue et al., 2007), and inhibitors of PI3K can abrogate both chemotaxis and cytokine production. The transcriptional response of cultured Th2 and Tc2 cells to PGD_2 has been explored by our laboratory previously using microarrays (Hilvering et al., 2018; Xue et al., 2015), identifying differential expression of thousands of genes including upregulation of type 2 cytokines. Recent data suggest that PGD_2 also stimulates its own autocrine production by type 2 lymphocytes (W. Chen et al., 2021; Luna-Gomes et al., 2011; Maric et al., 2019).

TSLP is an epithelial cytokine that promotes type 2 inflammation and is implicated in pathology including asthma (Corren & Ziegler, 2019). It binds to the TSLP receptor complex of TSLPR and IL7R-α (CD127) expressed by a range of immune cells including T cells (Park et al., 2000). TSLP promotes differentiation to Th2 either directly through induction of IL-4 (Omori & Ziegler, 2007) or indirectly via DCs (Soumelis et al., 2002). The receptor becomes specifically enriched on Th2 cells due to IL-4 action (Kitajima et al., 2011). After receptor ligation, multiple signalling pathways are activated including JAK1/2

mediated STAT5 signalling (Zhong et al., 2014). STAT5 refers to two proteins (STAT5a and STAT5b) which are most commonly activated by phosphorylation, and go on to translocate to the nucleus and exert effects via sequences termed GAS sites (Rani & Murphy, 2016). Activated STAT5 promotes survival, proliferation, and both production of and response to type 2 cytokines (Kitajima et al., 2011; Rochman et al., 2010, 2018; J. Zhu et al., 2003).

Interleukin 25 is one of the IL-17 family of cytokines and is associated with type 2 immunity, allergic inflammation, and asthma (Monin & Gaffen, 2018). It can be produced by cells including activated eosinophils or basophils (Y. H. Wang et al., 2007), respiratory epithelium (Monin & Gaffen, 2018) or Th2 cells themselves (Fort et al., 2001). IL-25 signals through a dimeric receptor consisting of IL-17RA and IL-17RB (Rickel et al., 2008) observed on a wide range of cells (Monin & Gaffen, 2018) and enriched on the surface of activated Th2 cells (Y. H. Wang et al., 2007) and ILC2s (Salimi et al., 2013). Downstream signalling partially involves both NF-κB (Maezawa et al., 2006) and STAT5 activation (Monin & Gaffen, 2018; L. Wu et al., 2015). The effects include enhanced production of type 2 cytokines by activated Th2 cells (Y. H. Wang et al., 2007).

Interleukin 33 is an alarmin cytokine released by damaged tissues with broad stimulatory effects on T cell subsets (Peine et al., 2016). IL-33 binds to the receptor ST2 to form a binary complex, which then recruits Interleukin-1 receptor accessory protein (IL-1RAcP) (X. Liu et al., 2013). The toll/interleukin-1 receptor (TIR) domains of ST2 and IL-1RAcP interact to activate NF-κB and MAPK pathways (Schmitz et al., 2005) as with other IL-1 family cytokines (Dunne & O'Neill, 2003). Expression of IL-1RacP is widespread in immune cells (The Human Protein Atlas, 2021a). In contrast, ST2 expression is more specific (The Human Protein Atlas, 2021b; Uhlén et al., 2015), including the surface of

human type 2 lymphocytes Th2 (D. Xu et al., 1998) and ILC2 (Mjösberg et al., 2011), and is positively regulated by canonical Th2 transcription factor GATA3 (L. Guo et al., 2009). IL-33 can augment Th2 differentiation (Peine et al., 2016), but also directly stimulates type 2 cytokine production by ILC2 (Salimi et al., 2013) or Th2 in conjunction with TCR triggering (Schmitz et al., 2005), or TSLP stimulation (L. Guo et al., 2009).

Together, the three aforementioned cytokines (TSLP, IL-25, and IL-33) are collectively referred to as 'alarmin cytokines', and they are considered central regulators of type 2 immunity at epithelial barrier sites (Roan et al., 2019).

In this chapter, I present data regarding the activation of CRTH2$^+$ CD8$^+$ T cells, which represent the majority of Tc2 cells in human blood (see Chapter 3). Three stimulation modalities are compared: TCR triggering, PGD_2-CRTH2 interaction, and the concerted direct effect of alarmin cytokines. Populations of CRTH2$^+$ CD8$^+$ T cells were first generated by the rapid expansion method explored in Chapter 3. The large number of cells generated this way facilitated deep transcriptional profiling of activation products by bulk RNA sequencing. The primary question is whether these three different stimulation modalities have the same downstream effect on CRTH2$^+$ CD8$^+$ T cells. A secondary aim is to understand CRTH2$^+$ CD8$^+$ T cell activation and biology in more general terms.

4.2 Methods

4.2.1 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

To quantify expression of specific genes in an RNA sample I used two-step RT-qPCR.

4.2.1.1 RNA extraction by single column centrifugation

RNA extraction was performed using the RNEasy Mini Kit system (Qiagen, 74104) following the manufacturer's protocol (Qiagen, 2019). Fresh or frozen cell pellets were disrupted with lysis buffer RLT, containing guanidine isothiocyanate, a chaotropic agent that lyses cells and denatures protein/RNase (Chirgwin et al., 1979) and disrupts bonds between nucleic acids and water to improve silica binding (Boom et al., 1990). Lysate was homogenised by pipette and dehydrated with 70% ethanol in nuclease free water to improve silica binding (Qiagen, 2019).

The sample was centrifuged in a spin column with a silica membrane to capture nucleic acid. The membrane was washed and centrifuged with Buffer RW1 containing a guanidine salt and ethanol to dissolves contaminants (protein, carbohydrate, fatty acids) (Qiagen, 2020c). The membrane was further washed with buffer RPE, a mild ethanol-based buffer which removes salt traces of previous buffers (Qiagen, 2020b). Target RNA was eluted from the membrane by nuclease free water.

4.2.1.2 RNA quantification and purity check by spectrophotometry

RNA quantity and quality was assessed using a Nanodrop 2000 (Thermo scientific) following the manufacturer's protocol (Thermo Fisher Scientific, 2010). To conduct tests, the sample is held on a pedestal held between two optical fibres. A xenon beam is passed through the sample to a spectrometer. Concentration of RNA is derived from absorbance at 260 nm using the Beers-Lambert equation: Absorbance = absorptivity × path length × concentration. To assess RNA purity, I viewed the spectral image to compare with the expected shape. I checked that the 260/280 absorbance ratio was approximately between 1.8-2.0, and the 260/230 ratio between 1.8-2.2.

4.2.1.3 Reverse Transcription

Isolated RNA was converted to complementary DNA (cDNA) in preparation for qPCR using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (4374966, Applied Biosystems) following the manufacturer's protocol (Applied Biosystems, 2010b). Sample RNA was diluted to the lowest concentration of any sample, or 20 ng/μL, whichever greater. Samples below 20 ng/μL were considered insufficient.

For each reverse transcription reaction, I combined 10 μL of 2X reverse transcription master mix (Supplementary Table 13) with 10 μL of RNA template in a PCR reaction plate (STARLAB, E1403-5209) then mixed the reactions and sealed the plate with an optical adhesive cover (ThermoFisher, 4360954). I ran the plate in a thermal cycler as per protocol to facilitate the following events: random primer annealing, cDNA synthesis by the RT enzyme, and reaction termination and enzyme inactivation. Finally, I diluted each reaction five-fold in nuclease free water to bring the volume to 100 μL. This dilutes contaminants and increases cDNA volume available for qPCR (Nolan et al., 2006). Diluted cDNA was stored at -20 °C.

4.2.1.4 Real-time polymerase chain reaction

For all reactions I used the 2X FAST SYBR Green Master Mix reagent (Supplementary Table 14) (ThermoFisher, 4385617) and followed the manufacturer's instructions (Applied Biosystems, 2010a). I vortexed the master mix and cDNA template before formulating individual RT-qPCR reactions (Table 9) in a PCR reaction plate. Three technical replicates of each reaction were plated. Each experiment also included three wells of no-template control (NTC) in which nuclease free H_2O was substituted instead of cDNA. This negative control helps to detect primer-dimers or contaminants (Life Technologies, 2015).

Table 9 Contents of RT-qPCR reaction

Volume	Reagent	Function
10 µL	Fast SYBR Green Master Mix (2X)	See Supplementary Table 14
1 µL	Forward primer (20X optimal concentration)	Binds to the start codon of the target gene's antisense strand.
1 µL	Reverse primer (20X optimal concentration)	Binds to the stop codon of the target gene's sense strand
4 µL	Diluted template cDNA	Represents RNA in original sample
4 µL	Nuclease free H_2O	brings the reaction volume to 20 µL

The plate was sealed with an optical adhesive cover and briefly centrifuged. It was loaded in a Bio-Rad CFX96 and qPCR was performed following the manufacturer's instructions (Bio-Rad, 2010a) using the following cycle conditions:

1. 95 °C for 20 seconds to activate the polymerase. It is supplied in an inhibited form to prevent premature enzyme activity (Life Technologies, 2015).
2. 95 °C for 5 seconds to denature DNA into single strands;
3. 60 °C for 30 seconds for annealing of primers and extension by polymerase.
4. Steps 2-3 repeated for another 39 cycles (40 total).

The fluorescence of each well is read at the end of every cycle. Cycle number (x) and relative fluorescence (y) are plotted against each other. The baseline fluorescence and threshold for significant increase in fluorescence (*t)* was set automatically but checked manually by looking at the amplification plot (Applied Biosystems, 2010a). The machine reports the number of PCR cycles for each reaction to reach this threshold (C_t) and this value was taken forward for analysis.

4.2.1.5 Primer design and validation

Primers to target genes were designed using the Roche Universal ProbeLibrary Assay Design Center (Roche, 2020) with default settings of length (18-27 nucleotides) and Tm (59-61 °C) (Supplementary Table 15). All primers were synthesised as Custom Standard DNA Oligos (ThermoFisher) using default options (25 nM, desalted purification).

Specificity of primer binding was assessed by melting (dissociation) curve analysis (Life Technologies, 2015) (Supplementary Figure 32). PCR product was heated to 95 °C with periodic fluorescence measurements. Fluorescence decreases as double stranded DNA PCR products melt into single stranded DNA. The negative of the change in fluorescence (y) was plotted against temperature (x). For pure PCR products, a narrow symmetrical peak is observed at the Tm of the target amplicon. Reactions with melting curve anomalies can indicate problems such as primer dimers and were excluded from analyses.

The reaction efficiency of each primer was measured by standard curve analysis, to ensure it was between an acceptable range of 90-110 % (Life Technologies, 2015) (Supplementary Figure 32). An efficiency of 100 % would mean that the PCR product exactly doubles after each cycle. I pipetted a twofold serial dilution from the initial 100 µL of cDNA and performed qPCR as described. Then, I plotted the Ct value from each reaction (y) against the dilution factor (x). Linear regression derived an equation for this line. Efficiency was calculated as $10^{(-\frac{1}{m})}$-1 where m is the slope of the regression line. Primer sensitivity was determined by considering the maximal dilution that fit the standard curve (Life Technologies, 2015).

4.2.1.6 Analysis of gene expression by the $\Delta\Delta C_t$ method

Relative gene expression was analysed by the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). This method compares the difference in gene expression between two samples, normalised to a uniformly expressed reference gene to control for variation introduced by the experiment itself. For each sample, I measured one gene of interest and one reference gene, in triplicate, taking the median value forward for analysis. To calculate the ΔC_t, I subtracted the C_t of the reference gene from the C_t of the gene of interest. To calculate the $\Delta\Delta C_t$ I subtracted the ΔC_t of a negative control sample (e.g., unstimulated) from the sample of interest (e.g., stimulated). Fold difference in expression was calculated as $2^{-\Delta\Delta Ct}$.

To select reference genes I first consulted relevant literature for T cells (Ledderose et al., 2011). Then, I tested candidates in my experimental system and assessed stability of expression by comparing the C_t values across the samples (Supplementary Figure 33).

4.2.2 T cell stimulation assay

For each condition, cultured CRTH2$^+$ CD8$^+$ T cells (see Chapter 2) were stimulated at a concentration of 1×10^6 cells/mL in RPMI + 2 % human serum. The number of cells was 2×10^5 in a 96-well U-bottom plate for qPCR optimisation experiments, or 1×10^6 in a 48-well plate for RNA-Seq experiments. Stimuli were used at concentrations derived from other studies (Table 10). Cells were incubated at 37 °C, 5 % CO_2 for the indicated amount of time. Finally, cells were centrifuged at 300 *g* for 8 minutes. Supernatants were aspirated and frozen at -20 °C. Cell pellets were frozen at -80 °C until RNA extraction (4.2.3.2).

Table 10 Reagents for T cell stimulation

Condition	Reagent	Supplier (#)	Final Conc.	Rationale for chosen concentration
TCR	Anti-CD3	BioLegend (317302)	5 µg/mL	Previously used to induce type 2 cytokine production by Th2 cells *in vitro* (Rao et al., 2005).
	Anti-CD28	BioLegend (302902)	5 µg/mL	
PGD_2	PGD_2	Cayman (12010)	200 nM	Optimal for type 2 cytokine production in cultured Th2 cells (Xue et al., 2005)(Hilvering et al., 2018).
Alarmins	IL-25	BioLegend (589902)	50 ng/mL	Within range reported to stimulate murine ILC2 (Angkasekwinai et al., 2017) and to augment human Th2 activation (Y. H. Wang et al., 2007).
	IL-33	BioLegend (581802)	50 ng/mL	Sufficient to induce or augment type 2 cytokine production by Th2 cells (L. Guo et al., 2009; Kurowska-Stolarska et al., 2008)
	TSLP	BioLegend (582404)	300 ng/mL	Reported to induce IL-4 production by Th2 cells (Tatsuno et al., 2015).

4.2.3 Bulk RNA Sequencing

4.2.3.1 Experimental design

My RNA-Seq experiment was quantitative and designed to identify gross differential gene expression, so I allocated resources to biological replication rather than technical replication or sequencing depth (Cresko Lab, n.d.). Considering my budget and previous optimisation studies (Y. Liu et al., 2014) I chose to sequence ~6 million reads per sample for five biological replicates.

4.2.3.2 RNA extraction by two-column centrifugation

Sample total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, 74134) following the manufacturer's protocol (Qiagen, 2010). The procedure uses Buffer RLT Plus, which contains the same ingredients as Buffer RLT but with added detergents that promote

binding of genomic DNA to the DNA eliminator column (Qiagen, 2020a). β-mercaptoethanol was added to Buffer RLT Plus to assist with denaturing of RNases (Qiagen, 2020d). In this protocol the cell lysate is first centrifuged through a gDNA eliminator column before the addition of ethanol, at which point the process is the same as for the RNeasy Mini Kit (4.2.1.1).

4.2.3.3 RNA quantification and quality control by Agilent TapeStation

RNA integrity was assessed by RNA ScreenTape Analysis on an Agilent 4200 TapeStation following the manufacturer's protocol (Agilent Technologies, 2015). The TapeStation performs automated electrophoresis of total RNA samples. It detects the position and quantity of RNA fragments in the gel by fluorimetry with an RNA-binding dye. This measurement generates a graph of fluorescence over time (electropherogram or EPG). From the EPG, an RNA Integrity Number (RIN) is derived. The RIN is a standardised value between 1-10 which indicates the degradation of RNA (higher RIN means higher integrity) (Mueller et al., 2016). It is generated by an algorithm based on a database of manually assessed electropherograms. A value of 6.8 was considered sufficient based on the requirements of the company performing sequencing (Novogene).

4.2.3.4 mRNA isolation, library preparation, and sequencing by Novogene

After quantification and quality control, total RNA was shipped to Novogene following their sample preparation and shipping instructions (Novogene, n.d.). All further laboratory work was performed by Novogene using their commercial protocol. mRNA was enriched from total RNA using oligo(dT)-conjugated magnetic beads which bind polyadenylated mRNA (Jakobsen et al., 1990). It was fragmented in fragmentation buffer so that long sequences could be shortened below the sequencer's maximum read length.

To prepare a cDNA library, the first strand of cDNA was synthesised from fragmented mRNA by reverse transcription with random hexamer primers. The second cDNA strand was synthesised from the first by nick-translation, in which RNase nicks the RNA and the resulting fragments act as primers for *E. Coli*-derived polymerase (Rigby et al., 1977). cDNA was purified and overhanging ends formed by second strand synthesis were repaired by a polymerase. Repaired cDNA was dA-tailed, in which adenine bases are ligated to the 3' ends of each strand to provide a binding site for adapter sequences. The adapter sequences included, from the target cDNA towards the terminus: sample-specific barcodes for identification of sample origin; a sequence for priming of the sequencing reaction; and terminal 'P5' and 'P7' sequences for attachment to the Illumina flow-cell within the sequencer (Cresko Lab, n.d.). Adaptor-ligated cDNA was amplified by PCR to produce the library.

Quality control of the library was performed by quantification with a Qubit 2.0 fluorimeter using a DNA-specific dye, and by qPCR. Insert size was measured by the Agilent 2100 Bioanalyzer automated gel electrophoresis system.

The library was sequenced using the NovaSeq platform to generate 150bp paired-end reads. The NovaSeq uses Illumina sequencing technology (Bentley et al., 2008). cDNA fragments are amplified in a flow cell by formation of bridges with sequences complementary to the adapters, forming a cluster for each cDNA fragment. To generate a sequence, fluorescently tagged nucleotides are incorporated into each cDNA strand within a cluster, the fluorescent tag terminates the extension and can be imaged, before chemical removal of the tag and addition of the next base. After the read length has been reached, the cDNA is amplified by bridge one more time and the forward strand cleaved, leaving the reverse strand which can be read similarly.

4.2.3.5 Data analysis and differential gene expression

4.2.3.5.1 Generation of read counts from raw sequencing data

Analysis of raw data was performed by bioinformaticians at Novogene. The images taken by the NovaSeq platform were converted to raw sequence reads by Illumina's CASAVA software, which stores files in the FASTQ format. The base calling software assigns a probability of incorrect call to each base from which an error rate and quality score can be derived. Novogene considers average error rate below 1 % as acceptable (equivalent of Q20). The overall GC content was expected to be approximately 50 % with each base evenly distributed along the read except for the initial 6-7 bases due to random primer binding bias. Reads were filtered to remove the following: reads containing P5/P7 adapter sequences; reads in which > 10% of bases could not be called; or reads in which > 50% of bases had average error rate above 1 % (below Q20) – remaining reads are 'clean reads'. Filtered reads were then mapped (by Novogene) using STAR (Dobin et al., 2013) version 2.6.1. The reference genome used was homo sapiens GRCh38 accession number GCA_000001405.15. A mapping rate of > 70 % was considered successful (Dündar et al., 2019) and the STAR output was stored as Binary Alignment Map (BAM) files. The proportion of reads mapping to exons, introns, or intergenic regions was checked (most reads were expected to map to exons).

4.2.3.5.2 Quality control of read counts and differential expression analysis

Read quantification was performed by Novogene using featureCounts 1.5.0 (Liao et al., 2014). The BAM files are the input, and the output is a table of gene IDs and numerical counts per sample. I loaded the read counts file into RStudio for subsequent analyses.

Before performing differential gene expression analysis, I first visualised the global read counts as an extra quality control step (Dündar et al., 2019). Read counts were normalised

by the DESeq2 R package's internal size factor method (Love et al., 2014), which adjusts for gene size, sequencing depth between samples, and expression of other genes within samples. Normalised counts were log-transformed by the regularised logarithm function of DESeq2 which transforms to the $\log_2$ scale whilst shrinking the variances of genes with low read counts to improve stability of variance.

Normalised, transformed read count data were inspected as a quality control step (Dündar et al., 2019). Distribution of counts per gene was compared between replicates by violin plot to check for similarity between biological replicates. Pearson correlation analysis of read counts between samples indicated similarity between replicates - biological replicates should exhibit > 90 % correlation (Dündar et al., 2019). The distribution of variance (i.e., homoscedasticity) was examined by mean-SD plot using the R vsn package (W. Huber et al., 2002), as this is an assumption of DESeq2. Unsupervised hierarchical clustering was performed using the 'hclust' base R function using the results of Pearson correlation analysis. Principal component analysis (PCA) to reduce the global data to two dimensions was performed within DESeq2.

Differential expression of specific genes was calculated by DESeq2. This is a statistical tool which estimates both the effect size (in terms of fold change) and statistical significance (in terms of *p*-value) of the difference in gene expression between predefined groups of samples, for each individual gene to which reads have been mapped. The null hypothesis is that for each gene there is no difference between groups of samples being tested. Because the test is performed thousands of times, a multiple comparison correction is applied: DESeq2 generates adjusted *p*-values the Benjamin-Hochberg method with the false discovery rate as default set to 0.1. These adjusted *p*-values were used in downstream analyses. MA plots were used to check the ability of the analysis to generate significant

results (Dündar et al., 2019). From the MA plot I defined *very low* expression to mean < 100 reads after normalisation, and the terms *low*, *moderat*e, *high*, and *very high* such that each term reflects a tenfold increase over the previous term.

4.2.3.5.3 Identification of individual genes of interest

The bulk RNA-Seq experiment generated a very large number of differentially expressed genes (DEGs), and it was not possible to consider each result individually. Therefore, I required a system by which to rank DEGs in order of importance. There seems to be no consensus method of doing this, and several variables can be considered relevant. I decided to consider three variables for each result:

1) the mean expression of a gene across the experiment as a whole (output from DESeq2 as 'baseMean', a measure of the average normalised read count for a gene)

2) the magnitude of the effect size of the change in gene expression (measured by DESeq2 as the binary logarithm (log_2) of the fold-change or 'log_2FC' compared to the unstimulated condition)

3) the statistical significance of the change in expression (measured by DESeq2 as the Benjamin-Hochberg adjusted *p*-value (*padj*)).

To produce a final rank of DEGs, I first assigned rankings based on each of these three measurement variables, and then derived a final ranking from their mean average.

There are many other possible ways to rank or select important DEGs, for example by assigning thresholds or most commonly by simply picking what looks interesting - I will not discuss these alternatives here.

One exception to this method arose when comparing the mutual DEGs shared between two different stimulation conditions (i.e., differentially-differentially expressed genes or dDEGs). In this case, I did not measure the effect size relative to the unstimulated control, but relative to a curve of best fit describing how changes in gene expression were related between the two conditions.

Genes of interest were investigated by reviewing that gene's entry on the interactive GeneCards database (Stelzer et al., 2016), which includes information from the National Center for Biotechnology Information (NCBI) Entrez database (Agarwala et al., 2018) and the Uniprot Knowledgebase (Bateman, 2019). For hits which could not be matched to an Entrez entry (e.g. noncoding RNA), I reviewed that entry in the Ensembl genome browser (Yates et al., 2019).

4.2.3.5.4 Defining gene groups

Specific groups of genes are known to be important for T cell functions. The gene group 'CD molecules' was defined by the gene group index from the HUGO Gene Nomenclature Committee (HGNC) database (Braschi et al., 2019). Gene groups 'Cytokines' and 'Cytokine Receptors' were defined by The Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017). Type 1, type 2, and type 17 genes were identified by reviewing literature concerned with Th1 (Gause et al., 2020; Tim R. Mosmann & Sad, 1996), Th2 (Gause et al., 2020), and Th17 (Korn et al., 2009; Stockinger & Omenetti, 2017) responses (Supplementary Table 16). Genes associated with asthma (Demenais et al., 2018; El-Husseini et al., 2020) or COPD (Sakornsakolpat et al., 2019; Shrine et al., 2019) were identified in recent GWAS studies.

4.2.4 Pathway enrichment analysis

Genes often do not operate in isolation, but as part of functionally related groups. Changes to these groups can be missed if a researcher considers only individual DEGs. This problem gave rise to the technique of gene set enrichment analysis (GSEA) (Subramanian et al., 2005). In GSEA, predefined groups ('gene sets') are tested for association with one condition compared to another. A complete list of detected genes is first ranked by some method of association (e.g., fold-change) between two conditions. The position of individual genes from a set is tested by 'walking' down the ranked list and keeping a running score of target vs non-target genes. The resulting enrichment score (ES) represents how deviated the genes are to one end of the list. The ES can be normalised by gene set size (NES) and is compared to a null distribution to generate a *p*-value. In my dataset, these calculations were performed in R following the guide 'Biomedical Knowledge Mining using GOSemSim and clusterProfiler' (Yu, 2020a).

A key parameter of GSEA is how the researcher defines groups of related genes. Generally, these groupings are drawn from large databases. I considered multiple recommended databases (Reimand et al., 2019, Box 2), which improves coverage compared to relying on a single database (Mubeen et al., 2019). In all cases where an enriched gene set was identified, the analysis returns a group of 'core' genes that contributed to the enrichment score of the set (Subramanian et al., 2005).

The Gene Ontology (GO) database is an initiative to categorise genes by their functions and processes (Ashburner et al., 2000). For GSEA of GO terms, I first ranked the results of DESeq2 for each unique Ensembl ID by fold-change, and then analysed these with clusterprofiler package (Yu et al., 2012) version 3.18.0 using the 'gseGO' function (biological processes) on default settings. The GO database used was the version included

in Bioconductor package GO.db (Carlson, 2020). Redundant gene sets were discarded by the *c*lusterprofiler 'simplify' function (cut-off 0.7) (Yu et al., 2010). Network diagrams were drawn with the 'emapplot' function of enrichplot (Yu, 2020b). Clusters of related pathways (termed 'modules') were identified and interpreted manually.

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017) is a collection of manually drawn biological pathways derived from experimental findings. KEGG pathway enrichment analysis was performed with the 'gseKEGG' function of clusterprofiler (minimum gene set size 20 genes). The package accesses the most current KEGG database directly through its website. Enriched pathways were visualised using the pathview R package (W. Luo & Brouwer, 2013).

The Reactome (Jassal et al., 2019) database focusses exclusively on human biology considered in terms of molecular reactions. Reactome pathway enrichment analysis was conducted using the 'gsePathway' function of the ReactomePA R package version 1.34.0 (Yu & He, 2016). The settings were standard except gene set size range was adjusted to 20-500. The Reactome database consulted is built into Bioconductor 3.12 package reactome.db version 1.74.0 (Wolfgang Huber et al., 2015). Before plotting, I filtered the GSEA results to reduce redundancy by selecting pairs of enriched pathways which overlapped by a value greater than 0.8 according to the 'pairwise_termsim' function of enrichplot and eliminating the pathway in that pair with lesser significance.

4.2.5 Data visualisation

Unless indicated otherwise in the figure legends, plots were drawn using the ggplot2 R package (Wickham, 2016) with some functions from the extension package ggpmisc (Aphalo, 2020). Multiple plots within one figure were arranged using the patchwork R

package (T. L. Pedersen, 2020). Colour gradients were either set manually or using the viridis package (Garnier, 2018). Some of the packages referred to are part of the Bioconductor framework version 3.12 (Wolfgang Huber et al., 2015), as specified in the individual package citations. *P*-values are indicated with asterisks by which a value < 0.05, < 0.01, <0 .001, or < 0.0001 is represented by one, two, three or four asterisks respectively.

4.3 Results

The aim of this chapter is to characterise the response of CRTH2$^+$ CD8$^+$ T cells to stimulation. Specifically, I compared the response CRTH2$^+$ CD8$^+$ T cells to activation by TCR triggering, PGD_2-CRTH2 interaction, or alarmin cytokines.

4.3.1 Different response times to stimulation conditions

To determine the optimal length of time for maximal stimulation, I conducted a time course of stimulation of cultured CRTH2$^+$ CD8$^+$ T cells (Figure 42). Expression of the gene for type 2 cytokine IL-13 was used as an indicator of type 2 activation. In this experiment, PGD_2 stimulation was highest at the earliest time point of 1.5 hours, alarmin stimulation at the latest time point of 48 hours, and TCR triggering was highly stimulatory at all time points.

4.3.2 Differential gene expression analysis

To explore the transcriptomic response of CRTH2$^+$ CD8$^+$ T cells to stimulation, bulk RNA-Seq was performed on cultured CRTH2$^+$ CD8$^+$ T cells stimulated by TCR triggering, PGD_2-CRTH2 interaction, or alarmin cytokines. The method used is outlined in 4.2.3 and the stimulation conditions were drawn from the results shown in 4.3.1. Specifically, PGD_2 or TCR stimulation were carried out for two hours and alarmin stimulation for 24 hours.

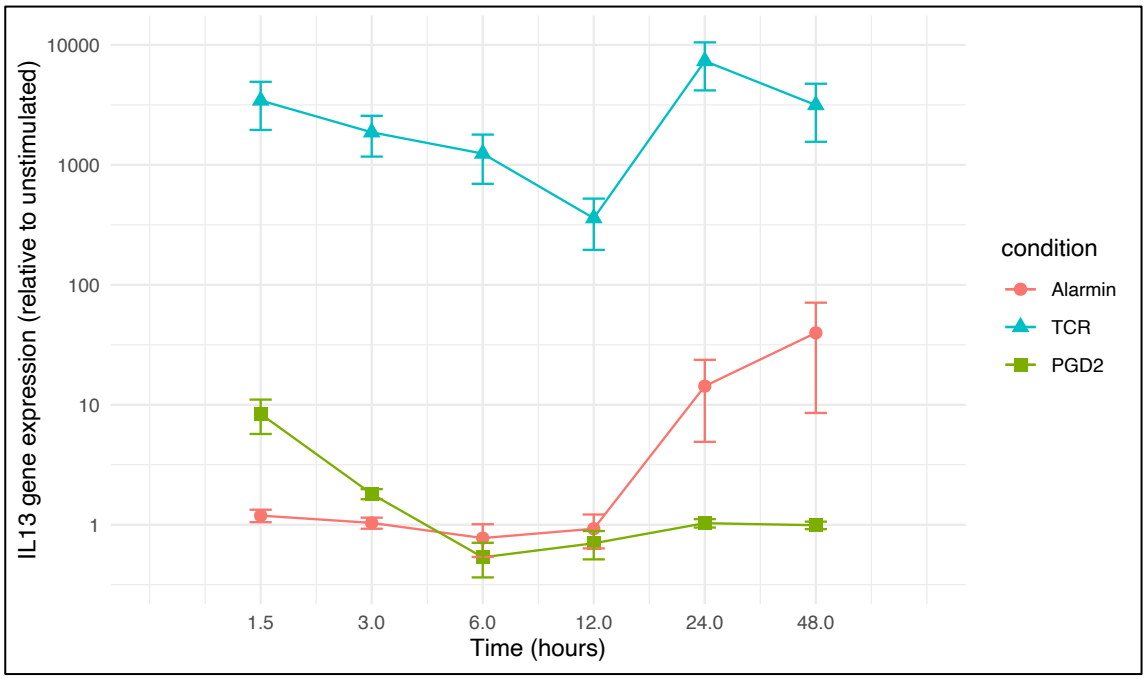


Figure 42 Time-course of *IL13* expression after stimulation

T cells were stimulated as described in 4.2.2. RT-qPCR was conducted as described in 4.2.1. The reference genes used were *GAPDH* for PGD_2 or alarmin stimulation and *TBP* for TCR stimulation, chosen for optimal stability of expression (Supplementary Figure 33). N = 3 donors for each combination of condition and time point. Error bars are standard error.

4.3.2.1 Quality control of RNA and raw sequencing data

Quality of input total RNA was assessed by spectrophotometry (4.2.1.2) and automated electrophoresis (4.2.3.3) (Supplementary Table 17). Quality of raw RNA-Seq data was assessed by Novogene (Supplementary Table 18). Error rate (estimated chance of incorrect base call) of raw reads was below 1% (Supplementary Figure 34A), GC content was evenly distributed (Supplementary Figure 34B), and > 97 % of reads passed filtering (see 4.2.3.5.1) across all samples (Supplementary Figure 34C). At least 96 % of clean reads for each sample were mapped to the reference genome, at least 88 % mapped uniquely, and at least 8 3% mapped to exon regions (Supplementary Figure 34D and Supplementary Table 19). Quality of read count data for input into DESeq2 was assessed in R. The distribution of normalised, transformed read counts was similar between biological replicates

(Supplementary Figure 35A), and the data were homoscedastic (Supplementary Figure 35B). The data were therefore appropriate for differential gene expression analysis.

4.3.2.2 Consistency of read count data between biological replicates

The transcriptomes of samples within each stimulation condition were at least 90% correlated between donors (Supplementary Figure 36A). The results of unsupervised hierarchical clustering showed that samples clustered based on stimulation condition for the TCR triggering or PGD_2 conditions (Supplementary Figure 36B). However, within the unstimulated or alarmin conditions, samples clustered based on biological donor. These findings are visualised as a Principal Component Analysis (PCA) (Figure 43).

4.3.2.3 Identification of DEGs in each condition

Differential gene expression analysis by DESeq2 reported 86 (60 up, 26 down) DEGs after stimulation by alarmin cytokines, 7775 (3990 up, 3742 down) after PGD_2 stimulation, and

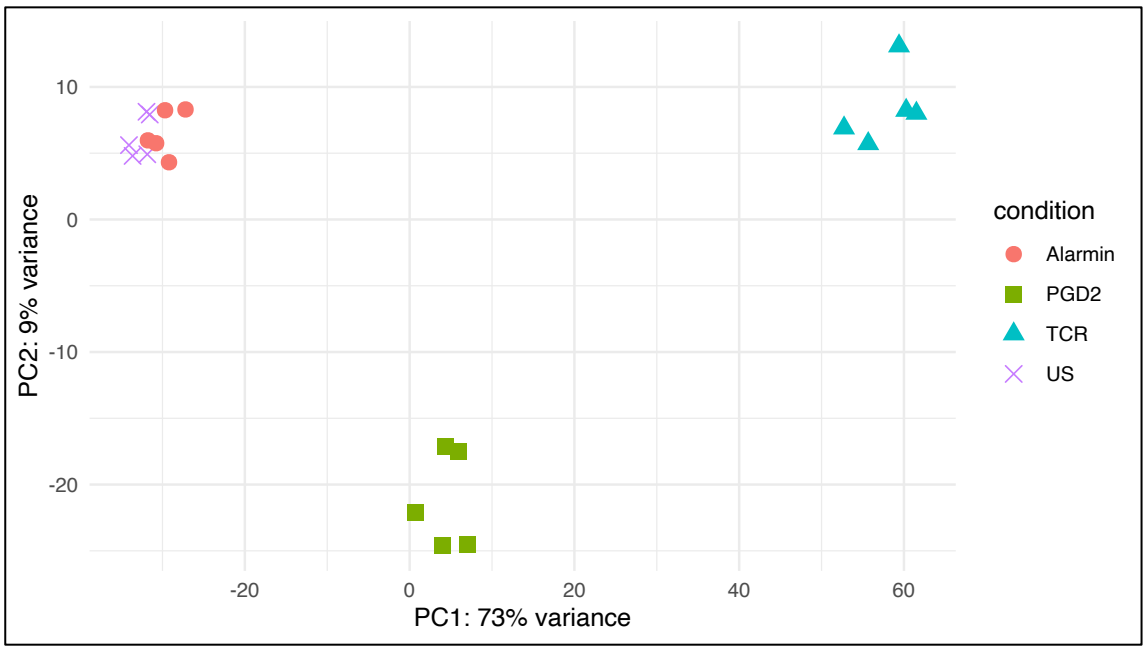


Figure 43 PCA plot of read count data for all genes

Global read count data were plotted in two dimensions using the 'PCA' function built in to the DESeq2 package. N = 5 donors per group. US = unstimulated. PC = Principal Component.

9141 (4820 up, 4275 down) after TCR triggering (Figure 44). The complete results of the analysis are available to download from the Oxford Research Archive (ORA) (Hoyle & Xue, 2021).

DEGs were identified across the full range of mean expression values (Supplementary Figure 37). The expression of DEGs by samples within the same group was consistent (Supplementary Figure 38). In line with the preliminary experiment (Figure 42), *IL13* was upregulated in all stimulation conditions (Figure 45).

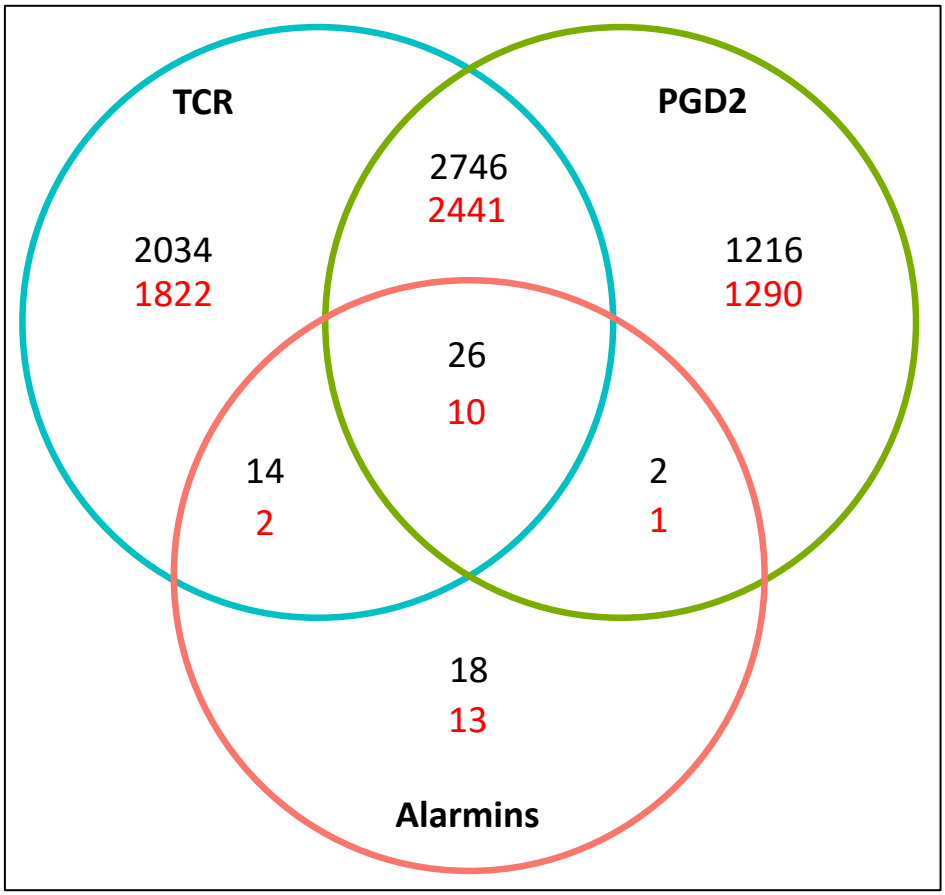


Figure 44 Venn diagram of DEGs detected by differential gene expression analysis
Results of DESeq2 analysis were calculated with the VennDiagram package in R (H. Chen, 2018). Upregulated genes are indicated above in black text, downregulated genes below in red.

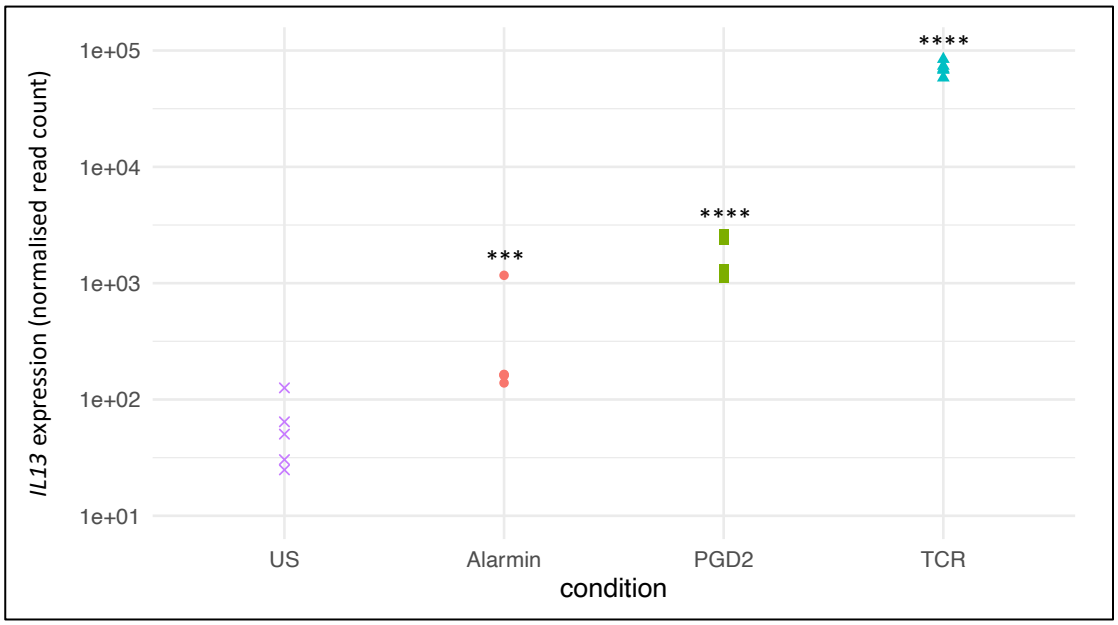


Figure 45 *IL13* was upregulated in all stimulation conditions

Data were extracted from the DESeq2 analysis, which normalises read counts by the size factor method. Asterisks refer to the adjusted p-value reported by DESeq2 analysis. Each condition is compared to the unstimulated (US) control. N = 5 biological replicates per condition.

4.3.2.4 Selecting DEGs of interest using a ranking system

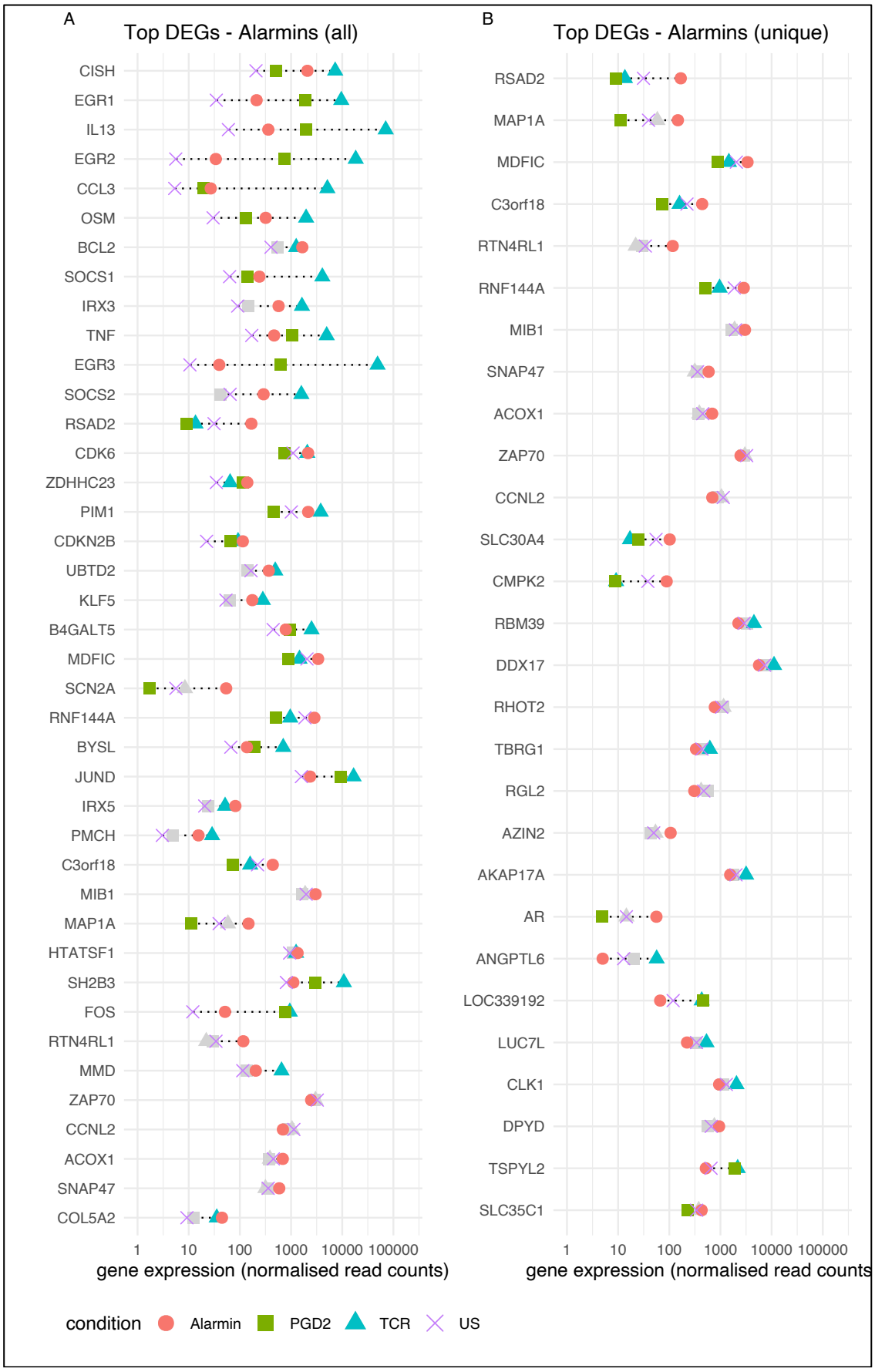
Too many DEGs were identified to consider each individually. To select the most extreme changes of gene expression, DEGs in each condition were ranked (see 4.2.3.5.3) considering fold-change vs unstimulated, statistical significance of the change, and mean absolute expression across samples (Supplementary Table 20 to Supplementary Table 22). Since this chapter concerns differences between the stimulation conditions, I was particularly interested in changes that were unique to one stimulation (Figure 44). Therefore, DEGs unique to one combination of condition and direction (e.g., upregulated only in the PGD_2 condition) were ranked separately (Supplementary Table 23 to Supplementary Table 26). For each condition, the top ranked DEGs overall, and the top ranked unique DEGs for that condition, were taken forward for further investigation and interpretation (Figure 46).

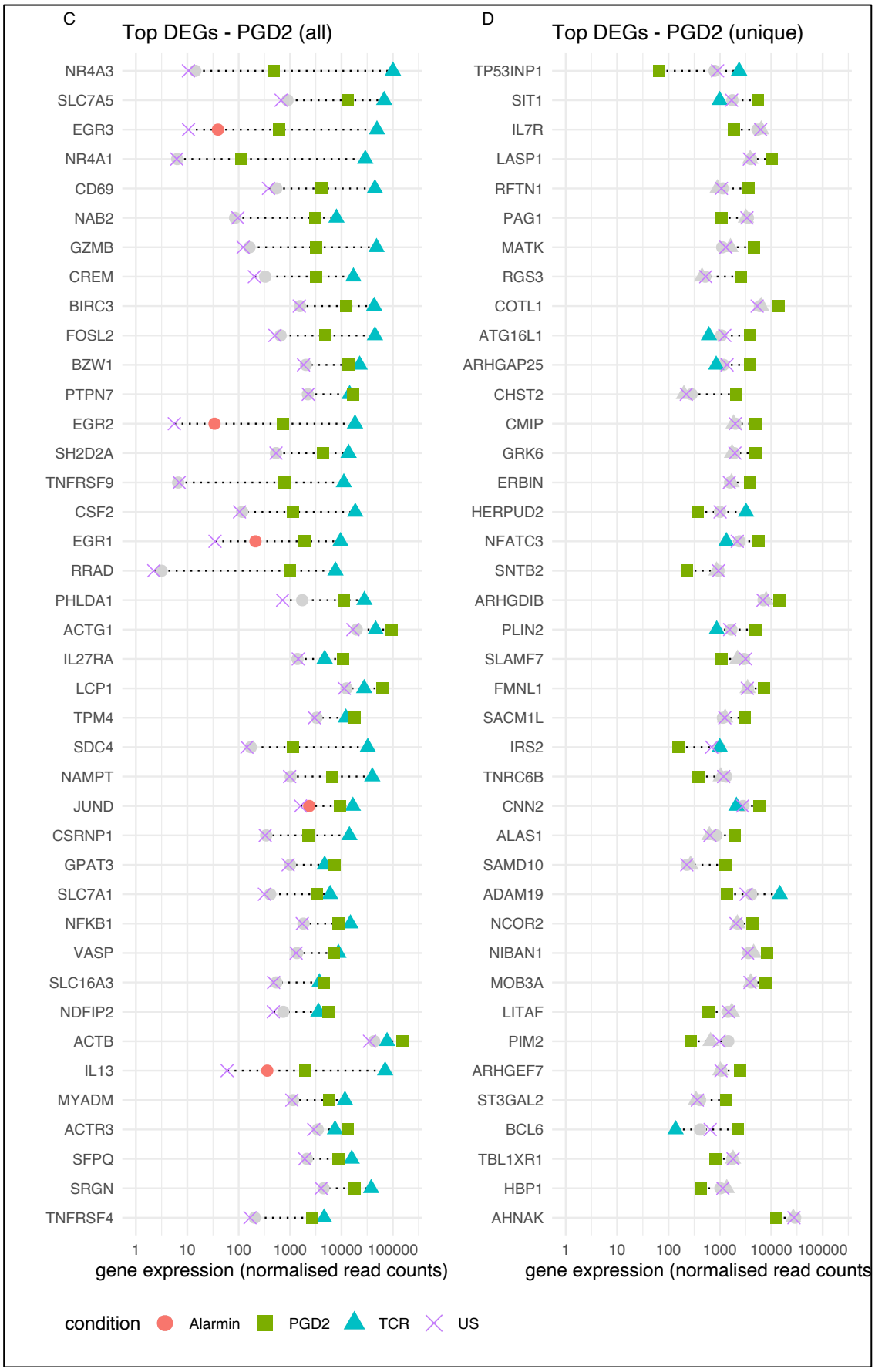
4.3.2.5 Quantitative comparison of shared DEGs by curve fitting

Many DEGs were shared between conditions (Figure 44) – e.g., IL13 was upregulated in all stimulated conditions compared to resting cells. I required a method to determine if a shared DEG was particularly changed in one condition (i.e., an outlier to the distribution of shared DEGs). To assess this, I plotted fold-change values of shared DEGs between each pair of conditions and drew a curve of best fit to approximate the pattern of common gene expression changes. This process was repeated for alarmin vs PGD_2 (Figure 47A), alarmin vs TCR (Figure 47B), and PGD_2 vs TCR (Figure 48) conditions. Each shared DEG was assigned a value 'e', its residual distance from the curve: for example, an e-value of 1 indicates gene expression twice as high as expected based on the fitted curve. Shared DEGs were then ranked in an analogous way to unique DEGs (see 4.2.3.5.3) except the magnitude of 'e' was substituted in for fold-change (Supplementary Table 26 to Supplementary Table 31). Given the large number of shared DEGs between PGD_2 and TCR conditions, only the top ranked shared DEGs were taken forward for further interpretation (Figure 49).

4.3.2.6 DEGs in gene groups of particular relevance to T cell biology

Certain groups of genes are particularly associated with T cell biology, and were therefore considered separately to the global dataset. I considered the following gene groups: genes relating to type 1, type 2, and type 17 'flavours' of the T cell response (Figure 50A to C); cytokines and cytokine receptors (Figure 50D and E); and CD molecules (Figure 50F), which together are important for the functions of T cells. For reference, I have also made similar figures of the most highly expressed genes in this dataset that have also been linked to asthma or COPD by GWAS studies (see 4.2.3.5.4 and Supplementary Figure 39).


A
Top DEGs - Alarmins (all)
CISH
EGR1
IL13
EGR2
CCL3
OSM
BCL2
SOCS1
IRX3
TNF
EGR3
SOCS2
RSAD2
CDK6
ZDHHC23
PIM1
CDKN2B
UBTD2
KLF5
B4GALT5
MDFIC
SCN2A
RNF144A
BYSL
JUND
IRX5
PMCH
C3orf18
MIB1
MAP1A
HTATSF1
SH2B3
FOS
RTN4RL1
MMD
ZAP70
CCNL2
ACOX1
SNAP47
COL5A2
1
10
100
1000
10000
100000
gene expression (normalised read counts)
B
Top DEGs - Alarmins (unique)
RSAD2
MAP1A
MDFIC
C3orf18
RTN4RL1
RNF144A
MIB1
SNAP47
ACOX1
ZAP70
CCNL2
SLC30A4
CMPK2
RBM39
DDX17
RHOT2
TBRG1
RGL2
AZIN2
AKAP17A
AR
ANGPTL6
LOC339192
LUC7L
CLK1
DPYD
TSPYL2
SLC35C1
1
10
100
1000
10000
100000
gene expression (normalised read counts
condition
Alarmin
PGD2
TCR
US


C
Top DEGs - PGD2 (all)
NR4A3
SLC7A5
EGR3
NR4A1
CD69
NAB2
GZMB
CREM
BIRC3
FOSL2
BZW1
PTPN7
EGR2
SH2D2A
TNFRSF9
CSF2
EGR1
RRAD
PHLDA1
ACTG1
IL27RA
LCP1
TPM4
SDC4
NAMPT
JUND
CSRNP1
GPAT3
SLC7A1
NFKB1
VASP
SLC16A3
NDFIP2
ACTB
IL13
MYADM
ACTR3
SFPQ
SRGN
TNFRSF4
1
10
100
1000
10000
100000
gene expression (normalised read counts)
D
Top DEGs - PGD2 (unique)
TP53INP1
SIT1
IL7R
LASP1
RFTN1
PAG1
MATK
RGS3
COTL1
ATG16L1
ARHGAP25
CHST2
CMIP
GRK6
ERBIN
HERPUD2
NFATC3
SNTB2
ARHGDIB
PLIN2
SLAMF7
FMNL1
SACM1L
IRS2
TNRC6B
CNN2
ALAS1
SAMD10
ADAM19
NCOR2
NIBAN1
MOB3A
LITAF
PIM2
ARHGEF7
ST3GAL2
BCL6
TBL1XR1
HBP1
AHNAK
1
10
100
1000
10000
100000
gene expression (normalised read counts
condition
Alarmin
PGD2
TCR
US

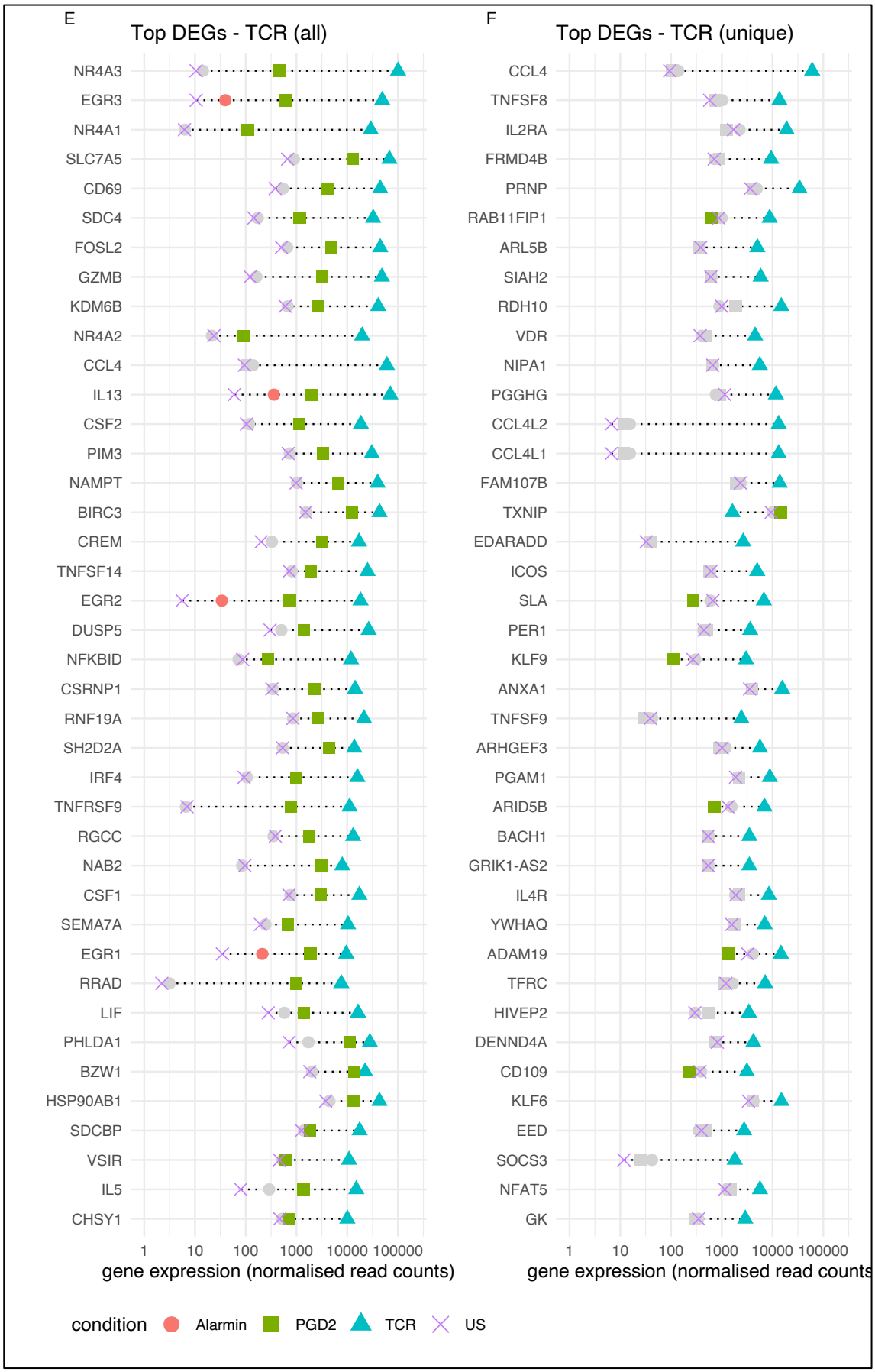

E
Top DEGs - TCR (all)
NR4A3
EGR3
NR4A1
SLC7A5
CD69
SDC4
FOSL2
GZMB
KDM6B
NR4A2
CCL4
IL13
CSF2
PIM3
NAMPT
BIRC3
CREM
TNFSF14
EGR2
DUSP5
NFKBID
CSRNP1
RNF19A
SH2D2A
IRF4
TNFRSF9
RGCC
NAB2
CSF1
SEMA7A
EGR1
RRAD
LIF
PHLDA1
BZW1
HSP90AB1
SDCBP
VSIR
IL5
CHSY1
1
10
100
1000
10000
100000
gene expression (normalised read counts)
F
Top DEGs - TCR (unique)
CCL4
TNFSF8
IL2RA
FRMD4B
PRNP
RAB11FIP1
ARL5B
SIAH2
RDH10
VDR
NIPA1
PGGHG
CCL4L2
CCL4L1
FAM107B
TXNIP
EDARADD
ICOS
SLA
PER1
KLF9
ANXA1
TNFSF9
ARHGEF3
PGAM1
ARID5B
BACH1
GRIK1-AS2
IL4R
YWHAQ
ADAM19
TFRC
HIVEP2
DENND4A
CD109
KLF6
EED
SOCS3
NFAT5
GK
1
10
100
1000
10000
100000
gene expression (normalised read counts
condition
Alarmin
PGD2
TCR
US

Figure 46 Top DEGs for each condition as identified by a ranking system

The x-axis represents absolute normalised read counts. The y-axis represents individual genes, ordered by ranking as described in 4.2.3.5.3. Each gene is represented by four points, one point per stimulation condition, linked by a dashed horizontal line, Each point represents the mean absolute read count for the replicates (n = five donors) within that condition. Points are coloured if *padj* < 0.05 compared with the unstimulated control (US), or greyed out if *padj* < 0.05. The greyed out points are therefore generally close to the unstimulated control values. (A), (C), and (E) show the highest ranked DEGs from the total pool of DEGs for that condition (compared to US). whereas (B), (D), and (F) only concern only DEGs that were uniquely upregulated or downregulated in that specified condition, compared to US.

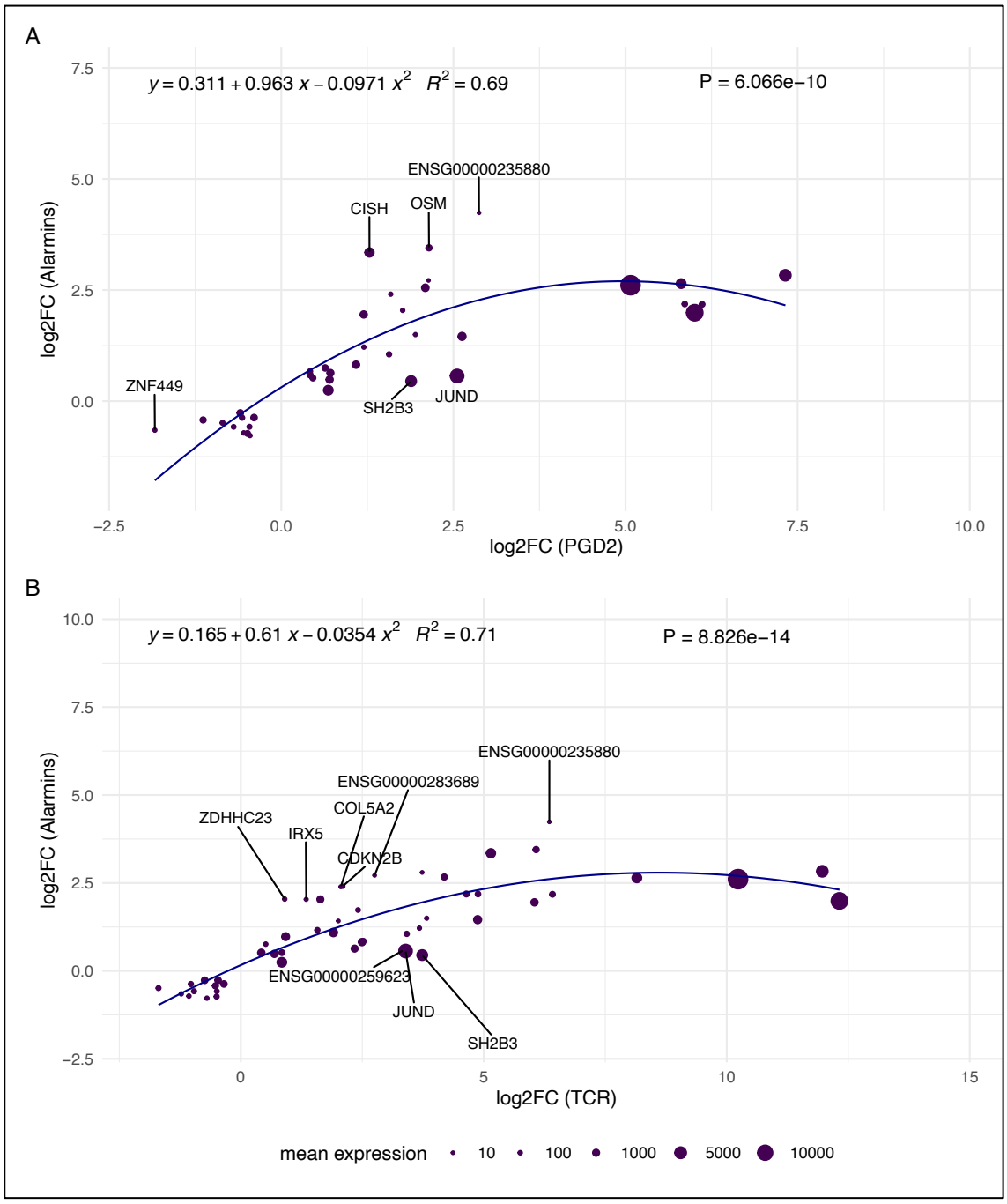


Figure 47 Outlier shared DEGs between Alarmin and PGD_2 or TCR stimulation

Plots of shared DEGs between alarmin condition and (A) PGD_2 or (B) TCR. Each data point represents one shared DEG, plotted in terms of the fold change in each condition. Point size is proportional to mean expression across the experiment. A curve of best fit and p-value were calculated in R, and points were labelled if they were a distance of at least 1 unit from the curve.

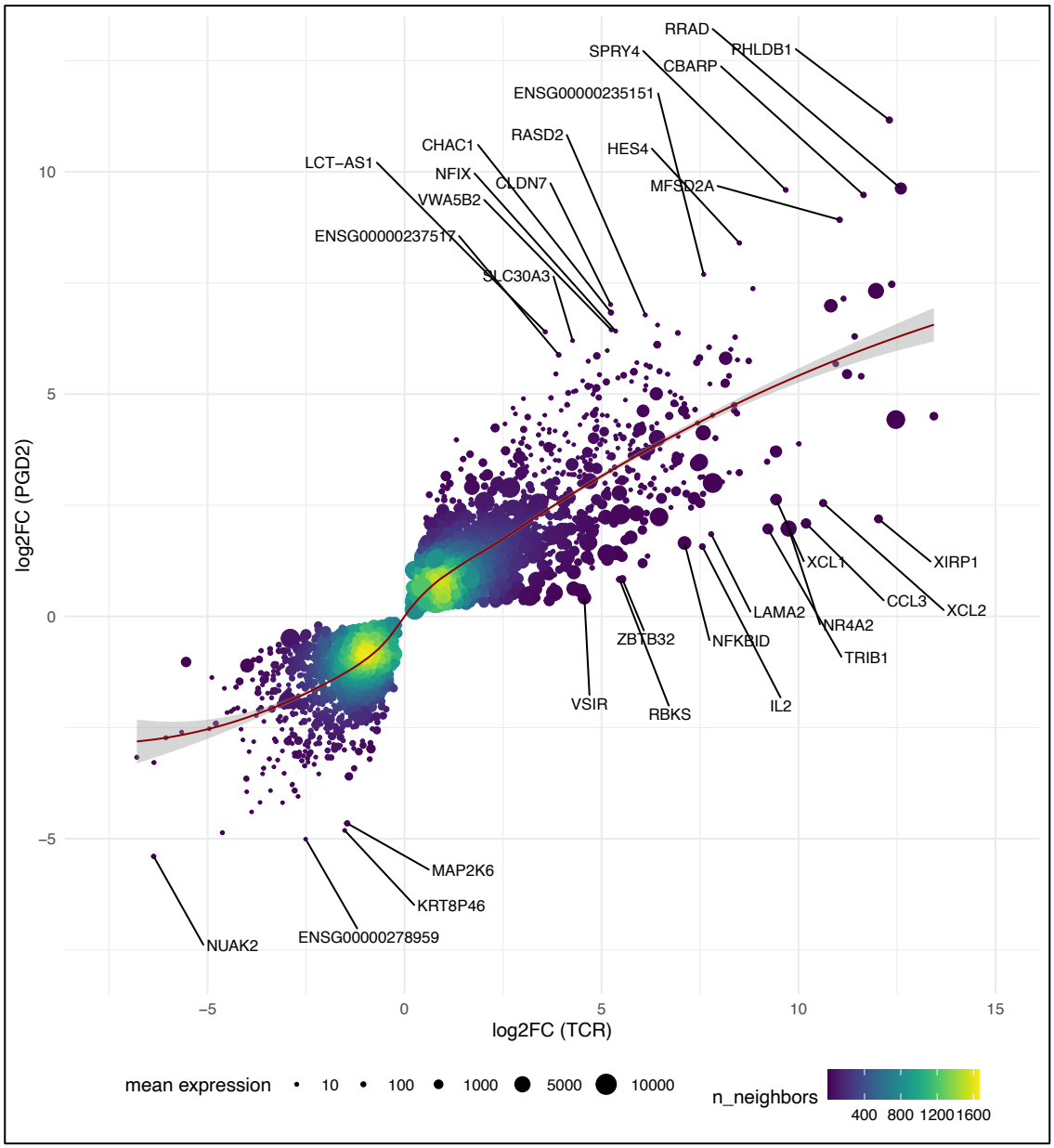


Figure 48 Outlier shared DEGs between PGD_2 and TCR stimulation

Plots of shared DEGs between PGD_2 and TCR conditions. Each data point represents one shared DEG, and the axes reflect fold change in each condition. The size of points is proportional to mean expression across the experiment. A curve of best fit was calculated in R by Loess regression, the grey shaded area either side of the curve represents standard error, and individual points were labelled farthest away from the curve. Colour is proportional to the number of adjacent points (n_neighbors) and therefore represents density of genes in that area of the graph, because there are too many to resolve all of them. This diagram was made using the R package ggpointdensity (Kremer, 2019).

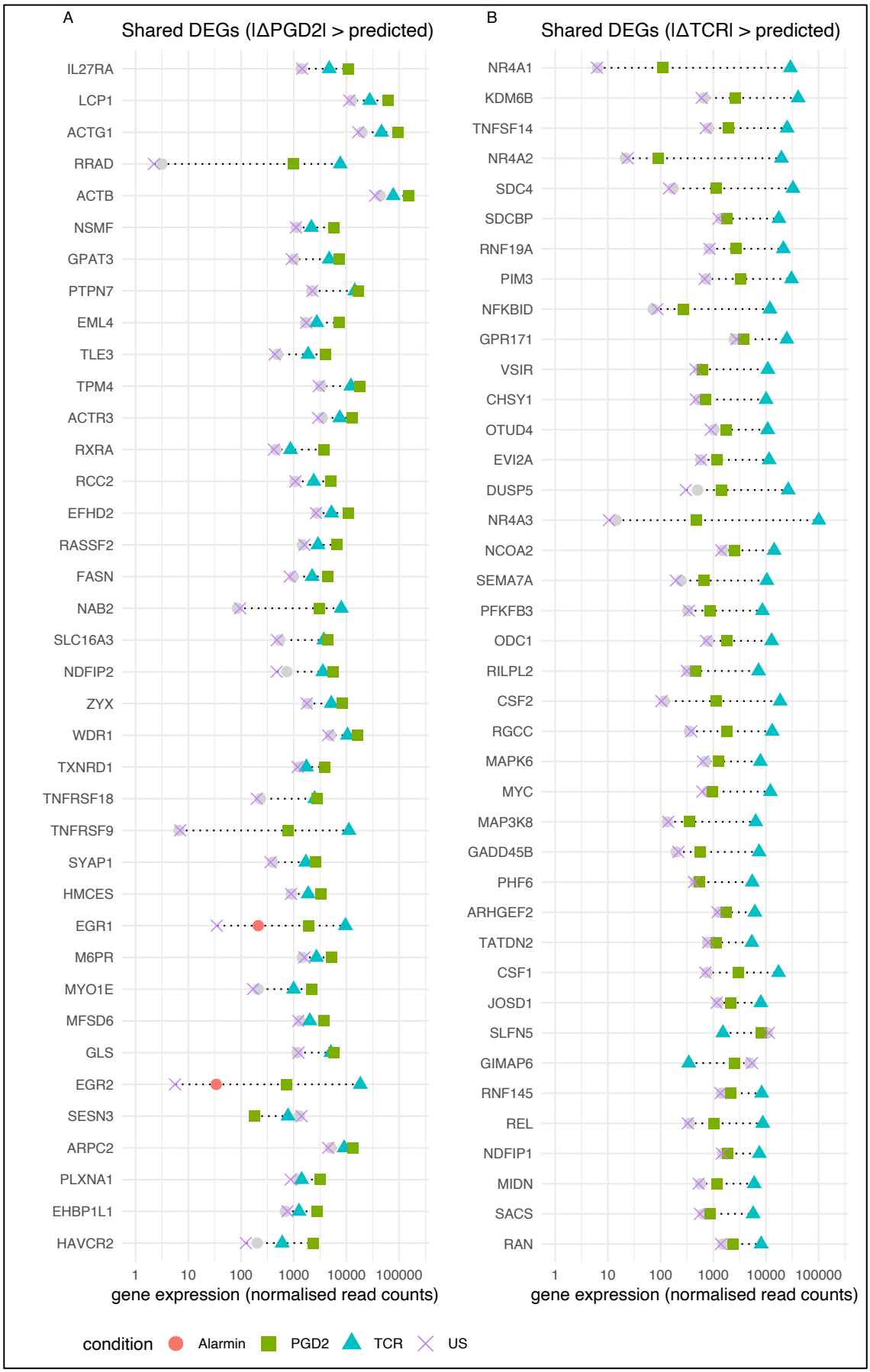

A
Shared DEGs (|ΔPGD2| > predicted)
IL27RA
LCP1
ACTG1
RRAD
ACTB
NSMF
GPAT3
PTPN7
EML4
TLE3
TPM4
ACTR3
RXRA
RCC2
EFHD2
RASSF2
FASN
NAB2
SLC16A3
NDFIP2
ZYX
WDR1
TXNRD1
TNFRSF18
TNFRSF9
SYAP1
HMCES
EGR1
M6PR
MYO1E
MFSD6
GLS
EGR2
SESN3
ARPC2
PLXNA1
EHBP1L1
HAVCR2
1
10
100
1000
10000
100000
gene expression (normalised read counts)
B
Shared DEGs (|ΔTCR| > predicted)
NR4A1
KDM6B
TNFSF14
NR4A2
SDC4
SDCBP
RNF19A
PIM3
NFKBID
GPR171
VSIR
CHSY1
OTUD4
EVI2A
DUSP5
NR4A3
NCOA2
SEMA7A
PFKFB3
ODC1
RILPL2
CSF2
RGCC
MAPK6
MYC
MAP3K8
GADD45B
PHF6
ARHGEF2
TATDN2
CSF1
JOSD1
SLFN5
GIMAP6
RNF145
REL
NDFIP1
MIDN
SACS
RAN
1
10
100
1000
10000
100000
gene expression (normalised read counts)
condition
Alarmin
PGD2
TCR
US

Figure 49 Top ranked shared DEGs between PGD_2 and TCR stimulation

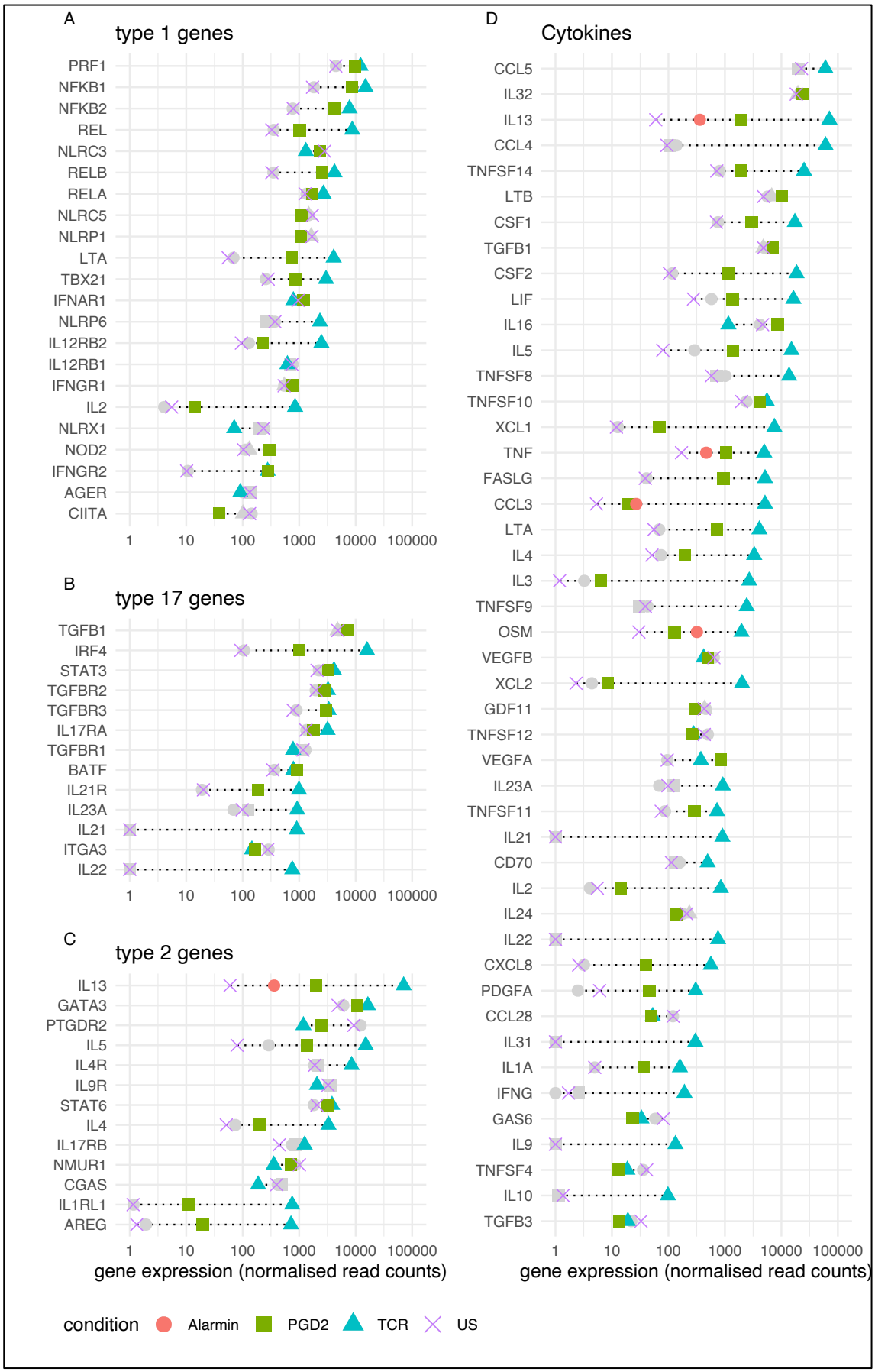
The x-axis represents absolute normalised read count. The y-axis represents individual genes, ordered by ranking as described in 4.2.3.5.3. Each gene is represented by four points, one point per condition, linked by a dashed horizontal line, Each point represents the mean absolute read count for the replicates (n = five donors) within that condition. Points are coloured if *padj* < 0.05 compared with the unstimulated control (US), or greyed out if *padj* > 0.05. The greyed out points are therefore generally close to the unstimulated control values. (A) shows the highest ranked shared DEGs between the PGD_2 and TCR conditions, where the change (Δ) in expression in the PGD_2 group was more extreme (higher |magnitude|) than expected based on the overall pattern of shared expression changes shown in Figure 48. (B) shows the inverse of this i.e., the highest ranked shared DEGs between the PGD_2 and TCR conditions, where the change in expression in the TCR group was more extreme than expected based on the overall pattern of shared expression changes shown in Figure 48.

4.3.3 Pathway enrichment analysis

Genes do not operate in isolation but cooperate in groups to exert biological effects. One way to investigate groups of functionally related genes is by pathway enrichment analyses such as GSEA. I looked for altered expression of known gene sets using established databases (see 4.2.4). The complete results can be downloaded from the Oxford Research Archive (ORA) (Hoyle & Xue, 2021)

4.3.3.1 Gene Ontology Knowledgebase (GO)

GO enrichment analysis for biological processes (BP) returned zero, 472 and 863 significantly enriched gene sets for Alarmin, PGD_2 and TCR stimulation respectively (Supplementary Figure 40A and Supplementary Table 33). To aid interpretation of the long list of enriched gene sets, I eliminated redundant gene sets (see 4.2.4) and then conducted a network analysis to cluster gene sets into modules. After PGD_2 (Figure 51) or TCR (Figure 52) stimulation, modules of upregulated gene sets were identified relating to T cell activation and wound repair. In the PGD_2 condition there was also a module related to regulation of vessel diameter, and in the TCR condition one related to epithelial cell migration. The 'core genes' responsible for the statistical enrichment of each gene set in selected modules were visualised in heatmaps (Supplementary Figure 41).


A
type 1 genes
PRF1
NFKB1
NFKB2
REL
NLRC3
RELB
RELA
NLRC5
NLRP1
LTA
TBX21
IFNAR1
NLRP6
IL12RB2
IL12RB1
IFNGR1
IL2
NLRX1
NOD2
IFNGR2
AGER
CIITA
1
10
100
1000
10000
100000
B
type 17 genes
TGFB1
IRF4
STAT3
TGFBR2
TGFBR3
IL17RA
TGFBR1
BATF
IL21R
IL23A
IL21
ITGA3
IL22
C
type 2 genes
IL13
GATA3
PTGDR2
IL5
IL4R
IL9R
STAT6
IL4
IL17RB
NMUR1
CGAS
IL1RL1
AREG
gene expression (normalised read counts)
D
Cytokines
CCL5
IL32
IL13
CCL4
TNFSF14
LTB
CSF1
TGFB1
CSF2
LIF
IL16
IL5
TNFSF8
TNFSF10
XCL1
TNF
FASLG
CCL3
LTA
IL4
IL3
TNFSF9
OSM
VEGFB
XCL2
GDF11
TNFSF12
VEGFA
IL23A
TNFSF11
IL21
CD70
IL2
IL24
IL22
CXCL8
PDGFA
CCL28
IL31
IL1A
IFNG
GAS6
IL9
TNFSF4
IL10
TGFB3
gene expression (normalised read counts)
condition
Alarmin
PGD2
TCR
US

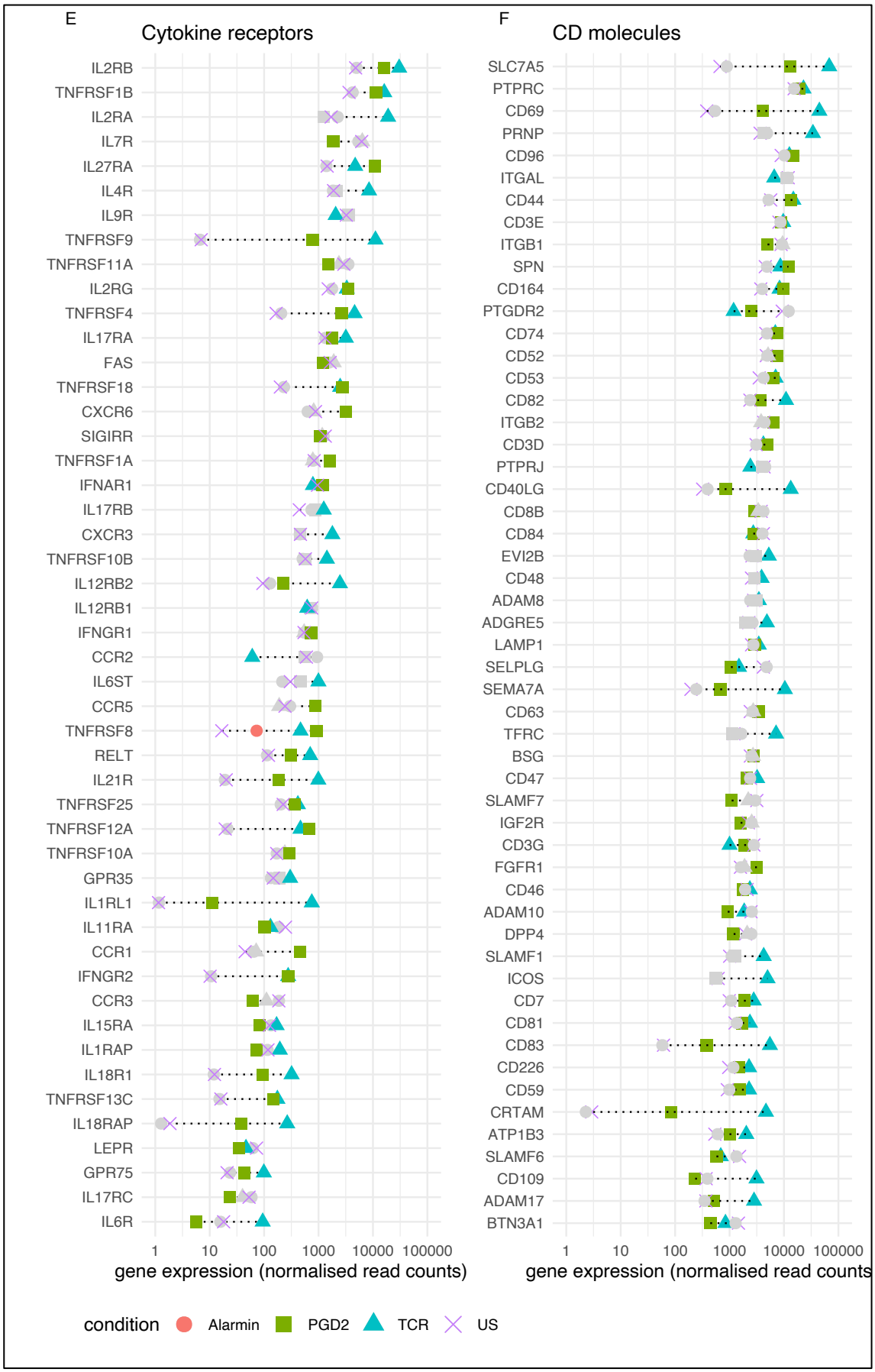
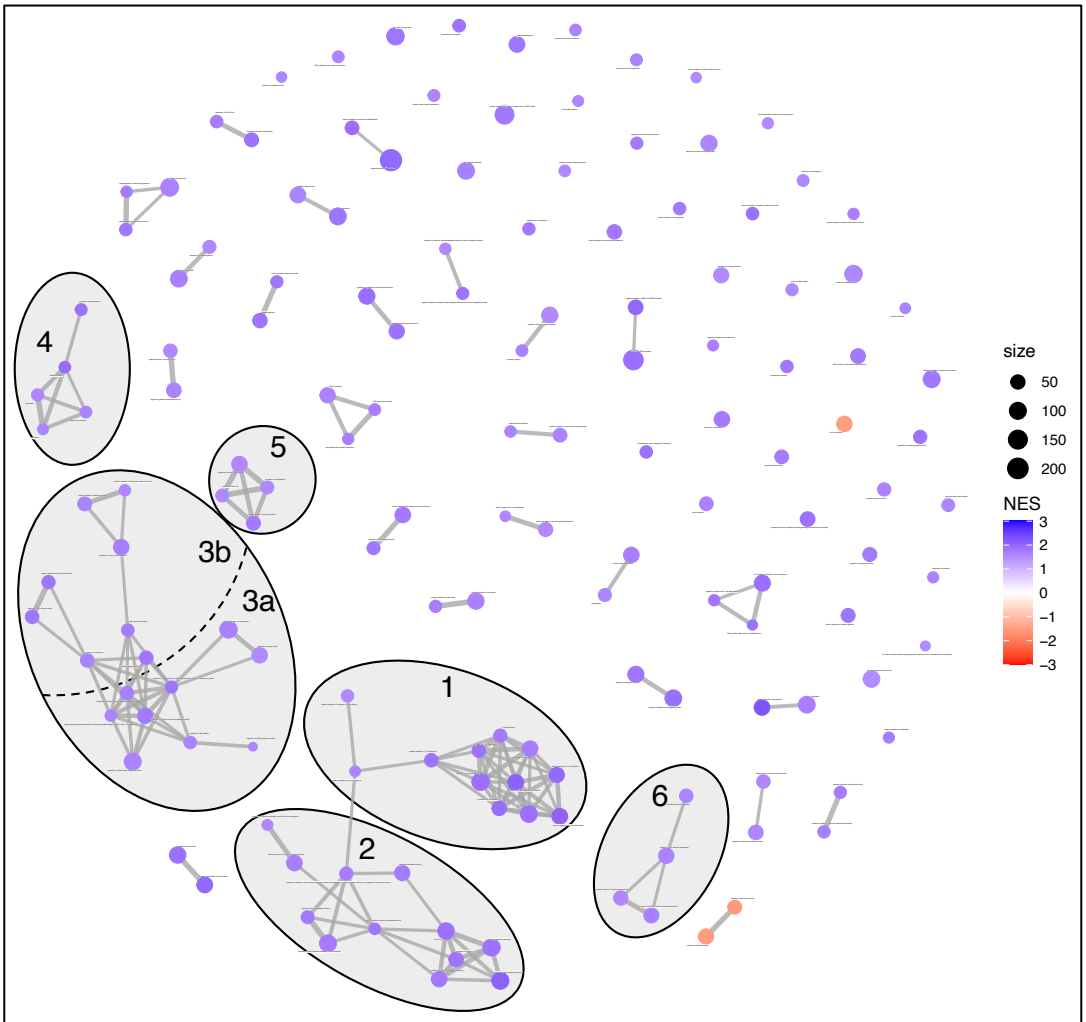

E
Cytokine receptors
IL2RB
TNFRSF1B
IL2RA
IL7R
IL27RA
IL4R
IL9R
TNFRSF9
TNFRSF11A
IL2RG
TNFRSF4
IL17RA
FAS
TNFRSF18
CXCR6
SIGIRR
TNFRSF1A
IFNAR1
IL17RB
CXCR3
TNFRSF10B
IL12RB2
IL12RB1
IFNGR1
CCR2
IL6ST
CCR5
TNFRSF8
RELT
IL21R
TNFRSF25
TNFRSF12A
TNFRSF10A
GPR35
IL1RL1
IL11RA
CCR1
IFNGR2
CCR3
IL15RA
IL1RAP
IL18R1
TNFRSF13C
IL18RAP
LEPR
GPR75
IL17RC
IL6R
1
10
100
1000
10000
100000
gene expression (normalised read counts)
F
CD molecules
SLC7A5
PTPRC
CD69
PRNP
CD96
ITGAL
CD44
CD3E
ITGB1
SPN
CD164
PTGDR2
CD74
CD52
CD53
CD82
ITGB2
CD3D
PTPRJ
CD40LG
CD8B
CD84
EVI2B
CD48
ADAM8
ADGRE5
LAMP1
SELPLG
SEMA7A
CD63
TFRC
BSG
CD47
SLAMF7
IGF2R
CD3G
FGFR1
CD46
ADAM10
DPP4
SLAMF1
ICOS
CD7
CD81
CD83
CD226
CD59
CRTAM
ATP1B3
SLAMF6
CD109
ADAM17
BTN3A1
1
10
100
1000
10000
100000
gene expression (normalised read counts
condition
Alarmin
PGD2
TCR
US

Figure 50 Expression of gene groups important to T cell biology

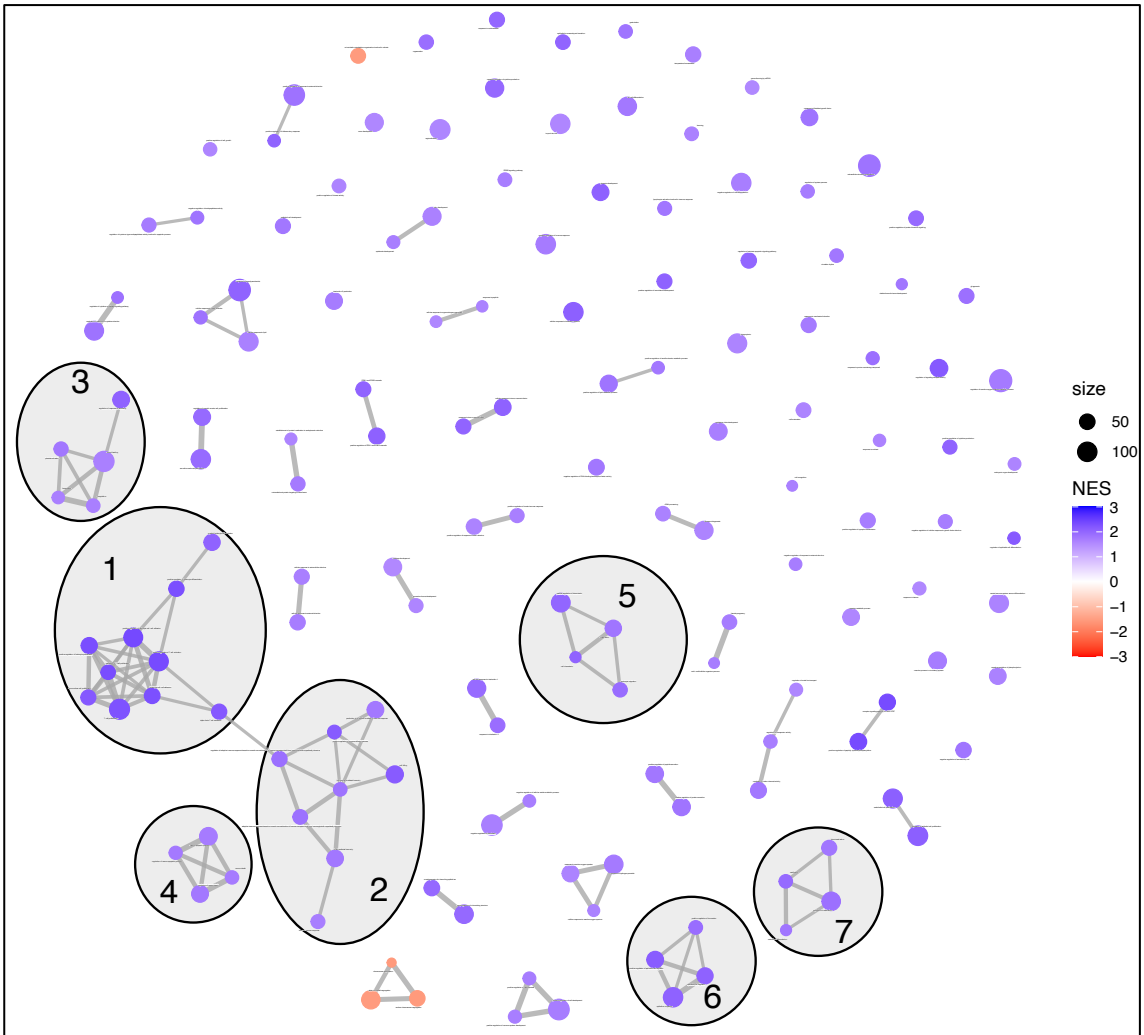
The x-axis represents absolute normalised read count. The y-axis represents individual genes, ordered by their average absolute expression across the whole experiment. Each gene is represented by four points, one point per condition, linked by a dashed horizontal line, Each point represents the mean absolute read count for the replicates (n = five donors) within that condition. Points are coloured if *padj* < 0.05 compared with the unstimulated control (US), or greyed out if *padj* > 0.05. The greyed out points are therefore generally close to the unstimulated control values. The minimum average number of reads for a gene to be included in the figure is 100 (A to C), 20 (D and E) 20, or 1000 (F) reads.



#	Interpretation of cluster	Pathway IDs (GO)
1	T cell activation	0050868, 0042129, 0032944, 0042098, 0007159, 0032943, 1903037, 0042110, 0050870, 0002696, 0002832, 0050777
2	Adaptive immune response	0002718, 0002367, 0002712, 0002889, 0002822, 0002705, 0002449, 0001910, 0001906, 0031341, 0001909, 0042667
3	a) Metabolic processes b) Innate defense mechanisms	a) 0071453, 0070482, 0061418, 1903311, 0043487, 0006521, 0010565, 0031145, 0002479 b) 0038061, 0038093, 0070555, 0071356, 0034612, 0045088, 0031349, 0032103
4	Wound repair	0030168, 0007599, 0050817, 0009611, 0034109
5	Regulation of vessel diameter	0035150, 0035296, 0003018, 0097746
6	Regulation of actin cytoskeleton	0030838, 0032273, 0110053, 0051017

Figure 51 GO (BP) GSEA - PGD_2 stimulation

Network diagram of GO (BP) terms enriched after PGD_2 stimulation. The top 150 most enriched gene sets are shown. Each gene set is a node and nodes are connected by an edge if the gene sets share > 30 % similarity. Clusters of nodes were circled and annotated manually. Size refers to number of genes in each set. Abbreviations: NES, normalised enrichment score.



#	Interpretation of cluster	Pathway IDs (GO)
1	T cell activation	0002573, 1902107, 0046631, 1903039, 0050870, 0007159, 0042098, 0032943, 0042129, 0070665
2	Adaptive immune response	0002440, 0002699, 0002822, 0001906, 0002449, 0002460, 0019724, 0006959
3	Wound repair	1903034, 0042060, 0030168, 0007599, 0050817
4	Neuronal cell death	0070997, 1901214, 0043523, 0051402
5	Chemotaxis	0050921, 0006935, 0060326, 0050900
6	Epithelial cell migration	0040017, 0010631, 0090132, 0010634
7	Bone deposition	0030278, 0001503, 0110148, 0001649

Figure 52 GO (BP) GSEA - TCR stimulation

Network diagram of GO (BP) terms enriched after TCR stimulation. The top 150 most enriched gene sets are shown. Each gene set is a node and nodes are connected by an edge if the gene sets share > 30 % similarity. Clusters of nodes were circled and annotated manually. Size refers to number of genes in each set. Abbreviations: NES, normalised enrichment score.

4.3.3.2 Kyoto Encyclopedia of Genes and Genomes (KEGG)

GSEA for KEGG pathways revealed zero, 55, and 85 significantly enriched pathways for alarmins, PGD_2, and TCR stimulation conditions respectively (Supplementary Figure 40B and Supplementary Table 34) The strongest enrichment of a pathway after TCR stimulation was 'cytokine-cytokine receptor interactions' which was also upregulated in the PGD_2 group (Figure 53). This reflects the widespread changes in expression of cytokines and their receptors after $CRTH2^+$ $CD8^+$ T cell activation. Other enriched pathways included asthma and inflammatory bowel disease, with possible involvement of type 17 immunity and tumour necrosis factor (TNF) signalling.

4.3.3.3 Reactome Pathway Database

Reactome pathway analysis resulted in zero significantly enriched pathways after alarmin stimulation, 165 after PGD_2 stimulation and 92 after TCR stimulation (Supplementary Figure 40C and Supplementary Table 35). As with GSEA for GO terms, clusters of nonredundant enriched pathways ('modules') were identified by network analysis (Figure 54 and Figure 55). After both PGD_2 and TCR stimulation, modules of related pathways could be seen involving NF-κB signalling, and general transcription and translation. In the TCR group, a module of gene sets related to ECM remodelling. In the PGD_2 group, one of the modules appeared to phagocytic responses to parasites. The core genes driving pathway enrichment in these modules are displayed as heatmaps (Supplementary Figure 42).

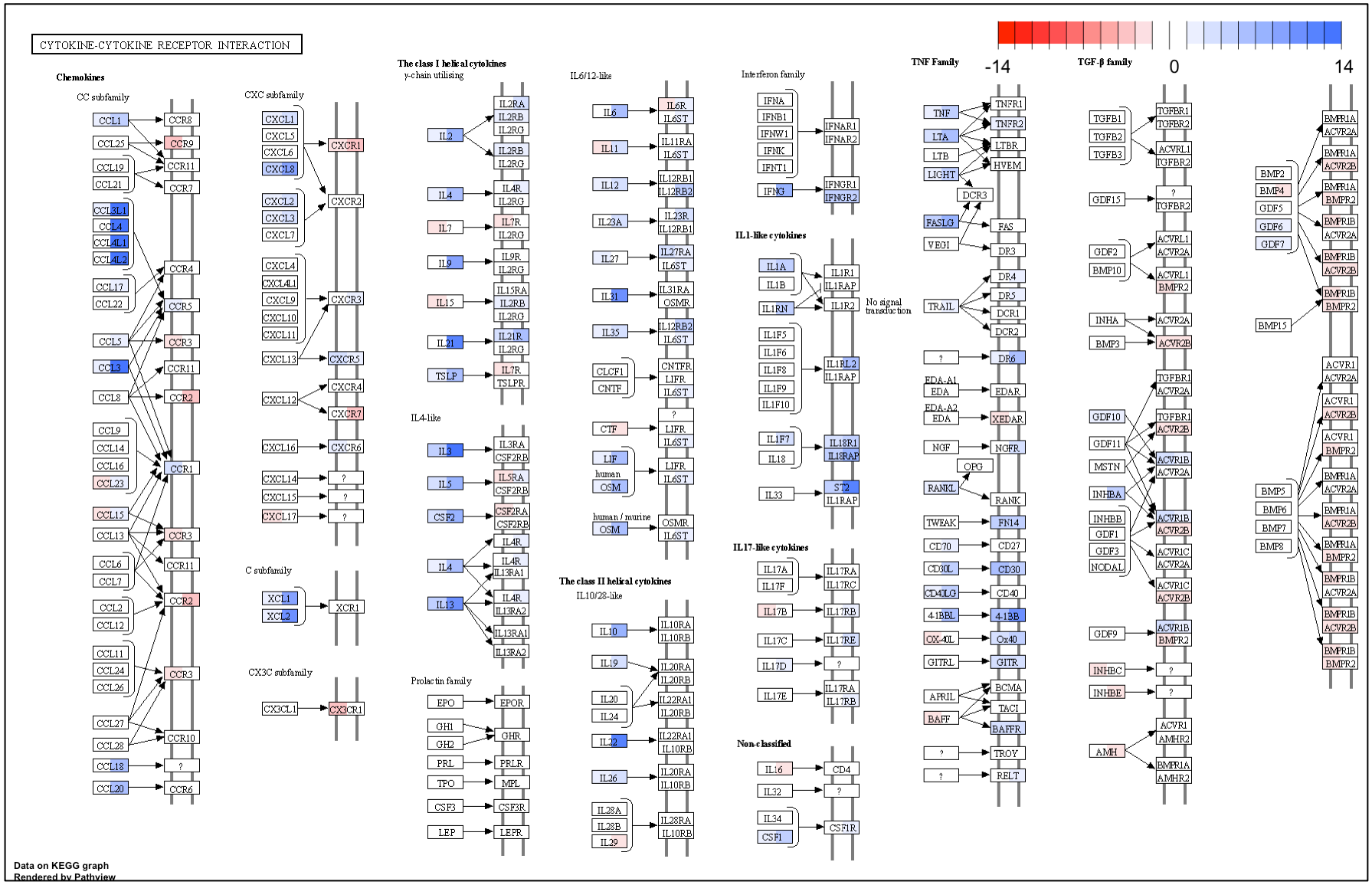
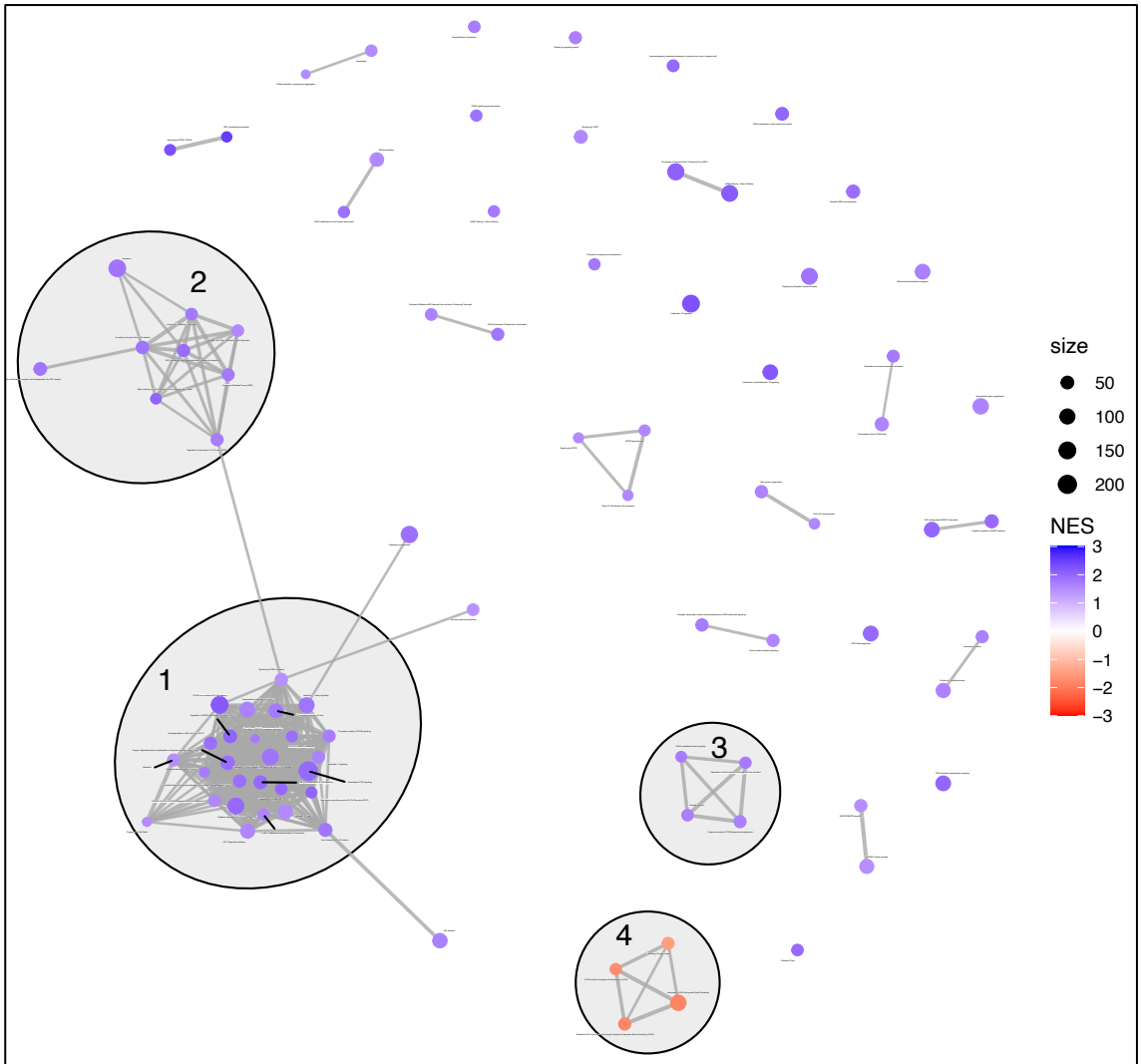

CYTOKINE-CYTOKINE RECEPTOR INTERACTION
Chemokines
CC subfamily
CXC subfamily
C subfamily
CX3C subfamily
The class I helical cytokines
γ-chain utilising
IL4-like
Prolactin family
IL6/12-like
The class II helical cytokines
IL10/28-like
Interferon family
IL1-like cytokines
No signal transduction
IL17-like cytokines
Non-classified
TNF Family
TGF-β family
-14
0
14
Data on KEGG graph
Rendered by Pathview

Figure 53 Cytokine-cytokine receptor KEGG pathway map – TCR and PGD_2 data overlay

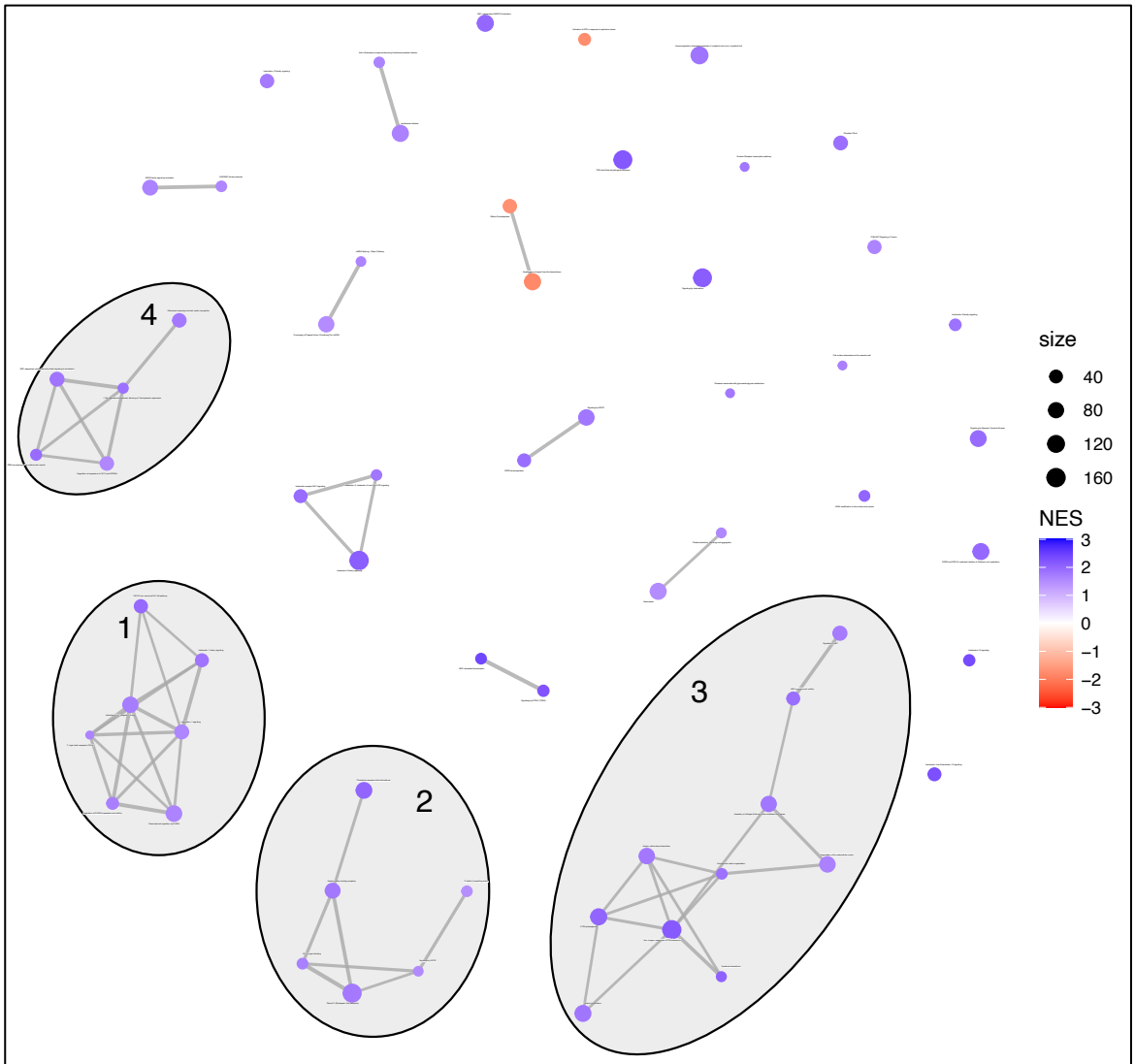
The colour legend refers to log_2FC of DEGs, vs US. For each gene, the left-hand side of the label represents fold-change in the PGD_2 condition, the right-hand side the TCR condition. Pathway maps were generated by the pathview package in R (W. Luo & Brouwer, 2013).



#	Interpretation of cluster	Pathway IDs (Reactome RA-HSA-#)
1	NF-κB and RUNX-related signalling, apoptosis, and metabolic pathways	376176, 5668541, 8878166, 5621481, 446652, 174084, 8941858 8939902, 5607764, 2454202, 109581, 8878159, 1234176, 450531, 446652, 202424, 5362768, 5676590, 351202, 1168372, 8852276, 9604323, 69202, 5632684, 5357801, 1236974, 162909
2	Transcription and translation	72766, 72764, 168273, 72695, 72689, 1799339, 927802, 6791226, 9010553
3	Phagocytic response to parasite infection	3928662, 2029482, 9664407, 2029480
4	DNA repair mechanisms	5693538, 5685942, 5693579, 5693554

Figure 54 Reactome GSEA network diagram for PGD_2 stimulation

Network diagram of Reactome terms enriched after PGD_2 stimulation. Each gene set is a node and nodes are connected by an edge if the gene sets share > 30 % similarity. Clusters of nodes were circled and annotated manually. Size refers to number of genes in each set. Abbreviations: NES, normalised enrichment score.



#	Interpretation of cluster	Pathway IDs (Reactome RA-HSA-#)
1	NF-κB-activation-related signalling pathways	5668541, 446652, 1169091, 9020702, 5621481, 8941858, 8878159
2	GPCR binding and signaling	9006934, 182971, 500792, 418594, 372790, 373076
3	Extracellular matrix interactions and remodeling	6806834, 8875878, 2022090, 1474228, 1474244, 216083, 3000178, 3000171, 3000170, 3000157
4	Transcriptional and translational machinery	72702, 156827, 1799339, 9010553, 8868773

Figure 55 Reactome GSEA network diagram for TCR stimulation

Network diagram of Reactome terms enriched after TCR stimulation. Each gene set is a node and nodes are connected by an edge if the gene sets share >30% similarity. Clusters of nodes were circled and annotated manually. Size refers to number of genes in each set. Abbreviations: NES, normalised enrichment score.

Individual enriched Reactome pathways can be visualised in terms of their structure on the Reactome database. I used this function to visualise the difference between PGD_2 and TCR stimulation in terms of the Interleukin-4 and Interleukin-13 signalling pathways (Figure 56). This shows broadly similar activation of these two pathways in both stimulation conditions, with the exceptions of some oppositely changed genes such as type 2 regulator *BCL6* (discussed in 4.4.4.4), and STAT5 signalling-associated *PIM1* (discussed in 4.4.3.1).

4.4 Discussion

The aim of this chapter is to understand the response of $CRTH2^+$ $CD8^+$ T cells to activation by three modes of stimulation. In this section I investigate the top DEGs as identified in the results figures. My interpretations are summarised in Figure 57.

4.4.1 Technical strengths and limitations of this dataset

This study considers only one cell type - $CRTH2^+$ $CD8^+$ T cells. This maximised the power of the study by limiting numbers of comparisons, and using purified cultures eliminated any need for *in silico* deconvolution of multiple cell types. The large number of purified cells grown by the rapid expansion protocol resulted in a high quality and quantity of input RNA for the RNA-Seq experiment, which would not have been practical using *ex vivo* cells. In future, a comparison could be made with other $CRTH2^+$ and $CRTH2^-$ T cell subsets, especially Th1, Th2, and Tc1 cells. This would resolve gene expression changes specific to $CRTH2^+$ $CD8^+$ T cell activation, from changes that are common to other T cells.

We do not know how closely expanded cultured T cells model with *in vivo* counterparts, especially lung T cells in their tissue context. To understand functions such as wound repair and ECM remodelling may require study of real lung tissue. It is also possible that $CRTH2^+$

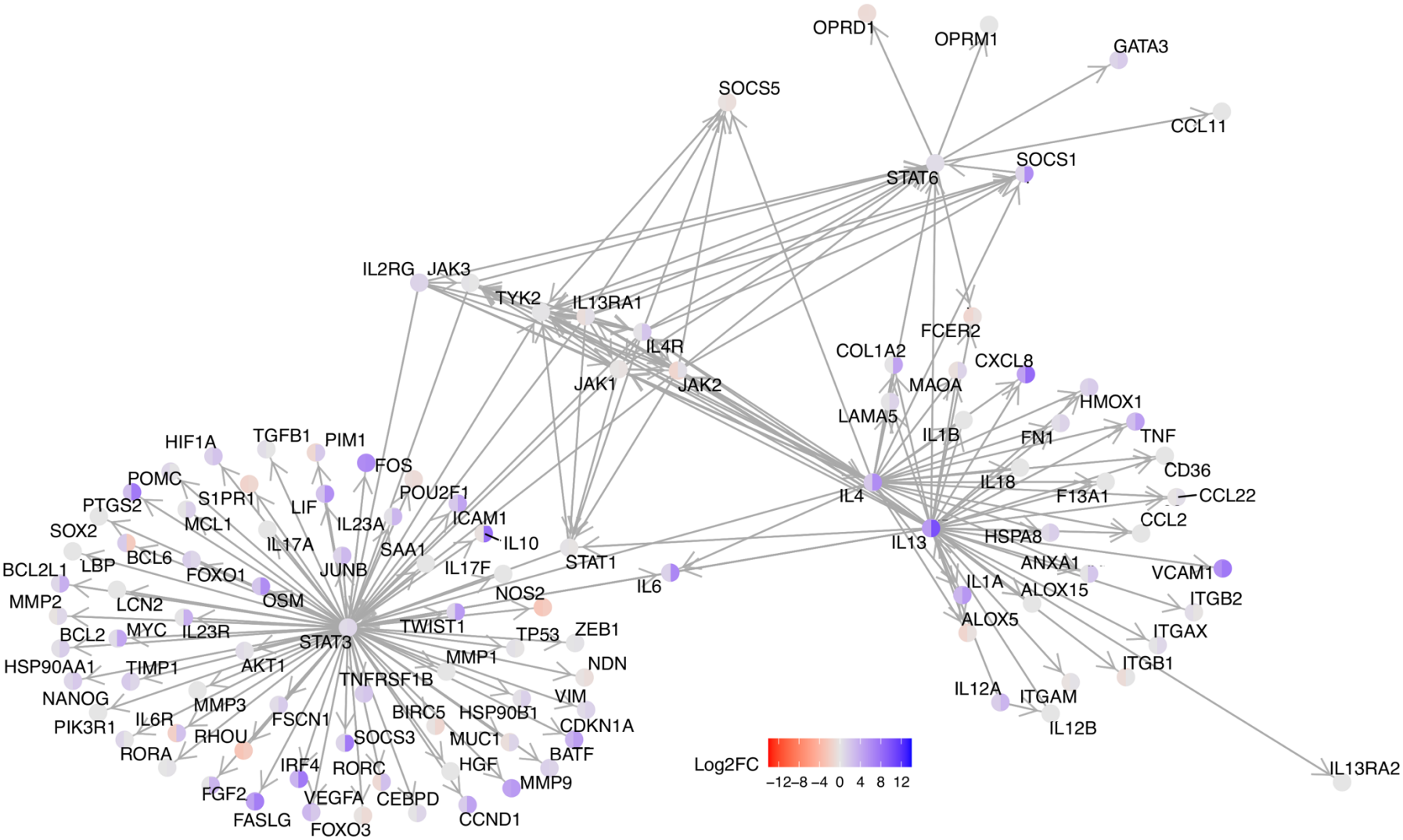

OPRD1
OPRM1
GATA3
SOCS5
CCL11
SOCS1
STAT6
IL2RG
JAK3
TYK2
IL13RA1
IL4R
JAK1
JAK2
COL1A2
FCER2
CXCL8
MAOA
HMOX1
LAMA5
IL1B
FN1
TNF
CD36
IL18
F13A1
CCL22
CCL2
IL4
IL13
HSPA8
ANXA1
VCAM1
IL1A
ALOX15
ITGB2
ALOX5
ITGAX
ITGB1
IL12A
ITGAM
IL12B
IL13RA2
HIF1A
TGFB1
PIM1
FOS
POMC
S1PR1
LIF
POU2F1
PTGS2
MCL1
IL23A
ICAM1
SOX2
IL17A
IL10
SAA1
BCL6
BCL2L1
LBP
FOXO1
JUNB
IL17F
STAT1
IL6
MMP2
OSM
NOS2
LCN2
BCL2
MYC
IL23R
STAT3
TWIST1
TP53
ZEB1
HSP90AA1
TIMP1
AKT1
MMP1
NDN
TNFRSF1B
NANOG
MMP3
VIM
IL6R
FSCN1
BIRC5
HSP90B1
PIK3R1
RHOU
SOCS3
MUC1
CDKN1A
RORA
IRF4
RORC
HGF
BATF
FGF2
VEGFA
CEBPD
MMP9
FASLG
FOXO3
CCND1
Log2FC
−12 −8 −4 0 4 8 12

Figure 56 Reactome pathway IL4 and IL13 signalling – overlay of PGD_2 and TCR data

Arrows indicate direction of cause and effect in the Reactome pathway. The left half of each datapoint is coloured by PGD_2 fold-change, the right half by TCR fold change. This plot was drawn in GIMP (The GIMP Development Team, 2019) by merging two individual plots.

$CD8^+$ T cells are not a homogenous population: this might be detectable with a single cell technique. This dataset concerns a single stimulation time point, but T cell activation is a dynamic process with events that take place over seconds to days (Obst, 2015). Alarmin cytokines could exert effects over long time periods in the human lung. In terms of the stimulation conditions, the alarmin cytokines were combined in this experiment to make the experiment more feasible. It may be informative to assess them individually, alongside other type 2 mediators such as IL-4, IL-5, leukotriene E_4 (Hilvering et al., 2018), adenosine, and neuromedin U. I did not block the other prostaglandin D_2 receptor DP_1 (*PTGDR1*), but its expression was not detected, so it is unlikely to have played a major role.

A clear limitation of this study is that it exclusively concerns mRNA. The transcriptome is one aspect of a cell but proteins, lipids, and metabolites carry out the majority of cell function. Advantages of transcript detection by RNA-Seq are that it is unbiased, comprehensive, sensitive, and specific even for rare transcripts. This is not true for detection of other cell components: for example, protein detection by antibodies is variable and there is often little or no supporting validation data for the detection reagents (Uhlen et al., 2016). Although RNA-Seq has been available for more than a decade (Mortazavi et al., 2008), aspects of its use lack consensus guidelines e.g. ordering of DEGs, comparing multiple lists of DEGs, and defining 'low' or 'high' expression. Because of the relatively low number of reads per sample in this study, some types of analysis such as isoform quantification could not be performed.

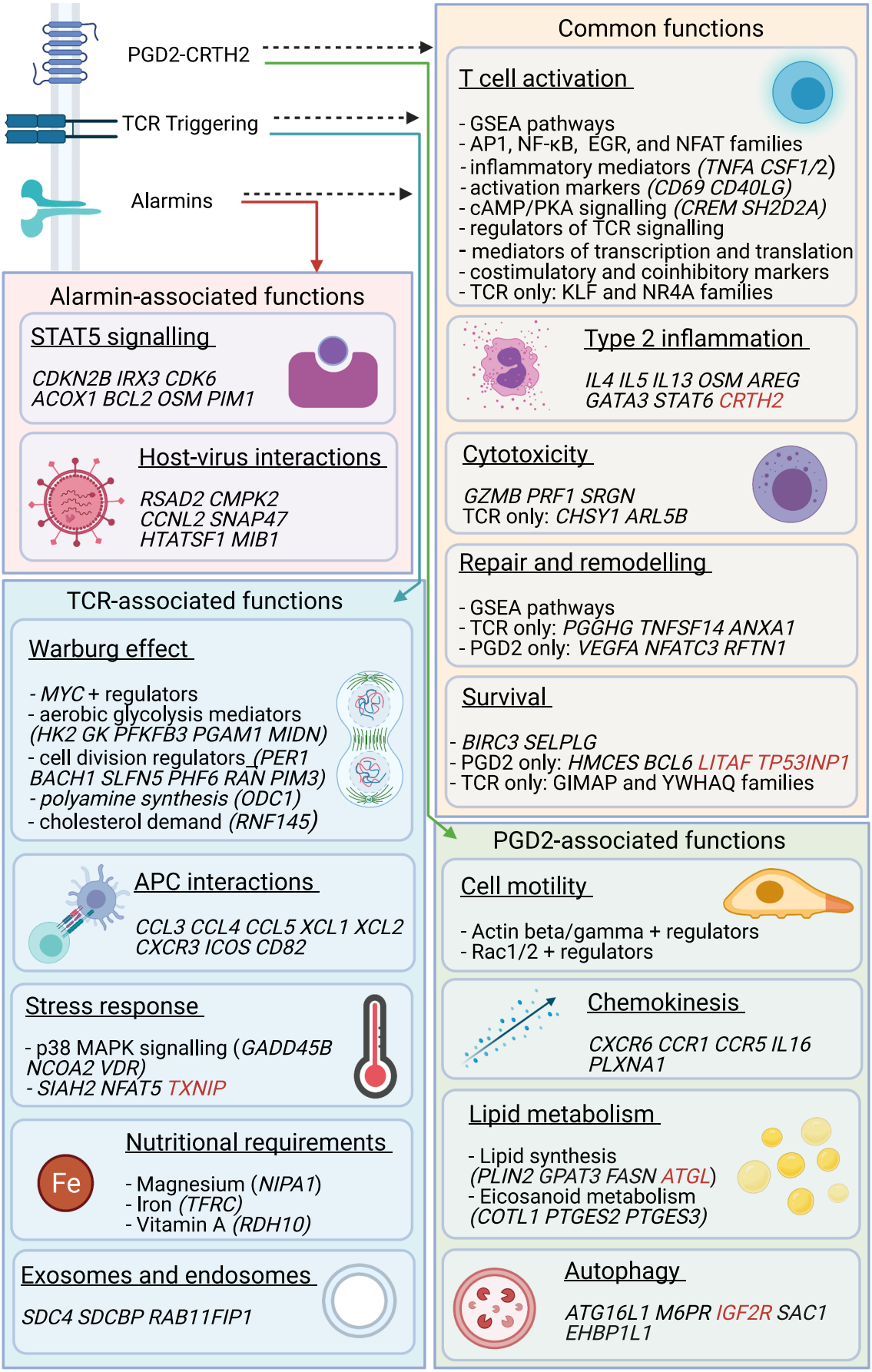

PGD2-CRTH2
TCR Triggering
Alarmins
Alarmin-associated functions
STAT5 signalling
CDKN2B IRX3 CDK6
ACOX1 BCL2 OSM PIM1
Host-virus interactions
RSAD2 CMPK2
CCNL2 SNAP47
HTATSF1 MIB1
TCR-associated functions
Warburg effect
- MYC + regulators
- aerobic glycolysis mediators
(HK2 GK PFKFB3 PGAM1 MIDN)
- cell division regulators (PER1
BACH1 SLFN5 PHF6 RAN PIM3)
- polyamine synthesis (ODC1)
- cholesterol demand (RNF145)
APC interactions
CCL3 CCL4 CCL5 XCL1 XCL2
CXCR3 ICOS CD82
Stress response
- p38 MAPK signalling (GADD45B
NCOA2 VDR)
- SIAH2 NFAT5 TXNIP
Fe
Nutritional requirements
- Magnesium (NIPA1)
- Iron (TFRC)
- Vitamin A (RDH10)
Exosomes and endosomes
SDC4 SDCBP RAB11FIP1
Common functions
T cell activation
- GSEA pathways
- AP1, NF-κB, EGR, and NFAT families
- inflammatory mediators (TNFA CSF1/2)
- activation markers (CD69 CD40LG)
- cAMP/PKA signalling (CREM SH2D2A)
- regulators of TCR signalling
- mediators of transcription and translation
- costimulatory and coinhibitory markers
- TCR only: KLF and NR4A families
Type 2 inflammation
IL4 IL5 IL13 OSM AREG
GATA3 STAT6 CRTH2
Cytotoxicity
GZMB PRF1 SRGN
TCR only: CHSY1 ARL5B
Repair and remodelling
- GSEA pathways
- TCR only: PGGHG TNFSF14 ANXA1
- PGD2 only: VEGFA NFATC3 RFTN1
Survival
- BIRC3 SELPLG
- PGD2 only: HMCES BCL6 LITAF TP53INP1
- TCR only: GIMAP and YWHAQ families
PGD2-associated functions
Cell motility
- Actin beta/gamma + regulators
- Rac1/2 + regulators
Chemokinesis
CXCR6 CCR1 CCR5 IL16
PLXNA1
Lipid metabolism
- Lipid synthesis
(PLIN2 GPAT3 FASN ATGL)
- Eicosanoid metabolism
(COTL1 PTGES2 PTGES3)
Autophagy
ATG16L1 M6PR IGF2R SAC1
EHBP1L1

Figure 57 Interpretation of the DEGs identified in this experiment

Individual genes are indicated in italics. Entries in black represents upregulation with stimulation, and red represents downregulation. Entries in the 'common functions' bubble include individual DEGs, groups of DEGs, or gene sets, that were identified in the figures and changed in the same direction in more than one stimulation condition (but not necessarily all three conditions). Entries in the 'TCR-associated functions' bubble and other equivalent bubbles were identified in the figures and are uniquely or especially changed in that specific condition only. Created in Biorender.com.

Since there were too many DEGs to explore all of them, I used GSEA to identify changes to sets of related genes. Although GSEA is useful for identifying biological changes that are not obvious from the initial DEG list, the widely used databases such as GO and Reactome define gene sets irrespective of cell type. Given that genes can play very different roles in different cell types (Lobo, 2008), it would be useful to develop a gene set database specific to T cell biology. For many of the DEGs I investigated, the available literature often focussed on cell types such as tumours or neurons, which may not reflect T cells. Further basic research specific to T cell activation and function will be required before 'omics' datasets can be fully interpreted.

4.4.2 General properties of activated CRTH2$^+$ CD8$^+$ T cells

4.4.2.1 Culture identity was confirmed by high expression of genes for the defining surface markers

Among the most highly expressed genes for CD molecules were those encoding subunits of the culture-defining CD3 (*CD3E*, *CD3D*, and *CD3G*), CD8 (*CD8B*), and CRTH2 (*PTGDR2*) surface markers, whereas *CD4* was not detected. This supports the stated identity of the cell cultures (CD3$^+$ CD8$^+$ CRTH2$^+$). *PTGDR2* was downregulated after PGD_2 and TCR stimulation, in line with the protein measurements shown in 3.3.1.1, but mean expression after alarmin stimulation increased, though not significantly. It would be worth checking on a larger number of donors whether alarmin stimulation increases the quantity of surface CRTH2, because this could represent a direct T2-enhancing effect.

4.4.2.2 CRTH2$^+$ CD8$^+$ T cells have a type 2 cytotoxic (Tc2) profile

Genes for canonical type 2 cytokines *IL4*, *IL5*, and *IL13* were among the most upregulated and highly expressed cytokines across all conditions. The defining Th2 transcription factors *GATA3* and *STAT6* were highly or very highly expressed throughout. Other type 2 mediators were expressed at a lower level: the genes for amphiregulin (*AREG*) (Morimoto et al., 2018; Zaiss et al., 2006) and Oncostatin M (*OSM*) (Richards, 2013, p. 10; Richards & Botelho, 2019). Many of these genes have been linked to asthma (Supplementary Table 32). Together, this confirms the type 2 capability of CRTH2$^+$ CD8$^+$ and their potential to contribute to type 2 inflammation in asthma.

The gene for canonical Th1 cytokine interferon gamma (*IFNG*) was not highly expressed in any condition. The ratio of genes for GATA3 (*GATA3*) to T-bet (*TBX21*) was dominated by *GATA3* in all conditions, suggesting T-bet was outcompeted (Oestreich & Weinmann, 2012). Genes encoding the canonical type 17 cytokine IL-17 (*IL17A*) and transcription factor ROR-γ (*RORC*) were undetectable. Although the gene for type 17-associated transcription factor *IRF4* (M. Huber et al., 2008; Mudter et al., 2011) was upregulated in PGD_2 and TCR conditions, it plays other roles in T2 responses (Honma et al., 2008) and Tc effector function in general (M. Huber & Lohoff, 2013; Mittrücker et al., 2017). Therefore, it appears that CRTH2$^+$ CD8$^+$ T cells were dominantly type 2 and did not present a mixed phenotype.

Nevertheless, CRTH2$^+$ CD8$^+$ T cells retain cytotoxic features. Genes related to killing, such as those encoding pore-forming perforin 1 (*PRF1*) and granzyme B (*GZMB*) (Voskoboinik et al., 2015), were among the top DEGs, as was the gene for serglycin (*SRGN*), a structural component of dense-core cytolytic granules in Tc cells (Sutton et al., 2016). Serglycin is modified by chondroitin sulphate side chains (Sutton et al., 2016), and

the gene for chondroitin sulphate synthase 1 (*CHSY1*) was very highly expressed after TCR triggering. *ARL5B*, also uniquely upregulated after TCR triggering, encodes Arl8 involved in movement and delivery of lytic granules in Tc cells (Kabanova et al., 2016; Khatter et al., 2015). A $CD8^+$ cytotoxic effector identity may be enforced by high expression of the gene for RUNX3 (*RUNX3*) (Shan et al., 2017), which is an asthma-associated gene (Supplementary Table 32), and increased further with stimulation. RUNX3 cooperates with TLE3, encoded by *TLE3* which was especially upregulated by PGD_2 stimulation, to maintain $CD8^+$ T cell identity by silencing CD4-associated genes (Xing et al., 2018). This is in agreement with a previous study that found Tc2 to express high levels of perforin and granzymes in cultured Tc2 cells (Hilvering et al., 2018), and with older functional data (Cerwenka et al., 1999; Dobrzanski et al., 2004). What is the role of cell killing in type 2 inflammation? One possibility is that a type 2 response to a viral infection is sometimes beneficial to the host, which may explain why viral infection of the airways often triggers release of type 2-skewing alarmin cytokines (Harker & Lloyd, 2021). Another possibility is that viruses may promote a type 2 response as a means of immune evasion, in which case it makes sense to ensure that cytotoxic T cells do not completely lose their function in a type 2 environment, or the immune system would be compromised in its ability to fight virus.

4.4.2.3 High expression of receptors for some type 2 mediators (IL-4, IL-9, IL-25, and NMU) but not others (IL-33 and TSLP)

The gene for the TSLP receptor (*CRLF2*) was undetectable across the experiment. *TSLP* is an asthma-associated gene (Supplementary Table 32) and the TSLP pathway has been intensively targeted in asthma. A phase 3 trial of anti-TSLP antibody tezepelumab was conducted and the primary outcome (exacerbations) was met in T2-high and T2-low groups, although improvements scaled with eosinophil count (Menzies-Gow et al., 2021). My data suggests that TSLP is unlikely to signal directly through Tc2 cells. I would propose

that tezepelumab operates through some other mechanism than direct targeting of type 2 T cells, perhaps by influencing DC activation trafficking to the lymph nodes.

The gene for IL-33 receptor subunit ST2 (*IL1RL1*) only became moderately expressed after TCR triggering. This may be in agreement with a previous paper that found Tc2 cells expressed very low ST2 in comparison to Tc1 cells, and that expression increases after TCR triggering (Q. Yang et al., 2011). The IL-33-ST2 axis is genetically implicated in asthma (Supplementary Table 32) but clinical trials have struggled and a phase III trial has not happened yet (Plieth, 2020). This data suggests that resting Tc2 cells would not be affected by IL-33 blockade, although perhaps activated Tc2 could express the receptor. Single cell RNA-sequencing data indicates *IL1RL1* expression is restricted to mast cells and endothelial cells (Travaglini et al., 2020), so I would propose that these are the major cell types involved in the IL-33 axis.

Both genes for components of the IL25 receptor (*IL17RA* and *IL17RB*) were expressed moderately or highly throughout. This suggests that resting Tc2 cells could respond directly to IL-25, and it is likely that the observed effects were due to IL-25 action. Of the three alarmin cytokines, IL-25 is the least well understood, although numerous studies have linked it to allergic asthma (Porsbjerg et al., 2020). Importantly, IL-25 expression is induced by rhinovirus infection of humans, and in the ovalbumin mouse model of asthma this in turn causes type 2 inflammation of the airways, including infiltration of IL17RB$^+$ Th cells (Beale et al., 2014). It is therefore plausible that CRTH2$^+$ CD8$^+$ are also able to respond to IL-25 in the context of rhinovirus infection. There is not yet trial data for targeting the IL-25 axis in asthma, but I would argue that it is most likely to benefit patients with lung-resident type 2 IL17RB$^+$ T cells at risk from virally-induced exacerbations.

Genes encoding receptors for other type 2 mediators (not alarmins) were highly expressed: IL4R (encoded by *IL4R*), IL-9 receptor (IL9R, encoded by *IL9R*), and neuromedin receptor 1 (NMUR1, encoded by *NMUR1*). IL4R is already the target of an approved drug for T2-high asthma, dupilumab (Chupp et al., 2020), and its action may partially occur through CRTH2$^+$ CD8$^+$ T cells. The IL-9 axis has also been targeted but so far unsuccessfully (Oh et al., 2013). However, a trial has not yet be constructed in which patients are stratified by IL-9 activity. Targeting of the NMU axis is still at early stages. Future work could seek to confirm the presence of these receptors on the cell surface of CRTH2$^+$ CD8$^+$ T cells, and to evaluate direct effects of these type 2 mediators.

There was also expression of genes for some receptors of type 1 mediators, such as interferon gamma (*IFNGR1* and *IFNGR2*) and IL-12 (*IL12RB1* and *IL12RB2*), especially after stimulation, indicating possible sensitivity to a type 1 environment. After TCR triggering, *NLRP6,* encoding the inflammasome and pathogen sensor NLRP6 (Ghimire et al., 2020) was highly expressed, perhaps in anticipation of infection.

4.4.2.4 Generic markers of CD8$^+$ T cell activation were upregulated across multiple stimulation conditions

GO pathway analysis identified clusters of pathways related to T cell activation in the PGD_2 and TCR conditions and this was supported by many of the top DEGs. Genes encoding general inflammatory and activation markers were upregulated in multiple conditions such as *TNF*, *LIF* (Nicola & Babon, 2015), *CSF1* (encodes M-CSF) and *CSF2* (encodes GM-CSF) (Ushach & Zlotnik, 2016), *CD69* (Ziegler et al., 1994) and *CD40LG* (Lederman et al., 1992).

Reactome pathway analysis suggested genes associated with transcription and translation were enriched in PGD_2 and TCR conditions, and some top upregulated DEGs were

involved in this function. *SFPQ* encodes PSF splice factor, which has widespread roles in transcription and translation (Yarosh et al., 2015). *BZW1* encodes BZW1 involved in translation initiation (Loughran et al., 2018). Members of the HSP90 (e.g. *HSP90B*) and HSP70 (e.g. *HSPA13*) families are involved in in protein folding and degradation (Biebl & Buchner, 2019; Rosenzweig et al., 2019). Protein synthesis requires amino acids, and genes for LAT1 (*SLC7A5*) and CAT-1 (*SLC7A1*) neutral and cationic amino acid transporters were highly upregulated (Fotiadis et al., 2013; Hayashi et al., 2013). In the TCR condition, the gene for Herp2 (*HERPUD2*) was specifically increased. Herp2 acts as a scaffold in ER-associated protein degradation (ERAD) (C. H. Huang et al., 2014), part of ER quality control (ERQC) mechanisms (Vembar & Brodsky, 2008) that regulate protein folding and turnover. Genes for negative regulators of transcription and translation such as the GW182 family (*TNRC6A*, *TNRC6B* and *TNRC6C*) components of mi-RISC (Eulalio et al., 2009) were suppressed. Overall, this suggests increased transcription and translation.

The transcriptional changes observed in this experiment were likely driven by canonical transcription factors associated with T cell activation. Genes for all members of the AP-1 transcription factors (i.e., Jun and Fos protein families) were highly upregulated in both the PGD_2 and TCR conditions, with *JUND* and *FOS* also upregulated by alarmin stimulation. Likewise, all NF-κB subunit genes were highly expressed and upregulated in both PGD_2 and TCR conditions. The gene for regulator IκBNS (*NFKBID*) was associated with TCR triggering – it is also linked to IL-2 induction so may explain why only TCR triggering induced *IL2* (Schuster et al., 2013). The NFAT family of transcription factors differentially changed between TCR and PGD_2 conditions. Both stimulated expression of the genes for NFAT1 (*NFATC2*) and NFAT2 (*NFATC1*), but only PGD_2 stimulation elevated NFAT4 (*NFATC3*). NFATs are regulated by calcium signalling except NFAT5 which is regulated

by osmotic stress (Müller & Rao, 2010) and *NFAT5* was specifically upregulated by TCR triggering. Calcium signalling may be regulated by the NF-κB target RRAD, and *RRAD* was a top DEG in both conditions (Finlin et al., 2003; G. Liu et al., 2020). Genes encoding the early growth response (EGR) family were among the top DEGs across conditions – they are broadly involved in T cell activation by influencing calcium signalling (Mookerjee-Basu et al., 2020), NF-κB (Wieland et al., 2005), AP-1 (S. Li et al., 2012) and NFAT (Decker et al., 2003). Genes for EGR regulators NAB1 (*NAB1*) and NAB2 (*NAB2*) (LaVallee et al. 2019) were also highly upregulated after PGD_2 or TCR stimulation.

Genes for further T cell activation markers were upregulated specifically after TCR triggering. Most of the KLF transcription family genes, with widespread roles including differentiation (Hart et al., 2012), were associated with TCR triggering. The NR4A family of transcription factors are early markers of T cell activation upregulated in proportion to the degree of TCR triggering and not by bystander activation (Odagiu et al., 2021). NR4A upregulation may be due to increased *KDM6B*, encoding lysine demethylase B which catalyses removal of H3K27me3 methylation at NR4A genes (Jasmine Li et al., 2021). One of the NR4A genes, *NR4A2,* is associated with COPD (Supplementary Table 32). *IL3* encodes IL3 (a.k.a. 'multi-CSF') which promotes a range of immune cell responses including eosinophil, basophil, plasmacytoid DC, and mast cells (Dougan et al., 2019). The gene *CD109* for the CD109 activation marker was upregulated with TCR triggering (D. R. Sutherland et al., 1991) but its role is not clear. *SEMA7A* encodes SEMA7A, a T cell activation marker which facilitates macrophage activation (Worzfeld & Offermanns, 2014). Prion protein PRPc encoded by *PRNP* is a T cell activation marker that can enhance cytokine production but the mechanism is unclear (Ingram et al., 2009).

4.4.2.5 Genes relating to cAMP-PKA signalling in the TCR and PGD_2 conditions

DEGs related to cAMP-PKA signalling were altered after TCR or PGD_2 stimulation. *CREM* was a top DEG in both conditions, and encodes over 20 isoforms which make up the CREM protein family and ICER (Rauen et al., 2013). These bind to cAMP responsive element (CRE) sequences and can have both stimulatory or inhibitory (e.g., ICER) effects on cAMP signalling. *CREM* itself can be upregulated by T cell activation including by the action of AP-1 proteins (Rauen et al., 2013). SH2D2A, highly upregulated in PGD_2 and TCR conditions, encodes TSAD adapter protein enriched in activated T cells (Spurkland et al., 1998). It can be induced by cAMP or PKA signalling but its function in T cells is not fully understood (Borowicz et al., 2020). As discussed in section 4.1, signalling through the Gai subunit of CRTH2 should inhibit cAMP-PKA signalling, and this has been confirmed in CRTH2-transfected cell lines (Sawyer et al., 2002). It is therefore plausible that during T cell activation, AP-1 proteins upregulate the ICER isoform of *CREM,* and this suppresses cAMP. Further research could quantify CREM isoforms after PGD_2 stimulation.

4.4.2.6 The three stimulation conditions upregulated different sets of pro-survival genes

Top DEGs in both PGD_2 and TCR conditions have pro-survival functions. cIAP2 (encoded by *BIRC3*) is part of the inhibitor of apoptosis protein family and operates by induction of canonical NF-$\varkappa$B signalling and inhibition of apoptotic caspases (Hrdinka & Yabal, 2019). P-selectin (encoded by *SELPLG*) is an inhibitory receptor that promotes T cell death so its downregulation may promote survival (Tinoco et al., 2016). However *FASLG*, an asthma-associated gene upregulated in both PGD_2 and TCR conditions, encodes FASLG which promotes apoptosis (Michael Croft & Siegel, 2017) through FAS

encoded by *FAS*, which was also highly expressed. FAS/FASL interactions are an established mechanism for apoptosis of activated T cells (Ju et al., 1995)

PGD_2 stimulation was specifically associated with upregulation of several survival genes. The proapoptotic *TP53INP1* was suppressed - it is a target gene of tumour suppressor transcription factors p53 and p73, but reciprocally activates them (Shahbazi et al., 2013; Tomasini et al., 2005). *HMCES* was particularly upregulated by PGD_2 stimulation and HMCES provides a unique mechanism for maintaining genomic stability (Mohni et al., 2019). Genomic stability and cell survival are closely linked (Zhivotovsky & Kroemer, 2004). The gene for TNF receptor TNFR1 (*TNFRSF1A*) was uniquely upregulated by PGD_2, the balance of TNFR1/TNFR2 affects whether TNF signalling promotes survival (Brenner et al., 2015). PGD_2 downregulated the *IL7R* gene for the IL7R-α (CD127) subunit of the IL-7 receptor; therefore PGD_2 may alter sensitivity to IL-7, a regulator of T cell population size (Carrette & Surh, 2012). *IL7R* is an asthma-associated gene (Supplementary Table 32). It has been linked to asthma with sensitivity to fungi, and administration of exogenous IL-7 induced type 2 inflammation in fungi-sensitized mice (Reeder et al., 2018). Therefore, this illustrates a possible scenario in which PGD_2 could have a paradoxical protective role.

A different set of survival genes were associated with TCR triggering. *TNFSF9* encodes 4-1BB ligand which binds to 4-1BB, the gene for which (*TNFRSF9*) was highly expressed after stimulation, to promote T cell proliferation and survival (Zapata et al., 2018). JOSD1 (*JOSD1*) can deubiquitinate and stabilise MCL1 (*MCL1* - also upregulated by TCR triggering) to suppress mitochondrial apoptosis in cell lines (X. Wu et al., 2020). The GIMAP family regulate T cell survival including after TCR engagement (Ciucci & Bosselut, 2014). TPL2 (*MAP3K8*) and MAP3K14 (*MAP3K14*) are upstream of the ERK1/2 pathway

(Arthur & Ley, 2013) and in $CD8^+$ T cells form part the MAP3K14-TRAF2-Tpl2-ERK1/2 axis that promotes T cell survival (Xie et al., 2020). Finally, 14-3-3 protein family (e.g. *YHWAQ*) are signalling hubs which regulated many cell functions and are generally pro-survival (Pennington et al., 2018).

In the alarmin condition, pro-survival genes associated with STAT5 signalling were especially upregulated (see 4.4.2.6). However, *RNF144A* was also upregulated – RNF144A reduces genomic stability by targeting elements of the DNA repair pathway for degradation (S. R. Ho et al., 2014; Y. Zhang et al., 2017).

The upregulation of survival genes is important because this indicates that a population of Tc2 cells may be sustained by stimulation through a number of different mechanisms. This effect could be confirmed by an *in vitro* survival assay. Because different survival genes were upregulated by the different stimuli, it is possible that they could act co-operatively to increase the competitiveness of Tc2 populations in the lung.

4.4.2.7 Changes in expression of co-stimulation and co-inhibitory markers across multiple stimulation conditions

Genes for costimulatory molecules were upregulated in both PGD_2 and TCR conditions. Two TNF receptors OX40 (*TNFRSF4*), and GITR (*TNFRSF18*) bind OX40L and GITRL produced by activated APCs (Michael Croft & Siegel, 2017). *LTA* was upregulated in both conditions and *LTB* was highly expressed: together their products form trimers involved in lymphoid tissue development (Calmon-Hamaty et al., 2011). CRTAM (*CRTAM*) is transiently expressed on activated $CD8^+$ T cells and binds CADM1 on certain DC and epithelial populations (Takeuchi et al. 2016). The α-subunit of IL-21 receptor (*IL21R*) senses IL-21, a gamma chain cytokine with stimulatory effects (Leonard & Wan,

2016). CD83 (*CD83*) is another costimulatory molecule expressed by activated T cells and is involved in interactions with other CD83+ cells, especially DCs (Grosche et al., 2020).

TCR triggering was specifically associated with several costimulatory molecules. CD30L (*TNFSF8*) binds CD30 (*TNFRSF8* - upregulated in all conditions) (van der Weyden et al., 2017), a receptor that promotes Tc memory and Tc2 responses (Del Prete et al., 1995; Manetti et al., 1994; Nishimura et al., 2005; Vukmanovic-Stejic et al., 2000). CD150 (*SLAMF1*) is expressed on activated T cells is also associated with type 2 immunity (Cannons et al., 2011). ICOS (*ICOS*) is a costimulator expressed on activated T cells that binds B7 on activated APCs (L. Chen & Flies, 2013; Dong et al., 2001). Two genes for inhibitory molecules were associated with TCR triggering: *VSIR* encoding VISTA, the checkpoint transiently upregulated on activated T cells (W. Xu et al., 2018), and *GPR171* encoding the GPCR GPR171 which binds neuropeptide BigLEN (Gomes et al., 2013) and may be a checkpoint (Fujiwara et al., 2019).

In contrast, PGD_2 stimulation induced several potentially inhibitory markers. The gene *TGFB1* for suppressive cytokine TGF-β was upregulated along with the gene *ERBIN* for one of its regulators ERBIN (Jang et al., 2021). Genes for all three receptors for TGF-β were highly expressed throughout. TIM3 (*HAVCR2*) can be stimulatory or inhibitory depending on context (Wolf et al., 2020). The IL-27 receptor α-subunit (*IL27RA*) binds IL-27, a pathway which can suppress Th2 but also augment $CD8^+$ T cell response (Yoshida & Hunter, 2015). The gene for SLAMF7 (*CD319*) was specifically reduced in PGD_2 stimulated T cells – but its role in $CD8^+$ T cells is unclear and could be stimulatory or suppressive (Awwad et al., 2021; Loyal et al., 2020; O'Connell et al., 2021). It therefore seems likely that the inflammatory context will modulate the effects of PGD_2 stimulation.

4.4.2.8 Wound repair and remodelling related genes were increased across multiple conditions

One of the established functions of type 2 immunity is tissue repair (Gause et al., 2020). GO pathway analysis identified gene sets related to wound repair enriched in both the TCR and PGD_2 conditions. TCR triggering was especially associated with ECM remodelling genes. Reactome pathway analysis identified multiple gene sets linked to ECM remodelling. One of the core genes identified was *TYRO3,* which is associated with COPD (Supplementary Table 32). Several individual DEGs specifically enriched in the TCR condition also have functions related to remodelling. *PGGHG* encodes an enzyme that removes a glucose residue from collagens (Hamazaki & Hamazaki, 2016) and can degrade basement membrane in rats (Sternberg & Spiro, 1980). CD258/LIGHT (*TNFSF14*), has been linked to remodelling including of the airways (Michael Croft & Siegel, 2017; Doherty et al., 2011) by interactions with its receptors HVEM and LTBR expressed on stromal cells (Doherty et al., 2011). Annexin A1 (*ANXA1*) promotes resolution of inflammation and repair (Gavins & Hickey, 2012; Leoni & Nusrat, 2016).

PGD_2 stimulation affected genes linked to control of blood flow and vascularisation. GO pathway analysis identified enrichment of gene sets related to vessel diameter. VEGFA (*VEGFA*) is particularly associated with pathological angiogenesis (Nagy et al., 2007) and binds VEGF receptors on endothelial cells to increase vascular permeability and diameter. PGD_2 also uniquely upregulated the gene for NFAT4 (*NFATC3*), associated with regulation of vascular development (Graef et al., 2001; Müller & Rao, 2010; Rengarajan et al., 2002). Secreted raftlin (*RFTN1*) is also associated with wounded tissue (Bilgen et al., 2019) and raftlin binds the complex VEGFR2 to promote angiogenesis (Bayliss et al., 2020). Interestingly, this all seems to contrast with data from the oncology field identifying PGD_2 as an antiangiogenic factor.

Tc2 seem clearly to have the capability of contributing to ECM remodelling and wound repair, as indicated by this data and a paper from our group (W. Chen et al., 2021). But the *in vivo* role will depend on the size and location of Tc2 populations in humans, which will require study of tissue by microscopy.

4.4.2.9 Enrichment of genes related to regulation of TCR signalling

TCR triggering specifically induced genes linked to TCR regulation. HIVEP2 (*HIVEP2*) is a transcription factor which modulates the T cell's perception of the strength of TCR signal (Staton et al., 2011) and is associated with both asthma (Dahlin et al., 2020) and COPD (Supplementary Table 32) . Src Like Adapter SLA (*SLAP1*) is a SH2/3 domain containing adapter that negatively regulates TCR signalling (Sosinowski et al., 2000). RNF19A (*RNF19A*) is a ubiquitin ligase which can tag TRAF6 for degradation (C. Wu et al., 2017) – itself involved in TCR signalling (Walsh et al., 2015). TCR stimulation may also negatively regulate TLR signalling, because OTUD4 (OTUD4 - particularly enriched in the TCR condition) negatively regulates MyD88 (Zhao et al., 2018).

PGD_2 stimulation enriched expression of genes for a different set of TCR regulators. C-maf inducing protein (*CMIP*) is an adapter protein recently shown to disrupt TCR signalling mediated by anti CD3/28 (Oniszczuk et al., 2020). Raftlin (*RFTN1*) is a lipid raft protein expressed by lymphocytes (Saeki et al., 2003, 2009) that positively regulates TCR signalling, possibly through supporting raft structures and recruiting Lck kinase (Saeki et al., 2009). SIT (*SIT1*) can negatively regulate TCR signalling via a mechanism proximal to PKC activation (Marie-Cardine et al., 1999; Pfrepper et al., 2001; Simeoni et al., 2008).

Protein tyrosine phosphatases are important regulators of TCR signalling (Rhee & Veillette, 2012) and several were upregulated in both PGD_2 and TCR groups. The gene for HePTP

(*PTPN7*) was particularly upregulated by PGD_2 stimulation, and the function of HePTP is to negatively regulate MAPK/ERK signalling downstream of the TCR (Saxena et al., 1999), suggesting that PGD2 could inhibit elements of the TCR signalling pathway. Genes for the nonclassical dual-specificity protein tyrosine phosphatases (DUSPs) were also upregulated. *DUSP5* was especially associated with TCR triggering and encodes another MAPK phosphatase DUSP5 (Castro-Sánchez et al., 2019).

4.4.2.10 Upregulation of Wnt signalling genes through different pathways in the TCR and PGD_2 conditions

CSRNP1, encoding AXUD1, was one of the top genes in both the PGD_2 and TCR conditions. Induction of AXUD1 is mediated by Wnt signalling elements (Ishiguro et al., 2001; Simões-Costa et al., 2015). Although classically linked to development, Wnt signalling is known to influence mature T cell biology (Staal & Arens, 2016), but the role of AXUD1 in this context is not known.

Wnt signalling can be classified as canonical or noncanonical (B. T. MacDonald et al., 2009). In the canonical pathway, activation of the signalling pathway results in β-catenin translocation to the nucleus where it coactivates Tcf-1 (*TCF7*) transcription factor, resulting in target gene expression. TCR triggering appeared to activate the canonical pathway. This is suggested by the unique upregulation of *TCF7*, and of target genes, *AXIN2*, and *MYC* (Staal & Arens, 2016).

PGD_2 could be more associated with noncanonical Wnt signalling, which can be induced by GPCRs and mediates remodelling of the actin cytoskeleton (Staal & Arens, 2016). The noncanonical pathway is especially associated with Rho GTPases including Rac1 (Schlessinger et al., 2009), which is likely to be a key target of PGD_2 stimulation (see 4.4.4.1). Supporting this hypothesis, genes encoding Dishevelled (*DVL1* and *DVL2*), part

of noncanonical pathway, were highest in the PGD_2 condition, though not significantly elevated.

4.4.3 Gene expression associated with alarmin stimulation

4.4.3.1 Alarmin stimulation specifically induced genes related to STAT5 signalling

Many of the genes uniquely or especially upregulated by alarmin stimulation encode known members of STAT5 signalling pathways. $p15^{INK4B}$ (*CDKN2B*) has been linked to STAT5 signalling in $CD8^+$ T cells (Grange et al., 2015). IRX3 (*IRX3*) has STAT5-responsive element in its promoter, and promotes survival (Persengiev, 2017). CDK6 (*CDK6*) is a binding site for STAT5 in murine $CD8^+$ T cells (P. Li et al., 2017), and is involved in the cell cycle and survival (Tigan et al., 2016). *ACOX1* is a STAT5 target (Coulter & Stephens, 2006) and oxidises very long chain fatty acids, which may be important for Tc memory (Gupta et al., 2020), BCL2 (*BCL2*) is a negative regulator of mitochondrial permeabilization in apoptosis (Hockenbery et al., 1990; Kale et al., 2018) and is upregulated by STAT5-signalling cytokines (Chong et al., 2020, p. 545) or by TSLP stimulation of Th2 cells (Kitajima et al., 2011). *OSM* can be induced by STAT5 activation (N. R. West et al., 2018, sec. 2.3). PIM1 serine/threonine kinase (encoded by *PIM1,* similarly upregulated by TCR triggering but downregulated by PGD_2) is associated with STAT5 signalling (Basham et al., 2008), survival, and BCL-2 interaction (Lilly et al., 1999).

Genes for regulators of STAT5 were also upregulated by alarmins. Genes for three SOCS family proteins: *CISH*, *SOCS1*, and *SOCS2*, were associated with alarmin and TCR stimulation. Their homonymous products are negative regulators of JAK/STAT signalling, including STAT5 (Alexander & Hilton, 2004). CISH and SOCS2 can directly bind and inhibit STAT5-targeting cytokine receptors such as IL4R, but it is not yet known if they

bind the IL-25 receptor (Alexander & Hilton, 2004; Linossi et al., 2013; Yoshimura, 2013). Furthermore, SOCS proteins restrain type 2 differentiation and their deficiency causes spontaneous T2 inflammation (Knosp et al., 2011; X. O. Yang et al., 2013). SH2B3 (*SH2B3*) is an SH2-domain containing adapter protein that acts as a negative regulator of cytokine and growth factor signalling (including STAT5) (Maslah et al., 2017) – however its gene was upregulated in all conditions.

In the PGD_2 condition genes encoding STAT5 signalling members were suppressed. The IRS2 adapter protein (*IRS2*) is associated with STAT-5 signalling cytokines (de Melo Campos et al., 2016; J. A. Johnston et al., 1995). PIM1 *(PIM1*) and HBP1 (*HBP1*) transcription factors cooperate to induce apoptosis in conditions of oxidative stress (Shuya Wang et al., 2017) and their genes were both suppressed. PIM2 has functional redundancy with PIM1 and its gene *PIM2* was also downregulated (Narlik-Grassow et al., 2014).

4.4.3.2 Alarmin stimulation was associated with genes involved in host response to virus

Alarmin stimulation was associated with genes related to antiviral response. Viperin (*RSAD2*) is an interferon inducible enzyme with broad antiviral properties (Rivera-Serrano et al., 2020). *In vitro*, viperin can inhibit intercellular transmission of RSV (Jumat et al., 2015), influenza A (X. Wang et al., 2007), and rhinoviruses (Proud et al., 2008). *In vivo,* viperin knockout mice are deficient in Th2 responses (Qiu et al., 2009). $CD4^+$ T cells from viperin-deficient mice could proliferate normally and produced equivalent levels of IL-2 and IFN-γ, but were significantly impeded in their ability to produce GATA3, IL-4, IL-5, and IL-13 (Qiu et al., 2009). This implicates viperin in the differentiation pathway for type 2 T cells. In my dataset, *RSAD2* was uniquely upregulated by alarmin stimulation, thus providing a plausible link between exposure to alarmins and Tc2 differentiation.

Furthermore, viperin is co-transcribed with the adjacent gene *CMPK2* (Rivera-Serrano et al., 2020), which was also uniquely increased in the alarmin condition. Alarmins downregulated the gene for cyclin L2 (*CCNL2*), a transcriptional cyclin regulating RNA polymerase (Quandt et al., 2020). Cyclin L2 can negatively regulate expression of restriction factor SAMHD1(Kyei et al., 2015), encoded by *SAMHD1* which was higher in the alarmin group (but not significantly higher). The gene encoding androgen receptor (*AR)* was uniquely increased in the alarmin condition, which suggests a link between alarmin signalling and sensitivity to testosterone. Testosterone has a protective effect in respiratory infection with influenza in mice, in part by contracting $CD8^+$ T cell populations (Vom Steeg et al., 2020). Expression of AR has very recently been shown to associate with better asthma outcomes in terms of lung functions and symptoms, and reduced FeNO, in a large study (Zein et al., 2021).

However, the alarmin condition was also associated with upregulation of pro-viral genes. Alarmins uniquely upregulated *SNAP47*, the product of which has an important role in replication of the respiratory enterovirus D68 (Corona et al., 2018). TAT-SF1 (*HTATSF1*) may be pro-viral in the context of HIV (Zhou & Sharp, 1996) and influenza A(Naito et al., 2007). MIB1(*MIB1)* is a ubiquitin ligase involved in Notch signalling (B. Guo et al., 2016) but is also a host factor for adenovirus (Bauer et al., 2019; Sarbanes et al., 2021). The gene for IL-32 *(IL32)* was stably very highly expressed across all conditions and upregulated by PGD_2. IL-32 may act as a viral restriction factor, but is poorly understood (Ribeiro-Dias et al., 2017).

Overall, these results suggest that genes associated with alarmin stimulation of Tc2 cells are involved in the host response to virus, but their effects are likely to be virus-dependent.

As discussed earlier (4.4.2.3), it is likely that these effects were due to IL-25. Given that IL-25 is released in response to certain viral infections (Beale et al., 2014), it is reasonable to hypothesise that type 2 T cells are involved in response to certain viruses, and that this response could become abnormal in the type 2 high environment common in asthma.

4.4.4 Gene expression associated with PGD_2 stimulation

4.4.4.1 PGD_2 induced expression of genes related to cell motion driven by Rac GTPases

The actin cytoskeleton is central to the motion of T cells (Dupré et al., 2015). *ACTG1* and *ACTB* encode closely related beta and gamma isoforms of non-muscular actin, and were especially upregulated by PGD_2 (Vedula & Kashina, 2018). Many genes for actin-associated structural proteins were also associated with PGD_2 stimulation. Zyxin (*ZYX*) repairs damaged actin stress fibres (Smith et al., 2010). Aip1 (*WDR1*), coronin 1A (*CORO1A*) and cofilin (*CFL1*) collaborate to disassemble actin filaments (Hao et al., 2008). LASP1 (*LASP1*) is a structural protein that can remodel of the actin cytoskeleton (reviewed in Butt and Raman 2018). ACTR2 (*ACTR2*), ACTR3 (*ACTR3*), and ARPC2 (*ARPC2*) are components of the ARP2/3 complex, a nucleation site for actin filaments (Goley & Welch, 2006). Calponin isoform CNN2 (*CNN2*) is an actin binding protein that improves cytoskeletal stability (R. Liu & Jin, 2016). LCP1 (*LCP1*), VASP (*VASP*), and swiprosin-1 (*EFHD2*) are actin-bundling proteins involved in cell motility (Freeley et al., 2012; Morley, 2012; Schirenbeck et al., 2006; Thylur et al., 2018). MYO1E (*MYO1E*) is part of the class I myosins that link actin cytoskeletal elements and cell membranes (Daniel Alberto Girón-Pérez et al., 2019). Tropomyosins 3 and 4 (*TPM3* and *TPM4*) are associated with cell movement by interactions with actin (reviewed in (Manstein et al., 2020). FMNL1 (*FMNL1*) facilitates deformation of the rigid T cell nucleus, required for transendothelial migration to inflamed tissue, via actin polymerisation (Thompson et al., 2020). In the TCR

condition, the discussed actin isoforms and most of the related genes were also upregulated but to a lower extent. This could be due to the formation of immunological synapses by the actin cytoskeleton and its regulators (e.g. LCP1) (Morley, 2012; Roy & Burkhardt, 2018).

PGD_2 also upregulated elements of cell motility beyond the actin cytoskeleton. AHNAK (*AHNAK*) was suppressed- it is a large protein with functions including regulation of cell motility and cell contacts, and calcium signalling (reviewed in Davis, Loos, and Engelbrecht 2014). EML4 (*EML4*) was associated with PGD_2 stimulation and has a stabilising function in the microtubule network (Pollmann et al., 2006).

PGD_2-mediated cell motility genes appeared to be driven by Rac1 (*RAC1*) and Rac2 (*RAC2*) members of the Rho small GTPase family, associated with cell movement and the cytoskeleton (Jaffe & Hall, 2005; Saoudi et al., 2014). *RAC1* and *RAC2* were uniquely and especially upregulated with PGD_2 stimulation respectively. Several genes for Rac-interacting proteins were associated with PGD_2 stimulation. RASSF2 (*RASSF2*) is an adapter protein that positively regulates Rac signalling (Stoner et al., 2020). GRK6 (*GRK6*) and GIT1 (*GIT1*) cooperate to activate Rac proteins in a macrophage model (Nakaya et al., 2013). Swiprosin-1 (*EFHD2*) enhances Rac1 activity (Thylur et al., 2018). MYO1E (*MYO1E*) promotes PI3K-AKT-Rac-1 signalling in B cells (Daniel A. Girón-Pérez et al., 2020). FMNL1 (*FMNL1*) also binds Rac1 in its role in cell motility (Kühn & Geyer, 2014). Syntrophin beta 2 (*SNTB2*) and genes for other syntrophins were downregulated in the PGD_2 condition: syntrophins bind to actin and Rac1 to regulate motility and adhesion (Bhat et al., 2019). MYADM (*MYADM* - similarly upregulated in the TCR condition) cooperates with Rac1 to form lamellipodia during cell migration (Aranda et al., 2011)

Rho GTPases such as Rac1 and Rac2 are regulated by Rho-GEFs, Rho-GAPS, and GDIs. Rho-GEFs promote the active GTP-bound form of Rho-GTPases. Each Rho-GEF has preferences for specific Rho proteins (Cook et al., 2014). The TCR-specific upregulated genes encode GEFs Lfc (*ARHGEF2*), XPLN (*ARHGEF3*) and LARG (*ARHGEF12*), which are specific to RhoA and RhoB. However, β-PIX (*ARHGEF7*), uniquely upregulated by PGD_2, prefers Rac1 (Cook et al., 2014; Symons, 2011). There were also several negative regulators of Rac linked to PGD_2 stimulation. ARHGAP25 (*ARHGAP25*) is a Rho-GAP that inactivates Rac proteins (Csépányi-Kömi et al., 2012), in turn affecting leukocyte migration (Csépányi-Kömi et al., 2012, 2016; Schlam et al., 2015). RCC2 (*RCC2*) binds Rac1 and negatively regulate access for Rho-GEFs, thus inhibiting Rac1 (Williamson et al., 2014). ARHGDIB (*ARGHDIB*) is a GDI which inhibits dissociation of Rho GTPases (including Rac1 or Rac2) from GDP, keeping the proteins an inactive state (DerMardirossian & Bokoch, 2005).

In summary, PGD_2 stimulation was particularly associated with increased expression of genes encoding two actin isoforms and a breadth of actin interactors and regulators. This is likely the result of increased Rac activity (especially Rac1), given the enrichment of both Rac genes themselves and a number of genes involved in Rac signalling. This finding is in line with previous functional studies showing that Rac-deficient T cells have impaired chemotaxis and migration (Faroudi et al., 2010). The purpose of this process is likely to be the movement of $CRTH2^+$ cells (including Tc2) towards sites of PGD_2 production, such as activated mast cells in the lungs.

4.4.4.2 PGD_2 stimulation modulated expression of chemotaxis receptors and ligands, including lung-associated CXCR6

PGD_2 stimulation was associated with upregulation of the gene for CXCR6 (*CXCR6*). The ligand CXCL16 *(CXCL16*) (Griffith et al., 2014) is produced constitutively by human lung epithelium (Day et al., 2009) and the CXCR6 receptor is enriched on the surface of lung T cells (A. J. Morgan et al., 2005), $CXCR6^+$ Th2 cells have increased potential to induce eosinophilic inflammation (Obata-Ninomiya et al., 2018). PGD_2 may therefore promote a lung tissue homing axis. Two genes for chemokine receptors CCR1 (*CCR1*) and CCR5 (*CCR5*), were upregulated by PGD_2 and mediate movement towards chemokines produced at sites of antigen recognition (see 4.4.5.2). Expression of the gene for IL-16 *(IL16*) was also specifically increased with PGD_2 stimulation. IL-16 acts as chemoattractant for CD4+ cells (Richmond et al., 2014) and can be induced by histamine (Mashikian et al., 1998). PGD_2 also particularly upregulated the gene for plexin A1 (*PLXNA1*), a receptor for class 3 semaphorins such as SEMA3A, which can halt migration of $CD8^+$ T cells (Barnkob et al., 2019). To summarise, PGD_2 could modulate the migratory behaviour of $CRTH2^+$ $CD8^+$ T cells, beyond the PGD_2-CRTH2 axis.

4.4.4.3 PGD_2 upregulated genes involved in lipid synthesis and eicosanoid metabolism, including prostacyclin and PGE_2 synthesis

PGD_2 stimulation was associated with genes involved in synthesis of lipids. *PLIN2* encodes perilipin 2 (adipophilin) that promotes the formation and stabilisation of lipid droplet organelles (Itabe et al., 2017). Degradation of perilipin 2 is associated with lipolysis and concurrent increase in the lipase ATGL (encoded by *PNPLA2*) (Kaushik & Cuervo, 2015). PGD_2 seems to have the inverse effect which may indicate reduction in lipolysis or indeed lipogenesis. The genes for enzyme GPAT3 (*GPAT3*) and three related AGPAT enzymes were associated with PGD_2 stimulation, including *AGPAT1* which is an asthma-associated

gene (Supplementary Table 32). Together these enzymes are part of the triacylglycerol synthesis pathway which produces DAG, TAG, and phospholipids from glycerol-3-phosphate (K. Takeuchi & Reue, 2009). These metabolites can enter other pathways including arachidonic acid metabolism. PGD_2 was also particularly associated with upregulation of the gene for fatty acid synthase (*FASN*), an enzyme in long chain fatty acid synthesis, known to be upregulated after TCR stimulation through a mechanism involving mTOR and SREBPs (Lochner et al., 2015).

Enzymes related to eicosanoid synthesis were differentially regulated between PGD_2 and TCR stimulation. Two enzymes supporting leukotriene synthesis were associated with the PGD_2 condition. *COTL1* encodes coactosin-like protein that interacts with and promotes 5-LO activity (Provost et al., 2001; Rakonjac et al., 2006). FLAP (*ALOX5AP*) is essential for 5-lipoxygenase activity (Dixon et al., 1990). 5-LO is the initial enzyme in the leukotriene synthesis pathway, but its gene *ALOX5* was not detectable.

The expression of genes for prostaglandin synthetic enzymes also differed between the two conditions. The gene for COX-2 (*PTGS2*) was particularly upregulated in the TCR condition, in agreement with other recent data (W. Chen et al., 2021), as was the gene for downstream prostacyclin synthase (*PTGIS*). The PGD_2 condition was associated with genes for prostaglandin E synthetic enzymes (*PTGES2* and *PTGES3*). Genes encoding enzymes for PGD_2 synthesis (*HPGDS* and *PTDGS*) were undetectable across the experiment. This contrasts with recently published data from our group which found that cultured Tc2 cells released PGD_2 after stimulation with CRTH2 agonist DK-PGD_2 or anti-CD3/28 antibodies (W. Chen et al., 2021). In that study, production peaked at four hours, so it is possible that I was looking too early for the detection of PGD_2 synthesis genes. In that study, *HPGDS* was detected by RT-qPCR, and the protein by flow cytometry.

However, the absolute level of *HPGDS* mRNA was not quantified. It is possible that HPGDS exists in Tc2 cells as a preformed enzyme with very little corresponding mRNA.

There are several explanations for these expression patterns. As PGD_2 induces chemotaxis and cell locomotion, it is possible that cell membrane needs to be synthesised at a higher rate, as has been indicated by study of the migration of single celled eukaryotes (M. Tanaka et al., 2017). This could explain the evidence for an increase in the triacylglycerol synthesis pathway. Alternatively, perhaps these building blocks are being synthesised for entry into arachidonic acid metabolism, for example to produce more PGD_2 (W. Chen et al., 2021). It is notable that *PTGES* (Supplementary Table 32) and *PTGES2* (C.-J. Xu et al., 2018) are asthma-associated genes, and that PGD_2 specifically induced *PTGES2* and *PTGES3*. All three genes encode enzymes that convert PGH_2 to PGE_2 (Samuelsson et al., 2007). The role of PGE_2 is complex and it operates through four receptors (Ricciotti & Fitzgerald, 2011). Even at the lung, PGE_2 appears to have both pro and anti-inflammatory roles depending on the receptor (Birrell et al., 2015). One widely cited function of PGE_2 is the suppression of Th1 but not Th2 cytokine profiles (in the presence of IL-2, which was in the culture medium) (Hilkens et al., 1995), so perhaps this contributed to type 2 cytokine production in the PGD_2 condition, given that *PTGER2* and *PTGER4* were both highly expressed.

4.4.4.4 PGD_2 stimulation increased the asthma-associated BCL6:LITAF ratio

BCL6, which was suppressed by TCR triggering but upregulated by PGD_2, encodes BCL6, which is associated with germinal centre lymphocytes (Crotty, 2014) but may also act as a brake on type 2 inflammation (Kusam et al., 2003; Ogasawara et al., 2017; Sawant et al., 2012; Ye et al., 1997). *LITAF* was downregulated by PGD_2, and studies of B cells show

that this is probably a consequence of increased BCL6 activity (Bertolo et al., 2013). Furthermore, higher BCL6:LITAF ratio promotes cell survival (Shi et al., 2016). Both *BLC6* and *LITAF* have been linked to asthma in large GWAS studies (Supplementary Table 32), but the mechanism by which they contribute to the disease seems to be unknown, and is surely a prime candidate for future research.

4.4.4.5 PGD_2 induced genes related to autophagy and lysosome activity

There was evidence for increased autophagy in the PGD_2 condition. ATG16L1 (*ATG16L1*) is an essential component of autophagy pathways including formation of the autophagosome (Gammoh 2021). Its gene was elevated after PGD_2 stimulation but reduced after TCR triggering. The gene for Mannose 6 phosphate receptor CD-MPR (*M6PR*) was especially upregulated by PGD_2 and the gene for CI-MPR (*IGF2R*) slightly downregulated. M6PRs bind degradative hydrolase enzymes to facilitate their delivery to lysosomes (Ghosh et al., 2003). There are functional differences between the M6P receptors (Munier-Lehmann et al., 1996), but how this balance affects T cells is not understood. The gene for SAC1 (*SACM1L*) was upregulated in the PGD_2 condition. It is an enzyme that dephosphorylates PI4P to replenish PI levels, thereby controlling intracellular PI gradients important for many functions related to vesicular transport and lipid metabolism (Del Bel & Brill, 2018). Sac1 is known to be active at the surface of secretory vesicles and lysosomes, and involved in autophagosome-lysosome fusion (Del Bel & Brill, 2018). The role of PGD_2 in autophagy has not been studied in humans, although the COX2-PGD_2 axis has been linked to reduced autophagy in the CNS in a rat model of type 2 diabetes (Y. Yang et al., 2019). PGD_2 and autophagy have been linked separately to silicosis of the lung, but not studied together (H. Liu et al., 2016; Pang et al., 2021). There are many functions of autophagy, and in this case it is plausible that its upregulation is part

of cell migration regulation, such as by degradation of focal adhesion proteins (Dikic & Elazar, 2018).

Upregulation of the gene for EHBP1L1 (*EHBP1L1*) in the PGD_2 condition suggests that the lysosomes may have become overloaded in the PGD_2 condition, as this protein forms part of the LRRK2-Rab8 pathway to maintain stressed lysosomes (Eguchi et al., 2018). In the TCR condition the gene for a different LRRK2-Rab8 effector, RILPL2 (*RILPL2*) (Waschbüsch et al., 2020), was particularly upregulated. It is associated with ciliogenesis but a function in T cells is not known.

4.4.4.6 Evidence of PI3K-Akt-mTOR signalling in multiple conditions but FOXO1 suppression specifically in the PGD_2 condition

The PI3K/Akt signalling pathway is a transducer of T cell activation affecting many aspects of $CD8^+$ T cell biology, with destinations including the mTOR complexes (Kim & Suresh, 2013). Broadly it appeared that the pathway was activated as expected (Figure 41.) PHLDA1 is a negative regulator of the PI3K-Akt pathway (Fearon et al., 2018) which acts by sequestering PIPs (Yu Chen et al., 2018), and its gene (*PHLDA1*) was a top gene in both PGD_2 and TCR conditions. *NDFIP1* (an asthma-associated gene) and *NDFIP2* were both upregulated, the former more associated with TCR triggering and the latter with PGD_2. NDFIPs activate NEDD4 (Foot & Kumar, 2018) which promotes degradation of PTEN, an inhibitor of Akt signalling (X. Huang et al., 2019). So increased NDFIPs may release a brake on Akt signalling. The gene for PTEN (*PTEN*) was also suppressed in both PGD_2 and TCR conditions, against suggesting a release on Akt signalling brakes. However, in the PGD_2 condition, the gene for negative Akt regulator ERBIN (*ERBIN*) was specifically upregulated (Jang et al., 2021).

One of the downstream targets of PI3K-Akt is the FOXO1 transcription factor. Activated Akt can cooperate with mTORC2 to phosphorylate FOXO1 and exclude it from the nucleus (Hedrick et al., 2012). mTORC2 is itself regulated by Rac1 (Saci et al., 2011) which as discussed earlier is likely to play a key role in the effects of PGD_2. A reduction in FOXO1 activity may explain why FOXO1 target genes *IL7R*, *KLF2*, *BCL2L11*, *CDKN1B*, and *SESN3* (Kim & Suresh, 2013; M. Wang et al., 2017) were all suppressed in the PGD_2 condition but not necessarily in the TCR condition. Loss of FOXO1 is linked to acquisition of $CD8^+$ T cell effector program (Delpoux et al., 2021), so it is possible that PGD_2 promotes the effector phenotype.

4.4.4.7 Regulators of Src family tyrosine kinases (SFKs) were associated with PGD_2 stimulation

The Src family tyrosine kinases (SFKs) are involved in signalling events including TCR triggering, GPCR signalling, and cell migration (Okada, 2012; Parsons & Parsons, 2004). Csk is a negative regulator of SFKs (Nada et al., 1993). *PAG1* encodes CBP which binds and promotes Csk (Kawabuchi et al., 2000). *PAG1* was suppressed by PGD_2 stimulation, so we might expect reduced activity of Csk and higher activity of SFKs. However, a Csk-related gene, *MATK* (Bennett et al., 1994), was uniquely upregulated by PGD_2 stimulation. Although initially linked to megakaryocytes, MATK (*MATK*) is also expressed in T cell lymphomas (Yunxin Chen et al., 2017; Tan et al., 2013) and negatively regulates SFKs. Furthermore, NDFIP2 (*NDFIP2*) can ubiquitinate SFKs for degradation (Mund & Pelham, 2010). The genes for both were associated with PGD_2 stimulation. On balance, PGD_2 seems to modulate regulators of SFKs but it is unclear in which direction, and whether this could affect the cell's responsiveness to TCR triggering or GPCR ligands.

4.4.4.8 PGD_2 stimulation positively regulated genes involved in the SMRT/NCoR-HDAC3 complex, which is associated with asthma

The gene for transcriptional repressor SMRT (*NCOR2*) was upregulated specifically by PGD_2. SMRT forms complexes with NCoR and HDAC3 (*HDAC3* is asthma-associated) which have widespread inhibitory effects on transcription through binding of unliganded nuclear transcription factors (Karagianni & Wong, 2007). The SMRT/NCoR-HDAC3 complexes can be degraded by TBL1XR1 (aka TBLR1) (Perissi et al., 2008; Yoon et al., 2003), the gene for which was downregulated by PGD_2 (*TBL1XR1*), and is another asthma-associated gene (Supplementary Table 32). This suggests increased SMRT/NCoR-HDAC3 activity is likely. However, genes for some of the key SMRT/NCoR-HDAC3 targets of inhibition (*RARA*, *RXRA*, and *PPARD*) were most highly expressed in the PGD_2 condition. Perhaps the upregulation of SMRT/NCoR-HDAC3 is itself a response to increased expression of these nuclear hormone receptors. Since multiple genes for components of the SMRT/HDAC3 complex are associated with asthma, it is reasonable to propose that this complex plays some role in the disease, but it is not clear how.

4.4.4.9 Negative regulation of GPCR signalling

Given that CRTH2 is a GPCR, one might expect GPCR regulators to be specifically changed in the PGD_2 condition. *RGS3* was specifically induced by PGD_2 and its product RGS3 is part of a family of RGS proteins involved in negative regulation of G protein signalling by promotion of Ga-mediated GTPase activity (Masuho et al., 2020, sec. Introduction). RGS3 is mostly selective for Gai and Gao (Masuho et al., 2020), which correspond to the signalling pathway for PGD_2 through CRTH2 (Sawyer et al., 2002).

4.4.5 Gene expression associated with TCR triggering

4.4.5.1 TCR triggering induced genes involved in clonal expansion (i.e., the Warburg effect)

When T cells are activated through the TCR, they undergo rapid growth and proliferation. This requires metabolic reprogramming controlled by the transcription factor c-Myc (R. Wang et al., 2011). C-Myc shifts cell metabolism towards aerobic glycolysis and glutaminolysis to meet immediate energy needs – better known as the Warburg effect (M. G. V. Heiden et al., 2009). The gene for c-Myc (*MYC*) was particularly upregulated in the TCR condition to become very highly expressed. How c-Myc is induced is not completely clear. The epigenetic regulator ARID5B, the gene for which (*ARID5B*) was also associated with TCR triggering, may contribute. ARID5B influences chromatin structure especially at AT-rich regions including *MYC* (Leong et al., 2017; P. Wang et al., 2020). *DENND4A* was also uniquely upregulated by TCR triggering – its product's function is not known but binds a sequence in *MYC* (Stasiv et al., 1994) so may regulate its expression. *MYC* is also an asthma-associated gene (Supplementary Table 32).

Multiple DEGs associated uniquely or especially with the TCR condition suggest a switch to aerobic glycolysis. Hexokinase-2 (*HK2*) catalyses the first step of glycolysis and is 'pivotal' in the Warburg effect (P. L. Pedersen, 2007). Glycerol kinase (*GK*) converts glycerol-3-phosphate to DHAP which can be used for glycolysis and other metabolic pathways (C. K. L. Ho, 2015). PFKFB3 (*PFKB3*) has a strong phosphofructokinase activity and its enzymatic product (fructose-2,6,-bisphosphate) is a potent glycolytic activator (De Bock et al. 2013). PGAM1 (*PGAM1*) is another enzyme in the glycolysis pathway and is associated with $CD8^+$ T cell effector function and proliferation (Sukumar et al., 2013; M. G. Vander Heiden et al., 2010). The gene for glucokinase (*GLK*) inhibitor midnolin (*MIDN*) (Hofmeister-Brix et al., 2013) was particularly upregulated by TCR triggering but

GLK was not expressed in this experiment - perhaps midnolin regulates other glycolytic enzymes.

In contrast, in the PGD_2 condition there was no evidence of aerobic glycolysis – *HK2* was indeed suppressed. Glutaminase (*GLS*), an enzyme which converts glutamine to glutamate, was especially upregulated. Glutamate can be used as a TCA substrate to meet the activated T cell's energy needs (Geltink et al., 2018). The gene for the NAMPT enzyme (*NAMPT*) was a top gene in both PGD_2 and TCR conditions. NAMPT catalyses production of NAD, a central cofactor in oxidative phosphorylation, but also a substrate for cell signalling and transcription enzymes (Audrito et al., 2020). Surprisingly, the gene for lactate shuttle MCT4 (*SLC16A3*) was particularly upregulated after PGD_2 stimulation. Lactate efflux occurs during aerobic glycolysis (Lunt & Vander Heiden, 2011) but lactate can also be transported into cells for lipogenesis (Halestrap, 2013) – it would be interesting to test experimentally whether PGD_2 induces efflux or influx of lactate.

C-Myc also upregulates the polyamine synthesis pathway to facilitate growth and proliferation (R. Wang et al., 2011). The first, rate-limiting step is catalysed by ODC1 (Pegg, 2016), the gene for which (*ODC1*) was especially upregulated in the TCR condition.

TCR triggering was associated with upregulation of regulators of cell division, another part of the Warburg effect. This included genes for cell cycle regulators RGCC (*RGCC*) (Tegla et al., 2015) and PER1 (*PER1*), also a clock gene (Gery et al., 2006). BACH1 (*BACH1*) is a transcriptional repressor but it can also play a structural role in mitosis (Jie Li et al., 2018). SLFN5 (*SLFN5*) is a negative regulator of proliferation of tumour cells (F. Liu et al., 2018), was particularly suppressed. PHF6 (*PHF6*) is another putative cell cycle regulator and DNA repair protein (J. Wang et al., 2013). RAN (*RAN*) is part of the Ras superfamily of small GTPases with a role as organiser of mitosis (Clarke & Zhang, 2008). PIM3 (*PIM3*) is

a direct target of c-Myc and cooperates with it to promote cell proliferation and survival (Forshell et al., 2011). SOCS3 (*SOCS3*) is associated with proliferation as well as STAT3 signalling and CD28-mediated cytokine production (Alexander & Hilton, 2004). The IL-2 receptor α-subunit (*IL2RA*) is a component of the heterotrimeric high affinity IL-2 receptor which regulates population expansion of activated Tc (Boyman & Sprent, 2012) (Boyman & Sprent, 2012) through IL-2 (*IL2)*, which was also uniquely upregulated by TCR triggering. Both *IL2* and *IL2RA* are asthma-associated genes (Supplementary Table 32). ERK3 (*MAPK6*) is an atypical kinase which might be involved in cell cycle progression (Cargnello & Roux, 2011), although it may be redundant (Ronkina et al., 2019). EED (*EED*) is a polycomb component of the PRC2 complex and transmits epigenetic signatures to daughter cells during cell division (Margueron et al., 2009).

Cholesterol demand is increased during clonal expansion (Kidani et al., 2013). In the TCR condition, *RNF145* was especially upregulated. It encodes an E3 ligase which is one of several negative regulators of HMG-CoA reductase of the cholesterol synthesis pathway (Menzies et al., 2018). These regulators increase in low sterol conditions, in anticipation of future activity when sterol levels return (Menzies et al. 2018).

Together, the gene expression changes strongly indicate that stimulation with anti-CD3/28 specifically induces the Warburg effect of cellular proliferation and metabolic reprogramming, driven by the transcription factor c-Myc.

4.4.5.2 TCR triggering induced genes involved in communication with activated APCs

Upregulation of genes for chemokines after anti-CD3/CD28 stimulations suggested an attempt by the cells to communicate with activated APCs. Genes encoding CCL3 (*CCL3*), CCL4 (*CCL4*), and CCL5 (*CCL5*) were all enriched by TCR triggering. These chemokines

are associated with T cell-DC interactions, to recruit more T cells via CCR1 and CCR5 (Castellino et al., 2006; Griffith et al., 2014). Genes encoding lymphotactin α (*XCL1*) and β (*XCL2*) were also upregulated. Although their functions are unclear, both lymphotaxins signal through XCR1 expressed on DCs and are involved in antigen-specific $CD8^+$ T cell responses (J. C. Fox et al., 2015; Fuchs et al., 2019). The gene for receptor CXCR3 (*CXCR3*) also became highly expressed only after TCR triggering; it is involved $CD8^+$ T cell positioning within the lymph node during activation by an APC (Griffith et al., 2014). The *ICOS* gene for costimulator ICOS was specifically upregulated: ICOS competes with CD28 and CTLA4 to bind ICOSL/B7-H2 on the surface of professional APCs, and this is important for Th2 and ILC2 functions (L. Chen & Flies, 2013; Maazi et al., 2015). CD82 (*CD82*) is a tetraspanin involved in surface proteins and signalling molecule organisation (Termini & Gillette, 2017). It facilitates T cell adhesion to other T cells and APCs through LFA-1 (*ITGAL*) and ICAM1 (*ICAM1*) (Shibagaki et al., 1999)., the genes for which were both highly expressed. Together it appears that TCR triggering specifically induces a range of genes involved in communication with activated APCs and further T cell recruitment and/or activation.

4.4.5.3 TCR triggering induced genes related to the use of magnesium, iron, and vitamin A

NIPA1 was uniquely upregulated and highly expressed after TCR triggering. Most literature about this gene relates to its role in hereditary spastic paraplegia although it is widely expressed in all systems (The Human Protein Atlas, 2021d). Functionally it encodes the magnesium transporter NIPA1, upregulated and redistributed in conditions of low magnesium (Goytain et al., 2007; Romani, 2011). Magnesium flux is an feature of TCR induced T cell activation, and its importance is underscored by the rare 'XMEN' immunodeficiency that can arise from mutations to the magnesium transporter MAGT1

(F. Y. Li et al., 2011). It is therefore possible that *NIPA1* plays a role in early magnesium signalling after TCR triggering.

TFRC was linked to TCR triggering and encodes the transferrin receptor responsible for iron uptake. TFRC is a costimulatory activation marker of T cells after antigen recognition (Bayer et al., 1998) and is associated with proliferation (Brekelmans et al., 1994) and formation of the immunological synapse (Batista et al., 2004). The importance of *TFRC* is underscored by the combined immunodeficiency that can result from its mutation, including impaired response of T cells to TCR triggering (Jabara et al., 2015). It is also an asthma-associated gene. In contrast, Haem synthesis and haemoproteins may be relevant to PGD_2 stimulation. *ALAS1* encodes ALAS1, the first enzyme in haem synthesis (Kikuchi et al., 1958), and was highly expressed after PGD_2 stimulation.

There was differential expression of genes relating to retinoic acid, a metabolite of vitamin A which contributes to T cell differentiation, effector responses, and tolerance (Raverdeau & Mills, 2014). *RDH10* was associated with TCR triggering and encodes an enzyme that oxidises retinol to retinal (B. X. Wu et al., 2002). RALDH enzymes convert retinal to bioactive retinoic acid but their genes were not expressed by the $CRTH2^+$ $CD8^+$ T cells. However, subsets of respiratory epithelial and stromal cells do express RALDH enzyme *ALDH1A1* (Travaglini et al., 2020). In the PGD_2 condition the gene for RXR receptor for 9-*cis*-retinoic acid *RXRA* was upregulated (Raverdeau & Mills, 2014). KO studies suggest that RXRA may promotes T2-bias and survival (Stephensen et al., 2007).

4.4.5.4 Evidence for exosomes and recycling endosome production after TCR triggering

SDC4 and *SDCBP*, encoding syndecan-4 and syndecan binding protein (syntenin-1) respectively, were especially highly expressed after TCR triggering. Syndecan-4 is a cell

surface proteoglycan with wide ranging roles and binding partners (Elfenbein & Simons, 2013). Syndecans and syntenin cooperate to facilitate exosome production (Baietti et al., 2012). Other genes for exosomal components (Kalluri & LeBleu, 2020) such as *CD81*, *CD63*, *TSG101*, Hsp70 and Hsp90 members, flotillins (*FLOT1* and *FLOT2*), and ALIX (*PDCD6IP*) were moderately or highly expressed.

Rab GTPases coordinate vesicular transport including exosome production and Rab11a is associated with recycling endosomes (Stenmark, 2009), in which surface proteins are internalised and trafficked elsewhere or returned to the surface (Goldenring, 2015). Within the Rab11a recycling system, Rab11-family interacting proteins (Rab11-FIPs) play distinct roles at different locations and times (Baetz & Goldenring, 2013; Hales et al., 2001). The gene *RAB11FIP1*, was specifically upregulated by TCR triggering to become very highly expressed. The gene can produce eight different Rab-11FIP1 variants (A to H) which behave differently (Baetz & Goldenring, 2013; Jin & Goldenring, 2006). In $CD8^+$ T cells, endosomal recycling pathway is used to traffic newly synthesised perforin to the immunological synapse (Lesteberg et al., 2017).

4.4.5.5 Evidence of cellular stress after TCR triggering

Cellular stress appeared to be greater in the TCR condition. All members of genes encoding the Gadd45 family were specifically upregulated by TCR triggering. This family is expressed in a variety of stressful contexts e.g. medium depletion or oxidative stress (Schäfer, 2013). TCR triggering induced *SIAH2*, associated with hypoxia, (K. Nakayama et al., 2009) which may reflect higher oxygen consumption in the TCR condition. NFAT5 (*NFAT5*) is an indicator of osmotic stress (Müller & Rao, 2010) and its gene was specifically upregulated by TCR triggering. However, genes encoding the sestrin family of stress sensors were all suppressed in both PGD_2 and TCR conditions. They are stress-

related proteins which act as antioxidants and negative regulators of mTORC1 signalling (M. Wang et al., 2017). Thioredoxin is an antioxidant protein that reduces disulphide bonds formed under oxidative stress. Thioredoxin can be suppressed by binding to TXNIP (Yoshihara et al., 2013). *TXNIP* was downregulated with TCR triggering but upregulated with PGD_2 stimulation. This would suggest TCR triggering resulted in oxidative stress but PGD_2 did not. However, *TXNRD1* was particularly upregulated by PGD_2 stimulation and encodes TXNRD1, a positive regulator of thioredoxin (Collet & Messens, 2010).

There was evidence that p38 MAPK signalling, a key component of cellular response to stress (Canovas & Nebreda, 2021), was specifically associated with TCR triggering. Genes encoding P38 activators such as the GADD45 family (e.g. *GADD45B*) were especially upregulated in the TCR condition (Schäfer, 2013). Downstream effectors of p38 were also especially upregulated. Grip1/TIF2/SRC-2 (encoded by *NCOA2*) is activated by p38 MAPK signalling and is a coactivator for multiple nuclear hormone receptors (Dasgupta et al., 2014; Frigo et al., 2006; Hong et al., 1997). The *VDR* gene for Vitamin D receptor was similarly upregulated and previous studies have shown this occurs through a TCR-p38 axis (Provvedini et al., 1983; Von Essen et al., 2010). The function of VDR can be stimulatory or inhibitory depending on context (Lahore et al., 2020).

4.4.5.6 Induction of genes for ADAM metalloproteinases

Two genes for ADAM metalloproteinases, *ADAM19* and TACE (*ADAM17*) were associated with TCR triggering. ADAM proteins cleave membrane bound proteins including immune checkpoint molecules and cytokines, allowing them to be released from the cell (Lambrecht et al., 2018). Both ADAM19 and TACE are known to cleave many targets including TNF and amphiregulin (Lambrecht et al., 2018; Y. Zheng et al., 2004).

Variants of ADM19 have been linked to COPD (Pérez-Rubio et al., 2016; J. B. Wilk et al., 2012).

4.4.6 Many DEGs of interest remain poorly understood

The majority of human gene functions remain unknown (Brown & Lad, 2019). This is due to in part to the large number of genes in the human genome, but also that each gene can have many functions (pleiotropy). For many DEGs of interest, little or no information could be found in the literature.

Several genes were associated with alarmin stimulation but have unclear functions in T cells. MAP1A (*MAP1A*) is generally expressed in the brain (The Human Protein Atlas, 2021c) and its protein is known to interact with microtubules and actin filaments (Halpain & Dehmelt, 2006), and is a binding partner for osteopontin (Long et al., 2012). RTN4RL1 (*RTN4RL1*) can interact with chondroitin sulphate proteoglycans (Dickendesher et al., 2012). MDFIC (*MDFIC*) can bind and influence glucocorticoid receptors (Oakley et al., 2017); SCN2A (*SCN2A*) is a sodium channel (Sanders et al., 2018); IRX5 (*IRX5*) is a GATA-3 binding protein (Bonnard et al., 2012); Nidd (*ZDDHC23*) can regulate big potassium (BK) ion channels (Tian et al., 2012); and *C3orf18* is uncharacterized.

Likewise, the genes strongly associated with PGD_2 stimulation that are unstudied in the T cell context include: *MFSD6; SYAP1; SAMD10; MOB3A* and genes for other MOB kinases; *NIBAN1* encoding FAM129A, linked to steroid response in asthma and MAPK signalling (Yim et al., 2020); *CHST2* encoding GlcNAc6ST-1, an enzyme that can sulphate glycans (Taniguchi et al., 2014) and is involved in biosynthesis of keratan sulphate (Caterson & Melrose, 2018), a glycosaminoglycan present in the airways (Monzon et al., 2006). *ST3GAL2*, uniquely upregulated by PGD_2, encodes a sialyltransferase involved in

ganglioside glycosphingolipid synthesis (Taniguchi et al., 2014). Gangliosides may play a role in T cell activation (T. Zhang et al., 2019) but the specific gangliosides produced by ST3GAL2 and their function in T cells are not known.

Further genes were strongly associated with TCR triggering but have unclear functions in this context: *EVI2A; TATDN2; FAM107B*; *SACS* encoding the large multidomain protein sacsin (Ménade et al., 2018); and *EDARADD* encoding an adapter protein involved in EDAR-signalling (Cluzeau et al., 2011). FRMD4B (*FRMD4B*) has been linked to smoking (Kõks et al., 2015), COPD (Morrow et al., 2018), and steroid sensitivity (Shinohara et al., 2020). These may be candidates for future biochemical studies of TCR signalling.

4.5 Conclusion

This chapter characterised the transcriptome of CRTH2$^+$ CD8$^+$ T cells activated by three different stimulation modalities, and made biological inferences from the data. Cultured CRTH2$^+$ CD8$^+$ T cells expressed high levels of genes for their defining surface markers, and for key transcription factors GATA3 and STAT6, supporting their identity as Tc2 cells. Furthermore, the cells responded to stimulation through the TCR, PGD_2-CRTH2 interactions or by combined alarmin cytokines IL-25, IL-33 and TSLP, by upregulating *IL13* expression. Activation by PGD_2 was rapid and transient, by TCR was rapid and sustained, and by alarmins was delayed and sustained. This shows that the kinetics of CRTH2$^+$ CD8$^+$ T cell activation depends on how they are stimulated.

Genes for alarmin receptors at resting state could only be detected for IL-25, and not TSLP or IL-33. So, it is likely that IL-25 drove the observed changes in the alarmin stimulation condition. High expression of genes for the receptors for type 2 mediators IL-

4, IL-9, and NMU was also detected, and it is likely that CRTH2$^+$ CD8$^+$ T cells can also respond directly to these type 2 mediators. This knowledge is important because all of these pathways are linked to asthma and are targets for novel biological therapies, despite our limited knowledge of their biology.

There was significant overlap in the gene expression changes induced by the three different stimulation conditions. Studying the overlapping DEGs revealed a shared programme of CRTH2$^+$ CD8$^+$ T cell activation. This programme involves genes relating to: type 2 cytokines (*IL4, IL5,* and *IL13)* and other type 2 mediators; cytotoxic granules and their regulators; transcriptional and translational machinery; increased cAMP-PKA signalling; possibly Wnt signalling (but through different pathways); higher survival; tissue repair and remodelling; changes to surface receptors involved in co-stimulation or co-inhibition; regulation of TCR signalling; and increased generic T cell activation markers (e.g., CD69) and transcription factors (e.g., NF-κB, and NFAT). There was no evidence of concurrent type 1 or type 17 responses, further confirming their type-2 gene signature. Broadly speaking, the data imply that CRTH2$^+$ CD8$^+$ T cells can function in type 2 inflammation, tissue repair and remodelling, response to parasites, and probably direct killing of host cells (e.g., in the context of cancer or viral infection). Because these processes are known to be important in the context of asthma (and to a lesser extent COPD), it is likely that CRTH2$^+$ CD8$^+$ T cells can contribute to the T2-high endotype of these diseases, if they are present and active in the lung.

Activation by alarmin cytokines specifically induced genes related to the STAT5 signalling pathway and its regulators. This is likely to have occurred through IL-25 signalling, and may contribute to type 2 differentiation. Future research may look for direct interaction between the IL-25 receptor and regulatory elements of the signalling pathway. Alarmin

stimulation also induced several genes related to host interactions with viruses. Viperin (*RSAD2*) may be of particular interest because it appears to link antiviral response and type 2 differentiation. CRTH2$^+$ CD8$^+$ T cells may be involved in a pathway that involves viral infection (e.g., by rhinoviruses), release of IL-25 by the epithelium, and activation of IL-25 receptive type 2 T cells through STAT5 signalling, resulting in a type 2 mediated exacerbation of disease in certain patients. The alarmin-induced upregulation of *AR* is also worthy of further study due to the recently identified protective roles of testosterone in asthma and influenza, through contraction of CD8$^+$ T cell populations.

Activation by PGD_2-CRTH2 interactions specifically induced genes related to cell locomotion and chemotaxis, including a large number of actin and myosin associated genes. These changes appeared to be driven by Rac1 signalling, because Rac1 and a large number of its regulators were associated specifically with PGD_2 stimulation. Autophagy-related genes were enriched and this could also be a response to the increase in cell locomotion. There was specific upregulation of *CXCR6* associated with homing to the lung, suggesting that PGD_2 might indirectly promote migration to the lung regardless of the PGD_2 source location. Genes for negative regulation of GPCR signalling were increased, probably as a negative feedback mechanism against CRTH2.

PGD_2 stimulation induced expression of genes related to lipid synthesis and eicosanoid metabolism, including enzymes involved in PGE_2 synthesis, which are also associated with asthma. Elevation of *BCL6* and corresponding fall of *LITAF* were specific to PGD_2 and both of these genes are associated with asthma – clearly the BCL6:LITAF ratio is important in asthma but the reason why is not yet understood. PGD_2 may also alter sensitivity to IL-7, through downregulation of *IL7R*, which is genetically linked to asthma, and this may be due to reduced activity of FOXO1. Finally, PGD_2 was specifically

associated with genes relating to activity of the SMRT/NCoR-HDAC3 complexes, elements of which are associated with asthma. PGD_2 stimulation was also associated with genes related to vessel diameter and angiogenesis, which may relate to the protective role PGD_2 has been reported to play in some cancers. Together, the data increase the detail of our understanding of PGD_2-induced chemotaxis and activation of $CRTH2^+$ $CD8^+$ T cells, and introduce some novel specific links between PGD_2 stimulation and genes associated with asthma.

Activation of $CRTH2^+$ $CD8^+$ T cells through the TCR specifically induced a large number of genes involved in the Warburg effect of aerobic glycolysis and proliferation. There were also upregulated genes, especially for chemokines, involved in communication with activated APCs in tissues or lymph nodes, and recruitment of more T cells. TCR-triggering upregulated genes involved in magnesium and iron uptake, suggesting these are components of TCR signalling. The cells showed signs stress by upregulating p38 MAPK activator and effector genes. It is unclear whether increased stress is qualitatively due to TCR signalling, or simply because the stimulation in that condition was far stronger. Finally, there may be an increase in production of exosomes or use of recycling endosomes, but it is unclear what the role of these processes is in TCR signalling, although it may be to do with formation of the immunological synapse.

TCR triggering was specifically associated with *IL2RA* , *IL2, MYC* and *TFRC* - all asthma-associated genes involved in the clonal expansion (Warburg) process for T cells. Clearly then, expansion of T cell populations by antigenic stimulation is important in asthma. The gene for the receptor for IL-33, ST2 (*IL1RL1*) was only expressed with TCR stimulation, and this is another pathway involved in asthma. Notably fewer genes associated with COPD were identified in this study compared with asthma-associated genes. All five

COPD-associated genes discussed (*ADAM19, HIVEP2, TYRO3*, *FRMD4B* and *NR4A2*) were uniquely or especially upregulated by TCR activation, and not PGD_2 or alarmin stimulation. This suggests that the TCR signalling pathway (but not T cell activation in general) specifically plays some role in the susceptibility of individuals to COPD.

In conclusion, activated $CRTH2^+$ $CD8^+$ T cells have the potential to contribute to known functions (and pathologies) relating to type 2 inflammation. Different modes of stimulation appear to elicit a combination of shared and unique responses from the T cells, depending on the nature of the stimulant. Some of the genes specifically responsive to one stimulant are also associated with asthma or COPD, and this dataset may help to narrow down on why those associations exist. The most pressing question to address now is whether $CRTH2^+$ $CD8^+$ T cells are really enriched and/or activated in the context of chronic airways disease or other T2-high disease contexts. If that turns out to be true, then this dataset exists to help us understand their activation programme at a greater depth than single-cell sequencing would allow.

4.6 Appendix

4.6.1 RT-qPCR

Supplementary Table 13 Contents of reverse transcription 2X master mix

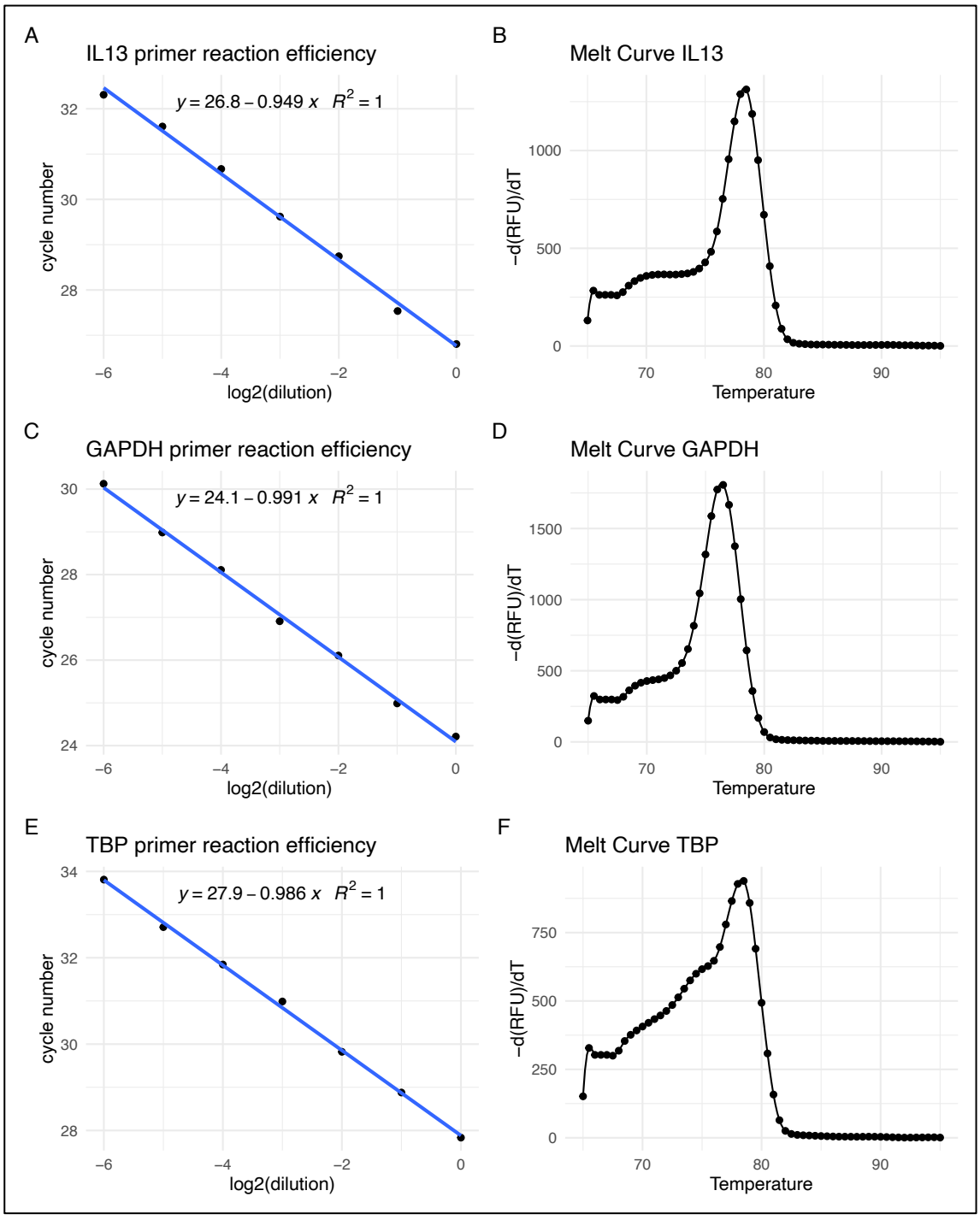
Reagent	Ratio	Function
RT Buffer	2	creates optimal conditions for the supplied enzyme.
dNTP Mix (100mM)	0.8	building block deoxynucleotides for cDNA.
RT Random Primers	2	oligonucleotides that bind template RNA. Randomness results in template-representative cDNA.
MultiScribe reverse transcriptase	1	catalyses cDNA synthesis by extension of bound primers.
RNase Inhibitor	1	inhibits enzymatic degradation of template RNA.
Nuclease free water	3.2	optimise the reaction volume and reagent concentrations.

Supplementary Table 14 Contents of RT-qPCR master mix

Reagent	Function
SYBR Green I Dye:	forms a fluorescent complex with dsDNA PCR product (Life Technologies, 2015).
AmpliTaq Fast DNA Polymerase:	thermostable polymerase to catalyse amplification of template cDNA in the presence of specific primers and nucleotides (Saiki et al., 1988).
Uracil-DNA glycosylase (UDG)	Prevents contamination from a previous PCR reactions by cleaving the dUTP used in PCR amplification, but not the dTTP used in cDNA synthesis (Longo et al., 1990). Deactivated by the first heating step of the PCR reaction.
ROX Passive Reference dye	A control dye to which the SYBR green signal can be normalised to control for external fluorescence variations (Life Technologies, 2015). It is not necessary to normalise to this in our qPCR system (Bio-Rad, 2010b).
dNTPs	the building blocks of DNA PCR product.
Buffer components (unspecified)	Optimisation of polymerase enzyme activity.

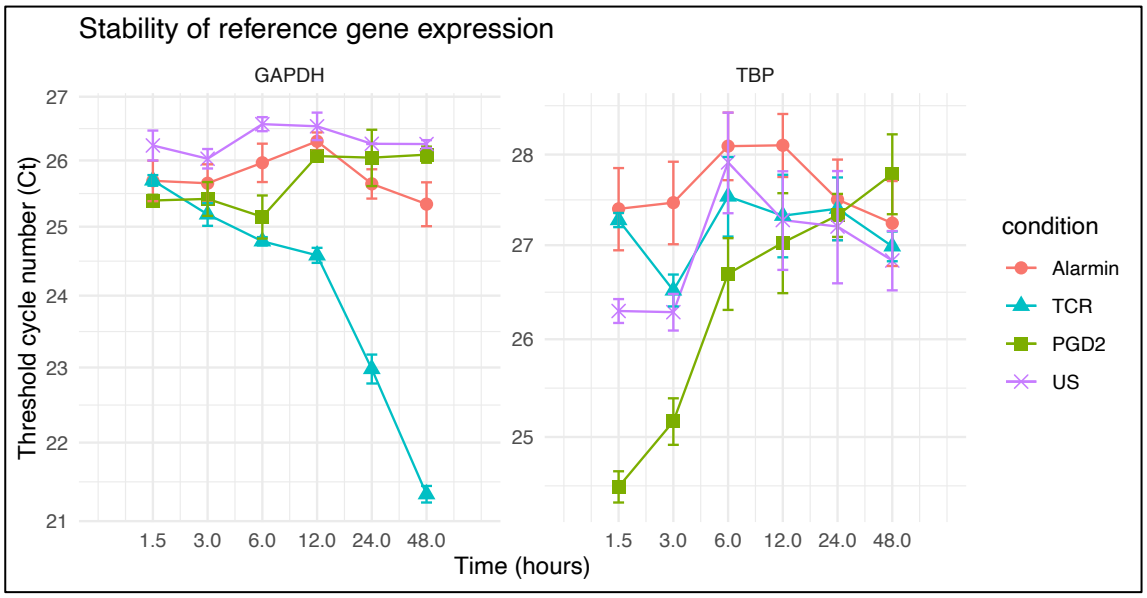
Supplementary Table 15 Primers used in this study

Gene	Accession	F/R	Position	Tm	Sequence
IL13	NM_002188.2	F	137 - 154	59	AGCCCTCAGGGAGCTCAT
		R	201 - 220	60	CTCCATACCATGCTGCCATT
GAPDH	NM_001289745.1	F	261 - 279	60	AGCCACATCGCTCAGACAC
		R	308 - 326	60	GCCCAATACGACCAAATCC
TBP	NM_001172085.1	F	501 - 518	59	CCCATGACTCCCATGACC
		R	586 - 608	59	TTTACAACCAAGATTCACTGTGG



Supplementary Figure 32 reaction efficiency and melting curves of primers

Reaction efficiency and melt curve data was obtained as described in 4.2.1.5. A reaction efficiency of 100% would produce a straight line of gradient -1, indicating that each additional cycle doubled the PCR product. A sharp melting curve peak at the expected Tm of the product, indicates purity of the PCR product.



Supplementary Figure 33 Reference gene stability between conditions over time

Cultured CRTH2+ CD8+ T cells were stimulated as described in 4.2.2. RT-qPCR was conducted as described in 4.2.1. A stable reference gene should not change over time.

4.6.2 RNA-Seq

Supplementary Table 16 List of genes associated with T cell immunotypes

Th1				**Th2**		**Th17**	
AGER	IFNG	NLRP11	PRF1	ADORA2B	IL33	BATF	IL23R
CIITA	IFNGR1	NLRP12	REL	AREG	IL4	CCL20	IL26
IFNA1	IFNGR2	NLRP13	RELA	CCR4	IL4R	CCR6	IL6
IFNA10	IL12A	NLRP14	RELB	CGAS	IL5	CCR6	IL6R
IFNA13	IL12RB1	NLRP2	STAT1	CRLF2	IL5RA	IL10RB	IRF4
IFNA14	IL12RB2	NLRP3	TBX21	EGFR	IL9	IL17A	ITGA3
IFNA16	IL2	NLRP4	TLR1	GATA3	IL9R	IL17F	RORA
IFNA17	LTA	NLRP5	TLR10	HPGDS	NMU	IL17RA	RORC
IFNA2	NAIP	NLRP6	TLR2	IL10	NMUR1	IL17RC	STAT3
IFNA21	NFKB1	NLRP7	TLR3	IL10RA	PTGDR2	IL1B	TGFB1
IFNA4	NFKB2	NLRP8	TLR4	IL13	PTGDS	IL20RA	TGFB2
IFNA5	NLRC3	NLRP9	TLR5	IL13RA1	STAT6	IL21	TGFB3
IFNA6	NLRC4	NLRX1	TLR6	IL13RA2	TSLP	IL21R	TGFBR1
IFNA7	NLRC5	NOD1	TLR7	IL17RB		IL22	TGFBR2
IFNA8	NLRP1	NOD2	TLR8	IL1RL1		IL22RA1	TGFBR3
IFNAR1	NLRP10	NOS2	TLR9	IL25		IL23A	

Supplementary Table 17 Quality control scores for total RNA for RNA-Seq

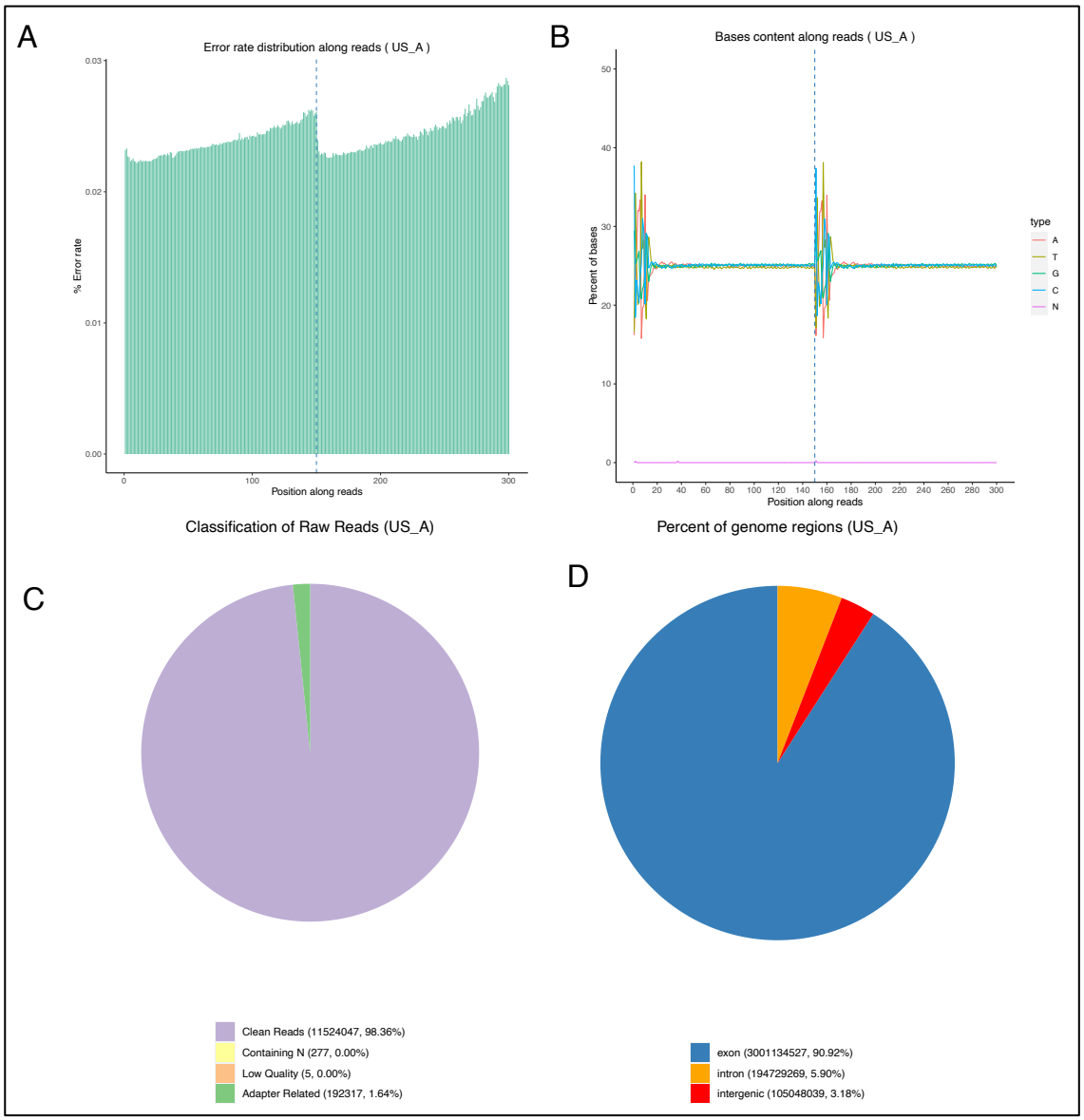
In the sample ID column, the letters A to E refer to the biological donor. Abbreviations: RIN^e, RNA Integrity Number equivalent.

Sample ID	Nanodrop Spectrophotometer			Agilent TapeStation	
	Concentration (ng/μl)	260/280	260/230	Concentration (ng/μl)	RIN^e
US_A	92.4	2.07	1.38	76	9.5
US_B	135.5	2.07	2.03	89.3	9.7
US_C	125.3	2.04	1.8	86.5	9.7
US_D	103.5	2.05	1.82	78.7	9.8
US_E	110	2.04	0.84	89.9	9.5
TCR_A	91.2	2.04	1.58	75.1	9.8
TCR_B	159.8	2.08	2.12	103	9. 8
TCR_C	107	2.05	1.92	95.2	9.5
TCR_D	101	2.06	1.77	81.7	9.7
TCR_E	103.3	2.05	0.82	59.5	9.5
PGD_2_A	97.9	2.06	1.65	66.3	9.9
PGD_2_B	148.6	2.09	0.89	95.2	9.8
PGD_2_C	92.1	2.02	1.32	61.3	9.8
PGD_2_D	85.1	2.03	0.81	75.6	9.8
PGD_2_E	110.3	2.07	0.75	80.5	9.7
Alarmin_A	65.2	2.02	1.77	68.4	10
Alarmin_B	130.1	2.07	1.48	103	9.9
Alarmin_C	64.7	2.02	1.62	53.5	9.6
Alarmin_D	93	2.05	1.03	64.9	10
Alarmin_E	129.8	2.07	0.87	49	9.7

Supplementary Table 18 Quality control scores for raw reads of RNA-Seq

Data provided by Novogene. In the sample ID column, the letters A to E refer to the biological donor. 'Clean reads' are those which passed the filtering process described in 4.2.3.5.1. Error rate is the estimated fraction of incorrectly called bases. Q20 is equivalent to less than 1% error rate on average for a read, and Q30 to 0.1%.

Sample ID	Raw reads	% clean reads	Error rate average (%)	Q20 (%)	Q30 (%)	GC content (%)
US_A	11716646	98.4	0.02	98.23	95.04	50.14
US_B	10658566	98.1	0.02	98.27	95.15	50.16
US_C	12358491	98.2	0.02	98.11	94.75	49.86
US_D	11804675	98.2	0.03	97.9	94.31	49.65
US_E	14100428	98.5	0.02	98.2	94.99	49.91
TCR_A	8719829	97.7	0.02	98.24	95.1	49.55
TCR_B	13611587	98.3	0.02	98.1	94.73	49.83
TCR_C	11310421	98.3	0.02	98.13	94.85	49.97
TCR_D	11384098	98.6	0.02	98.02	94.53	49.74
TCR_E	15421609	98.2	0.02	98.1	94.79	50.41
PGD_2_A	17186232	98.6	0.02	98.06	94.64	50.24
PGD_2_B	9528767	98.6	0.02	98.2	94.99	50.16
PGD_2_C	13713841	98.3	0.02	97.97	94.47	50.41
PGD_2_D	12537613	98.1	0.02	97.95	94.41	50.65
PGD_2_E	14105792	98.3	0.03	97.9	94.3	50.68
Alarmin_A	16888570	98.4	0.02	98.07	94.66	50.02
Alarmin_B	13169402	98.1	0.02	98.23	95.02	50.09
Alarmin_C	16686879	98.0	0.02	98.15	94.84	49.43
Alarmin_D	10644539	98.5	0.02	98.08	94.68	49.93
Alarmin_E	15143133	98.3	0.02	98.14	94.86	50.32



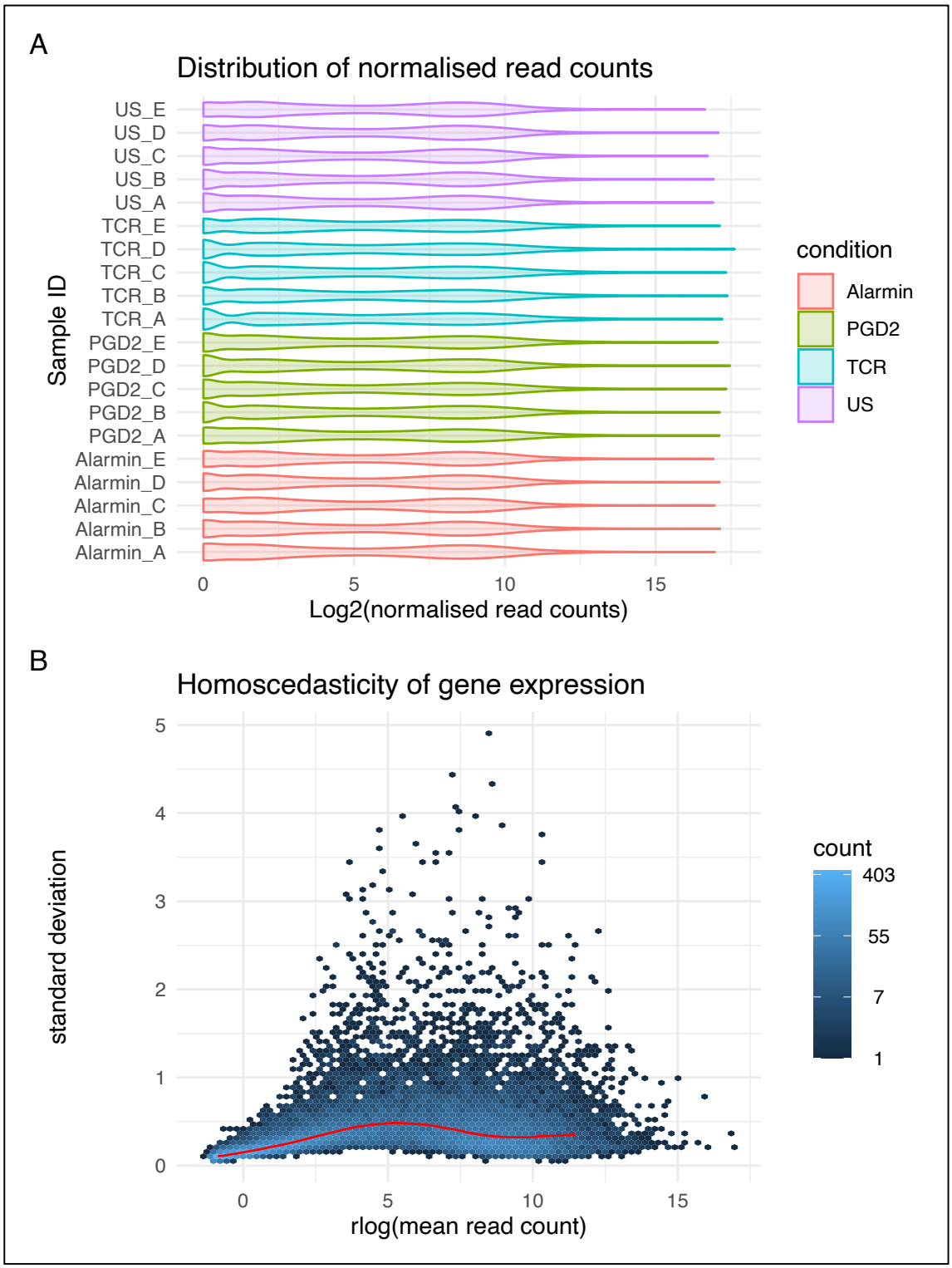
Supplementary Figure 34 Quality control of raw RNA-Seq data

Data provided by Novogene. (A) Representative plot of average error rate estimate per base along each read. A value below 1% was considered acceptable (B) Representative plot of individual base content along each read. The value should be approximately 25 % except for the first 6-7 bases of each read due to primer bias (C) Representative pie chart of read quality, more clean reads indicate a more successful sequencing run. (D) Representative pie chart of read mapping regions: most clean reads were expected to map to exonic regions (blue).

Supplementary Table 19 Read mapping data for RNA-Seq from Novogene

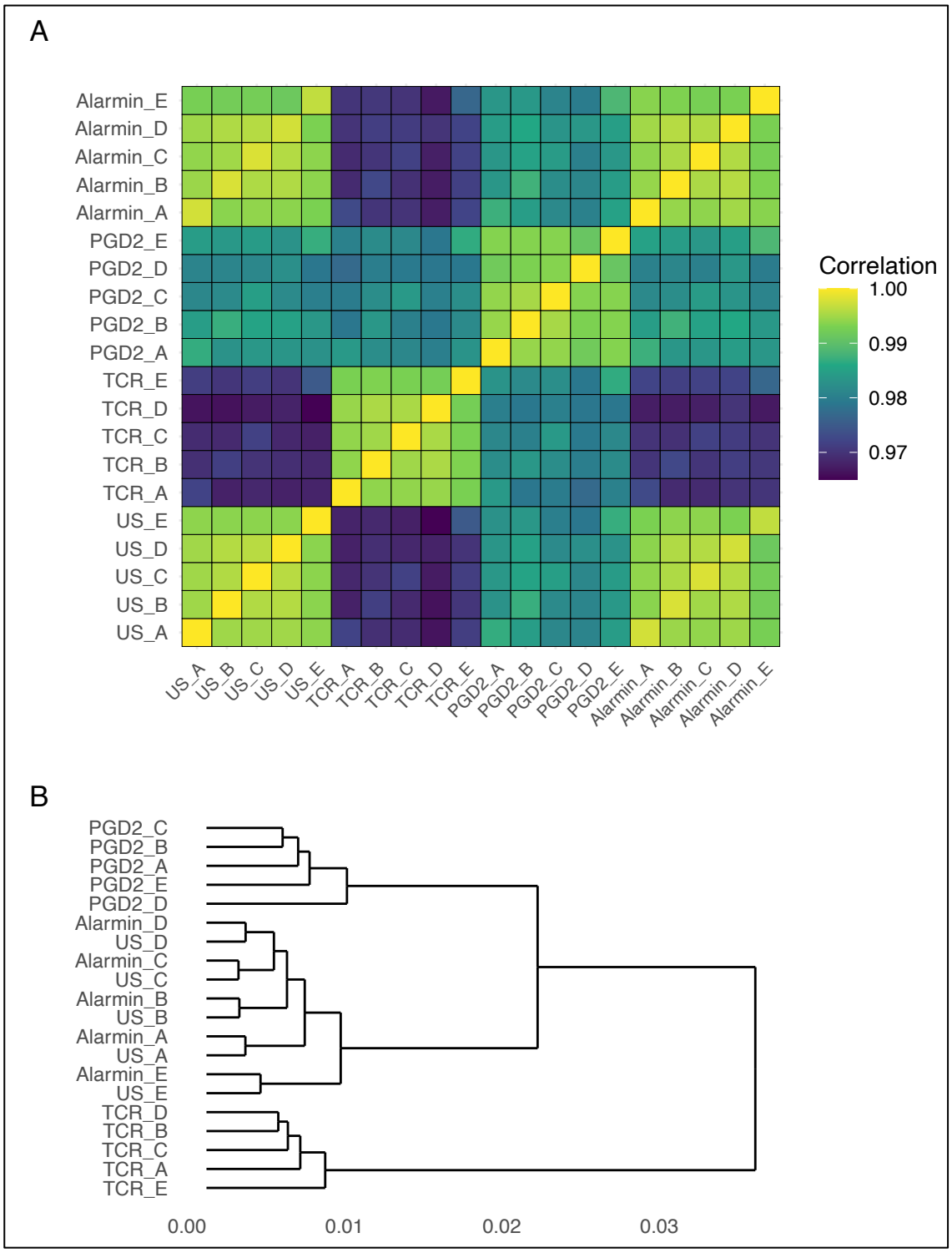
Data provided by Novogene. In the sample ID column, the letters A to E refer to the biological donor. Read mapping was performed by Novogene using STAR 2.6.1.

Sample ID	Total reads	Total mapping rate	Uniquely mapping rate	Multiple mapping rate	Mapped to exon (%)
US_A	23048094	96.61%	92.01%	4.60%	90.9
US_B	20919244	96.92%	91.84%	5.08%	90.7
US_C	24261852	96.47%	91.28%	5.18%	89.6
US_D	23176902	96.23%	91.85%	4.38%	88.3
US_E	27768858	96.83%	92.77%	4.07%	88.3
TCR_A	17044782	96.65%	92.07%	4.58%	89.3
TCR_B	26765868	96.77%	92.61%	4.15%	89.9
TCR_C	22231486	96.46%	91.44%	5.02%	90.7
TCR_D	22444244	96.68%	92.42%	4.25%	88.9
TCR_E	30273146	96.83%	88.10%	8.73%	83.3
PGD_2_A	33885344	96.52%	92.35%	4.17%	90.0
PGD_2_B	18784558	97.06%	93.24%	3.81%	90.5
PGD_2_C	26962560	96.34%	91.41%	4.93%	90.3
PGD_2_D	24609708	96.28%	90.82%	5.46%	91.2
PGD_2_E	27727990	96.47%	90.36%	6.11%	90.1
Alarmin_A	33220436	96.26%	91.45%	4.81%	90.5
Alarmin_B	25845278	96.96%	91.63%	5.33%	88.9
Alarmin_C	32720648	96.53%	92.32%	4.21%	87.0
Alarmin_D	20967550	96.54%	91.39%	5.15%	89.5
Alarmin_E	29766706	96.78%	91.65%	5.13%	89.1



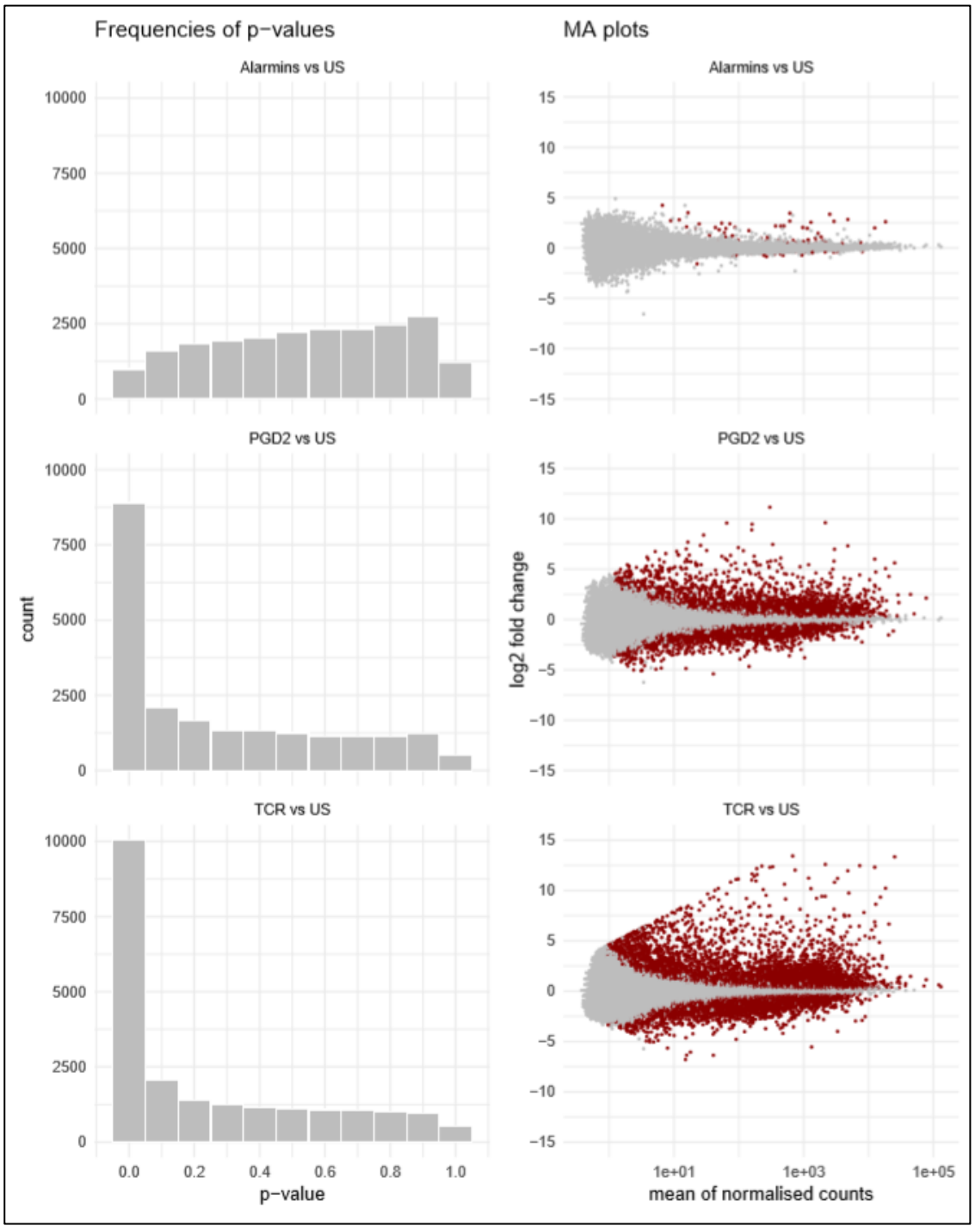
Supplementary Figure 35 Distribution and variance of normalised read counts

(A) Violin plot showing individual samples on the y-axis, and distribution of the number of normalised read counts per gene in the count data on the x-axis. (B) mean-SD plot showing transformed read count number on the x-axis and standard deviation on the y-axis. Each hexagon represents variable numbers of genes as indicated by the colour legend.



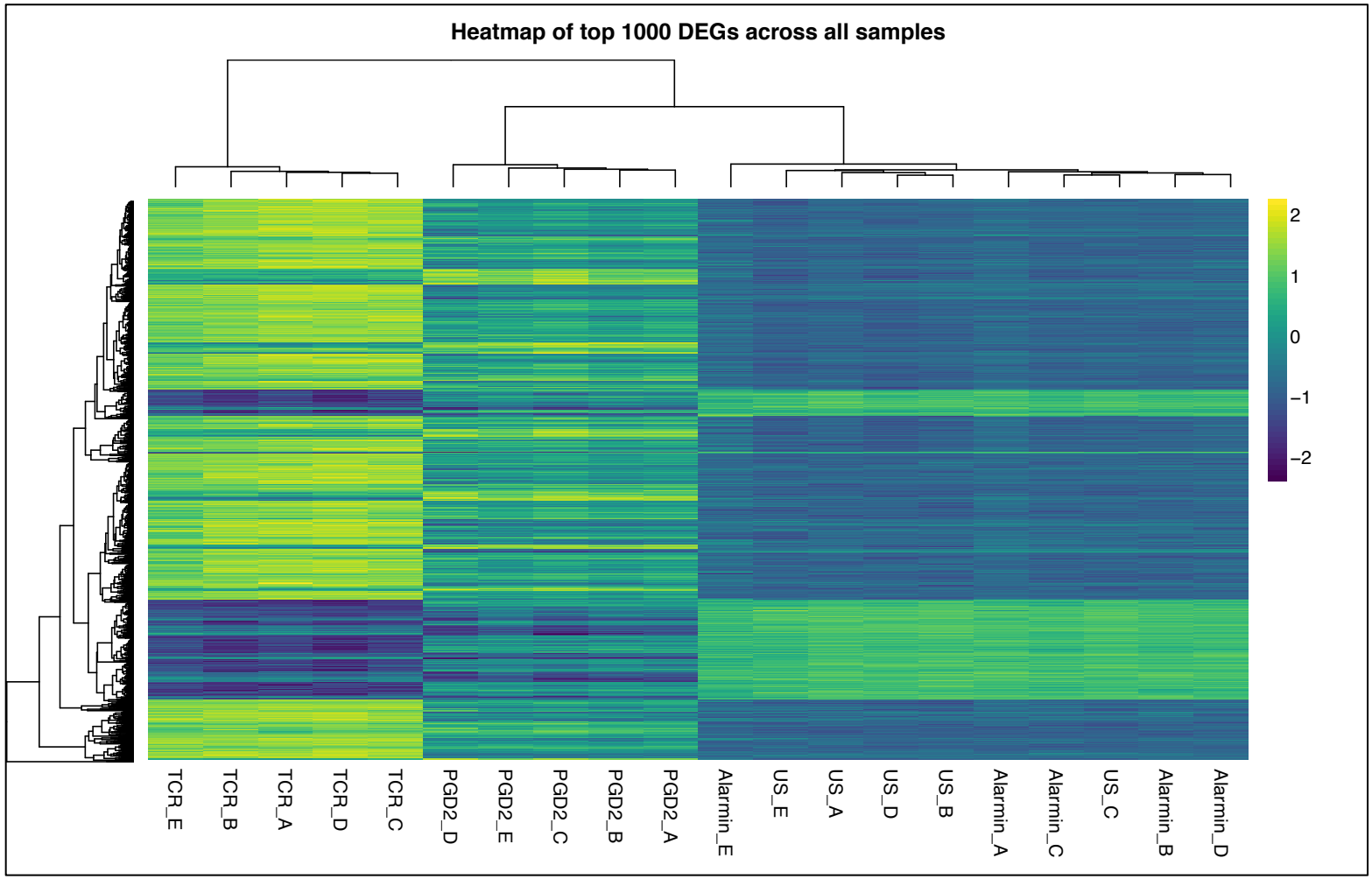
Supplementary Figure 36 Relatedness of global read count data between samples

On the y-axes letters A to E refer to the biological donor. (A) Heatmap of Pearson correlation analysis between all samples for all genes. (B) Dendrogram of unbiased hierarchical clustering of samples based on global read count data.



Supplementary Figure 37 MA plots and histogram of p-values for DESeq2 results

Histograms are of raw p-values from DESeq results. MA plots were constructed by plotting average normalised counts against fold-change (vs unstimulated condition). Each point represents a gene and points were coloured red if *padj* < 0.05



Supplementary Figure 38 Heatmap of 1000 most significant DEGs across all samples shows consistency of DEG results within conditions

Heatmap was drawn using the NMF package for R (Gaujoux & Seoighe, 2010). Samples and genes were clustered using hierarchical clustering based on Euclidean distance. Colour is proportionate to the deviation of each cell from the mean expression across all samples.

Supplementary Table 20 Top DEGs - Alarmin condition – All DEGs

The '#' column indicates gene rank (see 4.2.3.5.3). Abbreviations: Log2FC, binary log of fold change vs unstimulated; LogBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol;. Full dataset available (Hoyle & Xue, 2021).

#	SYMBOL	logBM	log2FC	*padj*	GENENAME
1	CISH	3.40	3.34	9.20E-20	cytokine inducible SH2 containing…
2	EGR1	3.47	2.64	5.51E-06	early growth response 1
3	IL13	4.26	2.61	3.14E-03	interleukin 13
4	EGR2	3.68	2.83	6.90E-03	early growth response 2
5	CCL3	3.11	2.55	9.55E-08	C-C motif chemokine ligand 3
6	OSM	2.79	3.45	7.71E-10	oncostatin M
7	BCL2	2.98	2.03	4.50E-24	BCL2 apoptosis regulator
8	SOCS1	3.05	1.95	4.63E-11	suppressor of cytokine signalling 1
9	IRX3	2.78	2.67	1.13E-04	iroquois homeobox 3
10	TNF	3.22	1.46	2.70E-04	tumor necrosis factor
10=	EGR3	4.09	1.99	1.44E-02	early growth response 3
11	SOCS2	2.69	2.19	6.72E-06	suppressor of cytokine signalling 2
12	RSAD2	1.74	2.46	4.61E-11	radical S-adenosyl methionine…
13	CDK6	3.18	0.97	1.08E-02	cyclin dependent kinase 6
14	ZDHHC23	1.94	2.04	1.80E-09	zinc finger DHHC-type…
14=	PIM1	3.27	1.09	1.54E-02	Pim-1 proto-oncogene…
15	CDKN2B	1.86	2.41	5.51E-06	cyclin dependent kinase inhibitor 2B
16	UBTD2	2.46	1.16	2.06E-06	ubiquitin domain containing 2
17	KLF5	2.15	1.73	8.91E-07	Kruppel like factor 5
18	B4GALT5	3.07	0.82	1.23E-02	beta-1,4-galactosyltransferase 5
18=	MDFIC	3.29	0.72	1.34E-02	MyoD family inhibitor domain…
19	SCN2A	1.22	3.50	1.93E-03	sodium voltage-gated channel…
20	RNF144A	3.19	0.61	1.02E-02	ring finger protein 144A
21	BYSL	2.44	1.05	2.70E-04	bystin like
22	JUND	3.87	0.57	1.54E-02	JunD proto-oncogene, AP-1…
23	IRX5	1.64	2.03	1.17E-04	iroquois homeobox 5
23=	PMCH	1.08	2.80	1.86E-03	pro-melanin concentrating hormone
24	C3orf18	2.35	0.99	1.38E-04	chromosome 3 open reading frame 18
24=	MIB1	3.33	0.61	1.80E-02	mindbomb E3 ubiquitin protein…
25	MAP1A	1.80	1.93	1.37E-03	microtubule associated protein 1A
25=	HTATSF1	3.06	0.52	5.15E-03	HIV-1 Tat specific factor 1
26	SH2B3	3.59	0.45	1.48E-02	SH2B adaptor protein 3
26=	FOS	2.65	2.18	3.68E-02	Fos proto-oncogene, AP-1…
27	RTN4RL1	1.70	1.81	4.00E-04	reticulon 4 receptor like 1
27=	MMD	2.44	0.85	3.88E-03	monocyte to macrophage…
28	ZAP70	3.46	-0.40	1.29E-02	zeta chain of T cell receptor…
29	CCNL2	2.97	-0.71	1.65E-02	cyclin L2
30	ACOX1	2.68	0.61	2.75E-03	acyl-CoA oxidase 1
31	SNAP47	2.60	0.72	9.52E-03	synaptosome associated protein 47
31=	COL5A2	1.39	2.39	1.08E-02	collagen type V alpha 2 chain
32	MCOLN3	1.19	2.10	7.04E-03	mucolipin 3

Supplementary Table 21 Top DEGs – PGD_2 condition – All DEGs

The '#' column indicates gene rank (see 4.2.3.5.3). Abbreviations: Log2FC, binary log of fold change vs unstimulated; LogBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol;. Full dataset available (Hoyle & Xue, 2021).

#	SYMBOL	logBM	log2FC	*padj*	GENENAME
1	NR4A3	4.40	5.63	2.01E-82	nuclear receptor subfamily 4…
2	SLC7A5	4.31	4.28	7.04E-96	solute carrier family 7 member 5
3	EGR3	4.09	6.01	1.89E-34	early growth response 3
4	NR4A1	3.86	4.43	1.06E-46	nuclear receptor subfamily 4…
5	CD69	4.09	3.41	4.44E-78	CD69 molecule
6	NAB2	3.45	5.01	1.83E-97	NGFI-A binding protein 2
7	GZMB	4.11	4.72	5.66E-31	granzyme B
8	CREM	3.72	4.01	3.90E-50	cAMP responsive element…
9	BIRC3	4.17	3.02	5.56E-115	baculoviral IAP repeat containing 3
10	FOSL2	4.10	3.26	2.29E-50	FOS like 2, AP-1 transcription…
11	BZW1	4.00	2.90	1.47E-130	basic leucine zipper and W2…
12	PTPN7	3.95	2.90	2.11E-142	protein tyrosine phosphatase…
13	EGR2	3.68	7.32	1.56E-28	early growth response 2
14	SH2D2A	3.68	3.04	9.06E-129	SH2 domain containing 2A
15	TNFRSF9	3.47	6.99	1.15E-35	TNF receptor superfamily member 9
16	CSF2	3.70	3.47	8.90E-44	colony stimulating factor 2
17	EGR1	3.47	5.81	1.40E-36	early growth response 1
18	RRAD	3.33	9.62	3.42E-58	RRAD, Ras related glycolysis…
19	PHLDA1	4.02	3.96	8.61E-28	pleckstrin homology like domain…
20	ACTG1	4.65	2.50	3.46E-99	actin gamma 1
21	IL27RA	3.66	2.91	2.42E-127	interleukin 27 receptor subunit alpha
22	LCP1	4.45	2.45	1.61E-119	lymphocyte cytosolic protein 1
23	TPM4	3.96	2.57	2.11E-90	tropomyosin 4
24	SDC4	3.93	3.00	1.88E-40	syndecan 4
25	NAMPT	4.08	2.76	1.38E-44	Nicotinamide…
26	JUND	3.87	2.55	4.61E-73	JunD proto-oncogene, AP-1…
27	CSRNP1	3.63	2.78	3.64E-108	cysteine and serine rich nuclear…
28	GPAT3	3.54	2.98	4.47E-83	glycerol-3-phosphate…
29	SLC7A1	3.40	3.42	1.85E-67	solute carrier family 7 member 1
30	NFKB1	3.83	2.34	1.43E-92	nuclear factor kappa B subunit 1
31	VASP	3.67	2.45	5.58E-117	vasodilator stimulated…
32	SLC16A3	3.36	3.23	9.79E-79	solute carrier family 16 member 3
33	NDFIP2	3.42	3.58	1.26E-39	Nedd4 family interacting protein 2
34	ACTB	4.89	2.13	2.36E-97	actin beta
35	IL13	4.26	5.07	1.38E-17	interleukin 13
36	MYADM	3.69	2.42	1.35E-62	myeloid associated differentiation…
37	ACTR3	3.83	2.18	1.21E-117	actin related protein 3
38	SFPQ	3.85	2.17	1.00E-104	splicing factor proline and…
39	SRGN	4.21	2.17	3.52E-58	Serglycin
40	TNFRSF4	3.28	4.01	2.63E-44	TNF receptor superfamily member 4

Supplementary Table 22 Top DEGs - TCR condition – All DEGs

The '#' column indicates gene rank (see 4.2.3.5.3). Abbreviations: Log2FC, binary log of fold change vs unstimulated; LogBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol;. Full dataset available (Hoyle & Xue, 2021).

#	SYMBOL	logBM	log2FC	*padj*	GENENAME
1	NR4A3	4.40	13.35	0.00E+00	nuclear receptor subfamily 4…
2	EGR3	4.09	12.32	1.58E-144	early growth response 3
3	NR4A1	3.86	12.47	0.00E+00	nuclear receptor subfamily 4…
4	SLC7A5	4.31	6.67	2.37E-233	solute carrier family 7 member 5
5	CD69	4.09	6.88	0.00E+00	CD69 molecule
6	SDC4	3.93	7.82	1.38E-275	syndecan 4
7	FOSL2	4.10	6.47	1.77E-198	FOS like 2, AP-1 transcription…
8	GZMB	4.11	8.63	4.29E-103	granzyme B
9	KDM6B	4.05	6.09	0.00E+00	lysine demethylase 6B
10	NR4A2	3.69	9.75	0.00E+00	nuclear receptor subfamily 4…
11	CCL4	4.18	9.34	1.13E-79	C-C motif chemokine ligand 4
12	IL13	4.26	10.23	8.11E-70	interleukin 13
13	CSF2	3.70	7.50	1.81E-204	colony stimulating factor 2
14	PIM3	3.95	5.48	3.75E-238	Pim-3 proto-oncogene…
15	NAMPT	4.08	5.34	1.33E-167	Nicotinamide…
16	BIRC3	4.17	4.85	5.84E-298	baculoviral IAP repeat containing 3
17	CREM	3.72	6.41	2.05E-128	cAMP responsive element…
18	TNFSF14	3.85	5.13	1.06E-219	TNF superfamily member 14
19	EGR2	3.68	11.97	8.31E-76	early growth response 2
20	DUSP5	3.86	6.46	7.99E-77	dual specificity phosphatase 5
21	NFKBID	3.49	7.11	0.00E+00	NFKB inhibitor delta
22	CSRNP1	3.63	5.45	0.00E+00	cysteine and serine rich nuclear…
23	RNF19A	3.81	4.66	5.27E-190	ring finger protein 19A, RBR E3…
24	SH2D2A	3.68	4.70	0.00E+00	SH2 domain containing 2A
25	IRF4	3.63	7.44	6.08E-68	interferon regulatory factor 4
26	TNFRSF9	3.47	10.82	1.82E-85	TNF receptor superfamily…
27	RGCC	3.59	5.10	5.50E-168	regulator of cell cycle
28	NAB2	3.45	6.39	1.12E-159	NGFI-A binding protein 2
29	CSF1	3.74	4.61	1.65E-130	colony stimulating factor 1
30	SEMA7A	3.46	5.74	2.10E-134	semaphorin 7A (John Milton…
31	EGR1	3.47	8.15	4.11E-72	early growth response 1
32	RRAD	3.33	12.60	3.47E-100	RRAD, Ras related glycolysis…
33	LIF	3.67	5.88	9.76E-56	LIF interleukin 6 family cytokine
33	PHLDA1	4.02	5.29	1.29E-49	pleckstrin homology like domain…
34	BZW1	4.00	3.63	1.20E-205	basic leucine zipper and W2…
35	HSP90AB1	4.21	3.52	2.15E-203	heat shock protein 90 alpha…
36	SDCBP	3.74	3.81	3.50E-259	syndecan binding protein
37	VSIR	3.49	4.57	4.55E-143	V-set immunoregulatory receptor
38	IL5	3.62	7.58	3.57E-44	interleukin 5
39	CHSY1	3.47	4.43	1.18E-194	chondroitin sulfate synthase 1
39	LMNA	3.72	4.00	7.06E-99	lamin A/C

Supplementary Table 23 Alarmin DEGs – unique to alarmin stimulation

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: Log2FC, binary log of fold change vs unstimulated; LogBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol.

#	SYMBOL	logBM	log2FC	*padj*	GENENAME
1	RSAD2	1.74	2.46	4.61E-11	radical S-adenosyl methionine…
2	MAP1A	1.80	1.93	1.37E-03	microtubule associated protein 1A
3	MDFIC	3.29	0.72	1.34E-02	MyoD family inhibitor domain…
4	C3orf18	2.35	0.99	1.38E-04	chromosome 3 open reading frame 18
5	RTN4RL1	1.70	1.81	4.00E-04	reticulon 4 receptor like 1
6	SCN2A	1.22	3.50	1.93E-03	sodium voltage-gated channel…
6=	RNF144A	3.19	0.61	1.02E-02	ring finger protein 144A
7	MIB1	3.33	0.61	1.80E-02	mindbomb E3 ubiquitin protein…
8	SNAP47	2.60	0.72	9.52E-03	synaptosome associated protein 47
8=	ACOX1	2.68	0.61	2.75E-03	acyl-CoA oxidase 1
9	ZAP70	3.46	-0.40	1.29E-02	zeta chain of T cell receptor…
9=	CCNL2	2.97	-0.71	1.65E-02	cyclin L2
10	MCOLN3	1.19	2.10	7.04E-03	mucolipin 3
11	SLC30A4	1.69	0.90	8.84E-03	solute carrier family 30 member 4
12	CMPK2	1.55	1.25	1.23E-02	cytidine/uridine monophosphate…
13	RBM39	3.51	-0.37	2.39E-02	RNA binding motif protein 39
13=	STN 1.00	2.60	0.48	8.84E-03	STN1 subunit of CST complex
14	DDX17	3.90	-0.36	3.68E-02	DEAD-box helicase 17
14=	RHOT2	3.01	-0.45	1.88E-02	ras homolog family member T2
15	TBRG1	2.66	-0.38	1.23E-02	transforming growth factor beta…
15=	RGL2	2.65	-0.62	3.68E-02	ral guanine nucleotide dissociation…
15=	AZIN2	1.79	1.11	2.75E-02	antizyme inhibitor 2
16	AKAP17A	3.33	-0.38	3.68E-02	A-kinase anchoring protein 17A
16=	AR	1.33	2.02	2.75E-02	androgen receptor
17	ANGPTL6	1.36	-1.57	2.51E-02	angiopoietin like 6
18	LOC339192	2.43	-0.85	4.33E-02	uncharacterized LOC339192
19	LUC7L	2.55	-0.62	4.19E-02	LUC7 like
19=	CLK1	3.13	-0.47	4.73E-02	CDC like kinase 1
20	DPYD	2.86	0.51	4.45E-02	dihydropyrimidine dehydrogenase
21	TSPYL2	3.12	-0.34	3.90E-02	TSPY like 2
22	SLC35C1	2.53	0.33	4.40E-02	solute carrier family 35 member C1

Supplementary Table 24 PGD_2 DEGs – unique to PGD_2 stimulation

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: Log2FC, binary log of fold change vs unstimulated; LogBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol.

#	SYMBOL	logBM	log2FC	*padj*	GENENAME
1	TP53INP1	3.01	-3.79	9.00E-59	tumor protein p53 inducible…
2	SIT1	3.39	1.69	9.28E-29	signaling threshold regulating…
3	IL7R	3.69	-1.75	1.07E-13	interleukin 7 receptor
4	LASP1	3.74	1.45	6.25E-56	LIM and SH3 protein 1
5	RFTN1	3.22	1.74	5.56E-33	raftlin, lipid raft linker 1
6	PAG1	3.44	-1.65	7.16E-17	phosphoprotein membrane…
7	MATK	3.34	1.84	8.00E-15	megakaryocyte-associated…
8	RGS3	3.01	2.29	1.15E-37	regulator of G protein signaling 3
9	COTL1	3.90	1.41	9.55E-22	coactosin like F-actin binding…
10	ATG16L1	3.23	1.64	1.07E-26	autophagy related 16 like 1
11	ARHGAP25	3.26	1.51	6.55E-31	Rho GTPase activating protein 25
12	CHST2	2.84	3.25	5.30E-29	carbohydrate sulfotransferase 2
13	CMIP	3.43	1.32	8.99E-38	c-Maf inducing protein
14	GRK6	3.42	1.33	4.06E-31	G protein-coupled receptor kinase 6
15	ERBIN	3.33	1.34	4.72E-35	erbb2 interacting protein
16	HERPUD2	3.15	-1.43	2.37E-38	HERPUD family member 2
17	NFATC3	3.46	1.38	7.63E-13	nuclear factor of activated T cells 3
18	SNTB2	2.87	-2.02	2.52E-59	syntrophin beta 2
19	ARHGDIB	3.96	1.09	4.09E-28	Rho GDP dissociation inhibitor beta
20	PLIN2	3.35	1.65	3.56E-09	perilipin 2
21	SLAMF7	3.37	-1.53	1.26E-09	SLAM family member 7
22	FMNL1	3.64	1.08	8.33E-50	formin like 1
23	SACM1L	3.22	1.27	3.14E-26	SAC1 like phosphatidylinositide…
24	IRS2	2.84	-2.13	5.10E-21	insulin receptor substrate 2
25	TNRC6B	2.99	-1.61	2.18E-20	trinucleotide repeat containing…
26	CNN2	3.53	1.08	9.39E-24	calponin 2
27	ALAS1	3.00	1.58	5.54E-15	5'-aminolevulinate synthase 1
28	SAMD10	2.70	2.51	8.20E-59	sterile alpha motif domain…
29	ADAM19	3.77	-1.24	9.13E-10	ADAM metallopeptidase domain 19
30	NCOR2	3.43	1.09	4.32E-18	nuclear receptor corepressor 2
31	NIBAN1	3.69	1.23	4.68E-10	niban apoptosis regulator 1
32	MOB3A	3.69	1.01	3.23E-21	MOB kinase activator 3A
33	LITAF	3.12	-1.30	2.92E-19	lipopolysaccharide induced…
34	PIM2	2.92	-1.85	1.33E-12	Pim-2 proto-oncogene…
35	ARHGEF7	3.15	1.25	3.06E-19	Rho guanine nucleotide exchange…
36	ST3GAL2	2.79	1.86	1.97E-58	ST3 beta-galactoside alpha-2,3-…
37	BCL6	2.93	1.76	1.69E-13	BCL6 transcription repressor
38	TBL1XR1	3.19	-1.12	9.35E-22	TBL1X receptor 1
39	HBP1	2.99	-1.42	8.95E-19	HMG-box transcription factor 1
40	AHNAK	4.38	-1.14	2.10E-09	AHNAK nucleoprotein

Supplementary Table 25 TCR DEGs – unique to TCR stimulation

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: Log2FC, binary log of fold change vs unstimulated; LogBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol.

#	SYMBOL	logBM	log2FC	*padj*	GENENAME
1	CCL4	4.18	9.34	1.13E-79	C-C motif chemokine ligand 4
2	TNFSF8	3.60	4.57	1.81E-34	TNF superfamily member 8
3	IL2RA	3.78	3.47	1.72E-58	interleukin 2 receptor subunit alpha
4	FRMD4B	3.47	3.73	1.21E-98	FERM domain containing 4B
5	PRNP	4.07	3.23	8.69E-50	prion protein
6	RAB11FIP1	3.45	3.33	3.02E-82	RAB11 family interacting protein 1
7	ARL5B	3.18	3.71	1.28E-81	ADP ribosylation factor like…
8	SIAH2	3.28	3.26	5.69E-185	siah E3 ubiquitin protein ligase 2
9	RDH10	3.67	3.90	1.50E-17	retinol dehydrogenase 10
10	VDR	3.16	3.60	6.40E-57	vitamin D receptor
11	NIPA1	3.28	3.08	1.55E-141	NIPA magnesium transporter 1
12	PGGHG	3.56	3.34	2.43E-22	protein-lucosylgalactosylhydroxyl…
13	CCL4L2	3.52	11.20	5.18E-11	C-C motif chemokine ligand 4 like 2
13	CCL4L1	3.52	11.20	5.18E-11	C-C motif chemokine ligand 4 like 1
14	FAM107B	3.71	2.58	4.82E-69	family with sequence similarity 107…
15	TXNIP	3.95	-2.53	7.43E-49	thioredoxin interacting protein
16	EDARADD	2.84	6.40	2.89E-106	EDAR associated death domain
17	ICOS	3.23	3.02	1.68E-87	inducible T cell costimulatory
18	SLA	3.32	3.33	4.92E-20	Src like adaptor
19	PER1	3.10	3.02	3.42E-127	period circadian regulator 1
20	KLF9	2.97	3.48	1.09E-91	Kruppel like factor 9
21	ANXA1	3.83	2.14	2.01E-89	annexin A1
22	TNFSF9	2.80	5.99	4.09E-37	TNF superfamily member 9
23	ARHGEF3	3.34	2.48	9.13E-52	Rho guanine nucleotide exchange…
24	PGAM1	3.57	2.24	6.20E-56	phosphoglycerate mutase 1
25	ARID5B	3.42	2.40	6.14E-40	AT-rich interaction domain 5B
26	BACH1	3.11	2.70	1.63E-99	BTB domain and CNC homolog 1
26	GRIK1-AS2	3.11	2.70	1.63E-99	GRIK1 antisense RNA 2
27	IL4R	3.55	2.17	1.17E-49	interleukin 4 receptor
28	YWHAQ	3.49	2.16	6.13E-62	tyrosine 3-monooxygenase…
29	ADAM19	3.77	2.18	9.78E-29	ADAM metallopeptidase domain 19
30	TFRC	3.44	2.57	2.33E-21	transferrin receptor
31	HIVEP2	3.06	3.54	5.46E-18	HIVEP zinc finger 2
32	DENND4A	3.21	2.35	1.87E-57	DENN domain containing 4A
33	CD109	3.01	3.08	6.58E-33	CD109 molecule
34	KLF6	3.82	2.15	2.29E-25	Kruppel like factor 6
35	EED	3.00	2.79	4.61E-105	embryonic ectoderm development
36	SOCS3	2.67	7.37	8.40E-42	suppressor of cytokine signaling 3
37	NFAT5	3.38	2.31	2.27E-27	nuclear factor of activated T cells 5
38	GK	2.99	3.06	2.06E-32	glycerol kinase
39	ZNFX1	3.55	1.98	2.63E-50	zinc finger NFX1-type containing 1

Supplementary Table 26 Alarmin DEGs - shared with PGD_2 but with a greater change in the alarmin condition relative to the fitted curve

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: e, residual distance (vertical) from the curve of best fit (Figure 47A); log2FC, binary log of fold change vs unstimulated; logBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol. A positive 'e' indicates greater increase than expected.

#	SYMBOL	logBM	log2FC	e	*padj*	GENENAME
1	CISH	3.40	3.34	1.96	9.20E-20	cytokine inducible…
2	OSM	2.79	3.45	1.52	7.71E-10	oncostatin M
3	EGR2	3.68	2.83	0.68	6.90E-03	early growth…
4	SOCS1	3.05	1.95	0.63	4.63E-11	suppressor of…
4=	CCL3	3.11	2.55	0.65	9.55E-08	C-C motif…
5	EGR1	3.47	2.64	0.02	5.51E-06	early growth…
5=	CDKN2B	1.86	2.41	0.81	5.51E-06	cyclin dependent…
6	ENSG00000235880	0.82	4.24	1.96	2.10E-02	NA
6=	RSRP1	2.68	-0.73	-0.54	2.10E-02	arginine and serine…
7	ZNF397	2.32	-0.58	-0.42	1.30E-04	zinc finger protein 397
8	ZDHHC23	1.94	2.04	0.34	1.80E-09	zinc finger DHHC-…
9	NPHP3	1.91	-0.78	-0.63	1.03E-02	nephrocystin 3
10	ENSG00000283689	0.95	2.72	0.79	2.77E-02	NA
11	TRIM38	2.87	-0.37	-0.29	3.68E-02	tripartite motif…
11	ADCK5	1.96	-0.72	-0.47	2.73E-02	aarF domain…
12	GOLGA1	2.39	-0.37	-0.11	2.39E-02	golgin A1
13	CENPT	2.20	-0.58	-0.18	3.60E-02	centromere protein T

Supplementary Table 27 Alarmin DEGs - shared with TCR but with a greater change in the alarmin condition relative to the fitted curve

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: e, residual distance (vertical) from the curve of best fit (Figure 47B); log2FC, binary log of fold change vs unstimulated; logBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol. A positive 'e' indicates greater increase than expected.

#	SYMBOL	logBM	log2FC	e	*padj*	GENENAME
1	CISH	3.40	3.34	0.98	9.20E-20	cytokine inducible…
2	BCL2	2.98	2.03	0.97	4.50E-24	BCL2 apoptosis…
3	OSM	2.79	3.45	0.89	7.71E-10	oncostatin M
4	ZDHHC23	1.94	2.04	1.36	1.80E-09	zinc finger DHHC-…
5	EGR2	3.68	2.83	0.44	6.90E-03	early growth…
6	IRX3	2.78	2.67	0.57	1.13E-04	iroquois homeobox..
7	CDKN2B	1.86	2.41	1.12	5.51E-06	cyclin dependent…
8	KLF5	2.15	1.73	0.30	8.91E-07	Kruppel like factor 5
8=	CDK6	3.18	0.97	0.28	1.08E-02	cyclin dependent…
8=	IRX5	1.64	2.03	1.11	1.17E-04	iroquois homeobox..
9	UBXN11	2.39	-0.73	-0.58	2.01E-02	UBX domain…
10	HTATSF1	3.06	0.52	0.10	5.15E-03	HIV-1 Tat specific…
10=	UBTD2	2.46	1.16	0.12	2.06E-06	ubiquitin domain…
10=	COL5A2	1.39	2.39	1.12	1.08E-02	collagen type V…
11	TRIM38	2.87	-0.37	-0.32	3.68E-02	tripartite motif…
11=	IRAK4	2.89	-0.27	-0.14	1.95E-02	interleukin 1…
12	PMCH	1.08	2.80	0.85	1.86E-03	pro-melanin…
12=	ENSG00000235880	0.82	4.24	1.63	2.10E-02	NA
12=	NPHP3	1.91	-0.78	-0.50	1.03E-02	nephrocystin 3
13	ZNF397	2.32	-0.58	-0.12	1.30E-04	zinc finger protein…
14	ENSG00000283689	0.95	2.72	1.14	2.77E-02	NA
15	CENPT	2.20	-0.58	-0.44	3.60E-02	centromere protein..
16	SFI1	2.77	-0.42	-0.26	4.58E-02	SFI1 centrin…
17	JMJD4	1.99	0.76	0.29	3.68E-02	jumonji domain…
18	ADCK5	1.96	-0.72	-0.19	2.73E-02	aarF domain…
19	PP2D1	1.21	1.42	0.17	4.39E-02	Protein…
20	ZNF449	1.86	-0.65	-0.02	3.68E-02	zinc finger protein…

Supplementary Table 28 PGD_2 DEGs - shared with alarmins but with a greater change in the PGD_2 condition relative to the fitted curve

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: e, residual distance (vertical) from the curve of best fit (Figure 47A); log2FC, binary log of fold change vs unstimulated; logBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol. A negative 'e' indicates greater increase than expected.

#	SYMBOL	logBM	log2FC	e	*padj*	GENENAME
1	TNF	3.22	2.63	-0.71	6.04E-19	tumor necrosis factor
2	JUND	3.87	2.55	-1.57	4.61E-73	JunD proto-…
3	SH2B3	3.59	1.89	-1.33	3.09E-64	SH2B adaptor…
4	EGR3	4.09	6.01	-0.60	1.89E-34	early growth…
5	B4GALT5	3.07	1.09	-0.42	2.38E-07	beta-1,4-galacto…
6	IL13	4.26	5.07	-0.09	1.38E-17	interleukin 13
7	BYSL	2.44	1.57	-0.53	3.52E-13	bystin like
7=	ARHGDIA	3.46	0.68	-0.68	1.92E-23	Rho GDP…
8	MAPKAPK3	3.06	0.70	-0.45	7.60E-07	MAPK activated…
8=	ETV3	3.00	0.71	-0.31	4.39E-05	ETS variant…
8=	EAF1	2.68	0.46	-0.21	1.18E-03	ELL associated…
9	LOC91370	1.78	1.95	-0.32	2.25E-07	Uncharacterized…
9=	ZNF449	1.86	-1.84	1.13	1.43E-19	zinc finger protein 449
9=	SFI1	2.77	-1.14	0.48	2.85E-20	SFI1 centrin binding…
10	FOS	2.65	6.11	-0.39	6.23E-25	Fos proto-oncogene...
11	RXRB	2.89	-0.60	0.03	3.44E-16	retinoid X receptor…
12	ENSG00000259623	2.74	0.42	-0.10	1.93E-02	NA
13	TNFRSF8	2.57	5.86	-0.44	2.39E-21	TNF receptor…
14	ATP7A	2.84	0.64	-0.14	6.35E-03	ATPase copper…
15	HSD17B7	2.32	-0.85	0.09	1.64E-10	hydroxysteroid 17…
16	TEX2	2.43	0.42	-0.03	3.49E-02	testis expressed 2
17	PTGIS	1.90	1.20	-0.11	1.19E-03	prostaglandin I2…

Supplementary Table 29 PGD_2 DEGs - shared with TCR but with a greater change in the PGD_2 condition relative to the fitted curve.

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: e, residual distance (vertical) from the curve of best fit (Figure 48); log2FC, binary log of fold change vs unstimulated; logBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol. A positive 'e' indicates greater increase than expected.

#	SYMBOL	logBM	log2FC	e	*padj*	GENENAME
1	IL27RA	3.66	2.91	1.59	2.42E-127	interleukin 27 receptor…
2	LCP1	4.45	2.45	1.38	1.61E-119	lymphocyte cytosolic…
3	ACTG1	4.65	2.50	1.32	3.46E-99	actin gamma 1
4	RRAD	3.33	9.62	3.31	3.42E-58	RRAD, Ras related…
5	ACTB	4.89	2.13	1.15	2.36E-97	actin beta
6	NSMF	3.40	2.39	1.51	7.52E-80	NMDA receptor…
7	GPAT3	3.54	2.98	1.32	4.47E-83	glycerol-3-phosphate…
8	PTPN7	3.95	2.90	1.05	2.11E-142	protein tyrosine…
9	EML4	3.53	2.08	1.41	1.23E-62	EMAP like 4
10	TLE3	3.23	3.19	1.66	5.82E-121	TLE family member 3…
11	TPM4	3.96	2.57	1.09	2.11E-90	tropomyosin 4
12	ACTR3	3.83	2.18	1.05	1.21E-117	actin related protein 3
13	RXRA	3.14	3.16	2.23	6.30E-106	retinoid X receptor alpha
14	RCC2	3.38	2.23	1.24	1.33E-153	regulator of…
15	EFHD2	3.73	2.05	1.16	1.57E-70	EF-hand domain…
16	RASSF2	3.50	2.05	1.24	2.09E-78	Ras association…
17	FASN	3.33	2.43	1.28	1.15E-109	fatty acid synthase
18	NAB2	3.45	5.01	1.15	1.83E-97	NGFI-A binding protein 2
19	SLC16A3	3.36	3.23	1.21	9.79E-79	solute carrier family…
20	NDFIP2	3.42	3.58	1.59	1.26E-39	Nedd4 family…
21	ZYX	3.63	2.24	1.02	5.17E-66	Zyxin
22	WDR1	3.96	1.88	0.84	3.10E-123	WD repeat domain 1
23	TXNRD1	3.30	1.70	1.13	5.65E-59	thioredoxin reductase 1
24	TNFRSF18	3.15	3.80	1.38	1.49E-56	TNF receptor…
25	TNFRSF9	3.47	6.99	1.27	1.15E-35	TNF receptor…
26	SYAP1	3.10	2.83	1.24	2.74E-98	synapse associated…
27	HMCES	3.24	1.88	0.93	7.76E-104	5-hydroxymethyl…
28	EGR1	3.47	5.81	1.14	1.40E-36	early growth response 1
29	M6PR	3.44	1.73	0.99	2.58E-43	mannose-6-phosphate…
30	MYO1E	2.95	3.73	1.94	2.03E-61	myosin IE
31	SOS1	3.82	-1.73	-0.96	1.22E-35	SOS Ras/Rac guanine…
32	MFSD6	3.32	1.63	0.91	5.39E-63	major facilitator…
33	GLS	3.52	2.21	0.72	1.16E-173	Glutaminase
34	EGR2	3.68	7.32	1.21	1.56E-28	early growth response 2
35	ERN1	3.90	-2.23	-1.19	4.43E-27	endoplasmic reticulum…
36	SESN3	2.96	-2.98	-2.10	1.50E-45	sestrin 3
37	ARPC2	3.90	1.58	0.67	1.07E-82	actin related protein…
38	PLXNA1	3.22	1.86	1.17	3.48E-41	plexin A1
39	EHBP1L1	3.14	1.88	1.16	3.05E-50	EH domain binding…

Supplementary Table 30 TCR DEGs - shared with alarmins but with a greater change in the TCR condition relative to the fitted curve

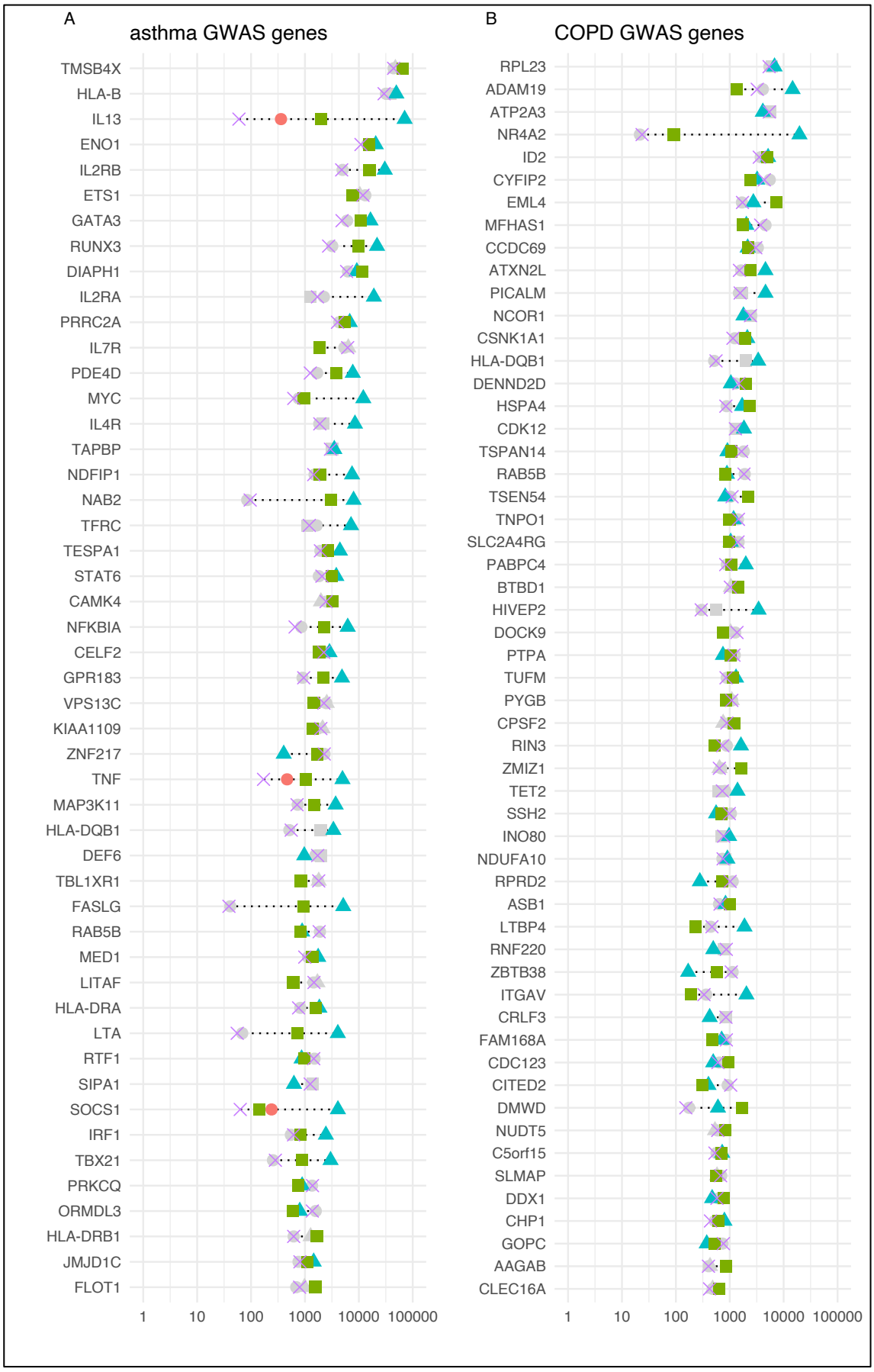
The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: e, residual distance (vertical) from the curve of best fit (Figure 47B); log2FC, binary log of fold change vs unstimulated; logBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol. A negative 'e' indicates greater increase than expected.

#	SYMBOL	logBM	Log2FC	e	*padj*	GENENAME
1	SH2B3	3.59	3.73	-1.50	4.27E-254	SH2B adaptor…
2	JUND	3.87	3.39	-1.26	1.97E-129	JunD proto-…
3	EGR3	4.09	12.32	-0.32	1.58E-144	early growth…
4	TNF	3.22	4.87	-0.84	3.12E-64	tumor necrosis factor
5	ENSG00000259623	2.74	3.35	-1.22	3.54E-109	NA
6	SOCS1	3.05	6.04	-0.61	2.72E-126	suppressor of…
6	CCL3	3.11	10.19	-0.15	1.10E-163	C-C motif…
7	EGR1	3.47	8.15	-0.14	4.11E-72	early growth…
8	ETV3	3.00	2.34	-0.77	4.56E-47	ETS variant…
9	B4GALT5	3.07	2.49	-0.65	1.44E-35	beta-1,4-galaco…
9	IL13	4.26	10.23	-0.09	8.11E-70	interleukin 13
10	ARHGDIA	3.46	0.84	-0.41	1.46E-35	Rho GDP…
11	BYSL	2.44	3.42	-0.78	4.86E-62	bystin like
12	MMD	2.44	2.52	-0.63	2.49E-40	monocyte to…
13	PTGIS	1.90	3.68	-0.71	5.19E-29	prostaglandin I2…
14	FOS	2.65	6.41	-0.44	1.03E-27	Fos proto-…
15	PIM1	3.27	1.91	-0.11	9.15E-12	Pim-1 proto-…
15	HSD17B7	2.32	-1.69	0.48	2.33E-32	hydroxysteroid 17…
16	SOCS2	2.69	4.64	-0.05	5.21E-34	suppressor of…
16	LOC91370	1.78	3.82	-0.48	1.88E-27	Uncharacterized…
17	MAPKAPK3	3.06	0.69	-0.08	9.68E-07	MAPK activated…
17	EAF1	2.68	0.85	-0.14	2.32E-10	ELL associated…
17	RXRB	2.89	-0.74	0.04	1.96E-23	retinoid X receptor…
18	TNFRSF8	2.57	4.88	-0.11	2.67E-15	TNF receptor…
19	GOLGA1	2.39	-1.03	0.12	1.17E-20	golgin A1

Supplementary Table 31 TCR DEGs - shared with PGD_2 but with a greater change in the TCR condition relative to the fitted curve

The '#' column indicates gene rank as described in 4.2.3.5.3. Abbreviations: e, residual distance (vertical) from the curve of best fit (Figure 48); log2FC, binary log of fold change vs unstimulated; logBM, log of mean normalised base count across experiment; *padj,* adjusted p-value; SYMBOL, HGNC gene symbol. A negative 'e' indicates greater increase than expected.

#	SYMBOL	logBM	Log2FC	e	*padj*	GENENAME
1	NR4A1	3.86	12.47	-1.84	0.00E+00	nuclear receptor…
2	KDM6B	4.05	6.09	-1.56	0.00E+00	lysine demethylase 6B
3	TNFSF14	3.85	5.13	-1.81	1.06E-219	TNF superfamily member 14
4	NR4A2	3.69	9.75	-3.34	0.00E+00	nuclear receptor subfamily…
5	SDC4	3.93	7.82	-1.52	1.38E-275	syndecan 4
6	SDCBP	3.74	3.81	-1.97	3.50E-259	syndecan binding protein
7	RNF19A	3.81	4.66	-1.30	5.27E-190	ring finger protein 19A…
8	PIM3	3.95	5.48	-1.11	3.75E-238	Pim-3 proto-oncogene…
9	NFKBID	3.49	7.11	-2.54	0.00E+00	NFKB inhibitor delta
10	GPR171	3.93	3.18	-1.68	1.42E-86	G protein-coupled receptor…
11	VSIR	3.49	4.57	-2.50	4.55E-143	V-set immunoregulatory…
12	CHSY1	3.47	4.43	-2.26	1.18E-194	chondroitin sulfate synthase 1
13	OTUD4	3.56	3.59	-1.40	1.95E-162	OTU deubiquitinase 4
14	EVI2A	3.54	4.30	-1.78	9.63E-121	ecotropic viral integration…
14	DUSP5	3.86	6.46	-1.65	7.99E-77	dual specificity…
15	NR4A3	4.40	13.35	-0.91	0.00E+00	nuclear receptor subfamily…
16	NCOA2	3.69	3.34	-1.42	2.59E-103	nuclear receptor coactivator 2
17	SEMA7A	3.46	5.74	-1.76	2.10E-134	semaphorin 7A (John Milton…
18	PFKFB3	3.40	4.63	-1.63	0.00E+00	6-phosphofructo-2-…
19	ODC1	3.61	4.13	-1.36	2.34E-101	ornithine decarboxylase 1
20	RILPL2	3.32	4.52	-2.35	3.85E-250	Rab interacting lysosomal…
21	CSF2	3.70	7.50	-0.90	1.81E-204	colony stimulating factor 2
22	RGCC	3.59	5.10	-0.98	5.50E-168	regulator of cell cycle
23	MAPK6	3.42	3.62	-1.40	2.45E-115	mitogen-activated protein…
24	MYC	3.56	4.29	-2.15	1.54E-63	MYC proto-oncogene…
25	MAP3K8	3.24	5.52	-2.07	5.15E-302	mitogen-activated protein…
25	GADD45B	3.32	5.10	-1.81	1.03E-124	growth arrest and DNA…
26	PHF6	3.24	3.67	-2.07	0.00E+00	PHD finger protein 6
27	ARHGEF2	3.41	2.35	-1.11	7.86E-158	Rho/Rac guanine…
28	TATDN2	3.31	2.76	-1.40	2.63E-192	TatD DNase domain…
29	CSF1	3.74	4.61	-0.86	1.65E-130	colony stimulating factor 1
30	JOSD1	3.49	2.79	-1.04	2.17E-111	Josephin domain containing 1
31	SLFN5	3.90	-2.90	1.40	5.11E-52	schlafen family member 5
32	GIMAP6	3.52	-3.99	1.17	2.10E-81	GTPase, IMAP family…
33	RNF145	3.52	2.59	-1.14	1.20E-83	ring finger protein 145
34	REL	3.42	4.73	-1.36	1.55E-91	REL proto-oncogene…
35	NDFIP1	3.49	2.36	-1.27	6.71E-75	Nedd4 family interacting…
36	MIDN	3.31	3.51	-1.18	4.44E-221	Midnolin
37	SACS	3.29	3.35	-1.63	1.21E-118	sacsin molecular chaperone


A
asthma GWAS genes
TMSB4X
HLA-B
IL13
ENO1
IL2RB
ETS1
GATA3
RUNX3
DIAPH1
IL2RA
PRRC2A
IL7R
PDE4D
MYC
IL4R
TAPBP
NDFIP1
NAB2
TFRC
TESPA1
STAT6
CAMK4
NFKBIA
CELF2
GPR183
VPS13C
KIAA1109
ZNF217
TNF
MAP3K11
HLA-DQB1
DEF6
TBL1XR1
FASLG
RAB5B
MED1
LITAF
HLA-DRA
LTA
RTF1
SIPA1
SOCS1
IRF1
TBX21
PRKCQ
ORMDL3
HLA-DRB1
JMJD1C
FLOT1
1
10
100
1000
10000
100000
B
COPD GWAS genes
RPL23
ADAM19
ATP2A3
NR4A2
ID2
CYFIP2
EML4
MFHAS1
CCDC69
ATXN2L
PICALM
NCOR1
CSNK1A1
HLA-DQB1
DENND2D
HSPA4
CDK12
TSPAN14
RAB5B
TSEN54
TNPO1
SLC2A4RG
PABPC4
BTBD1
HIVEP2
DOCK9
PTPA
TUFM
PYGB
CPSF2
RIN3
ZMIZ1
TET2
SSH2
INO80
NDUFA10
RPRD2
ASB1
LTBP4
RNF220
ZBTB38
ITGAV
CRLF3
FAM168A
CDC123
CITED2
DMWD
NUDT5
C5orf15
SLMAP
DDX1
CHP1
GOPC
AAGAB
CLEC16A
1
10
100
1000
10000
100000

Supplementary Figure 39 Expression of genes associated with asthma or COPD

The x-axis represents absolute normalised read count. The y-axis represents individual genes, ordered by their average absolute expression across the whole experiment (not all genes are shown in this figure – see Supplementary Table 32 for the full list). In this case the genes were identified in recent GWAS studies as being related to asthma or COPD (see 4.2.3.5.4). Each gene is represented by four points, one point per condition, linked by a dashed horizontal line, Each point represents the mean absolute read count for the replicates (n = 5 donors) within that condition. Points are coloured if *padj* < 0.05 compared with the unstimulated control (US), or greyed out if *padj* > 0.05. The greyed out points are therefore generally close to the unstimulated control values.

Supplementary Table 32 list of genes associated with asthma or COPD

List of genes copied from recent large GWAS studies of asthma (Demenais et al., 2018; El-Husseini et al., 2020) and COPD (Sakornsakolpat et al., 2019; Shrine et al., 2019)

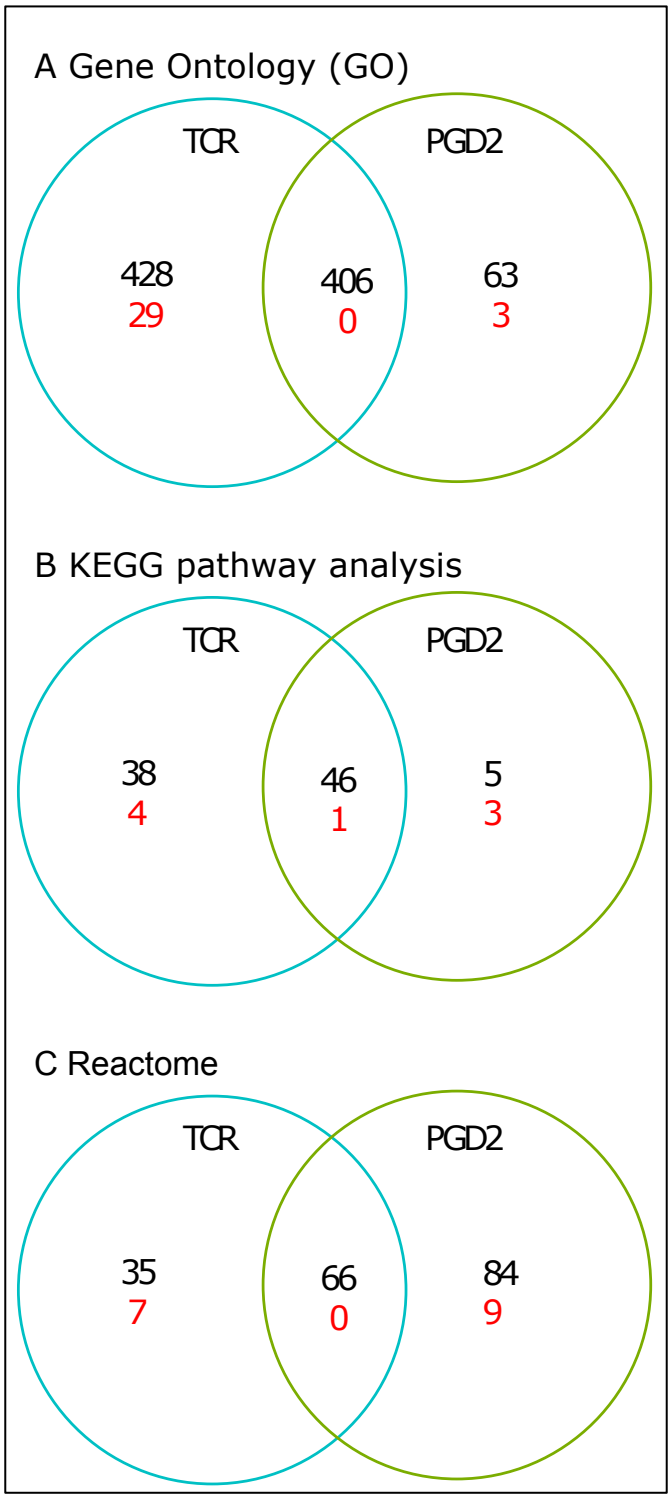
Asthma-associated genes					
AAGAB	EMSY	IKZF4	MAP3K11	PRKCQ	STARD6
ABCB5	ENDOG	IL13	MAP3K7	PRR5L	STAT6
AC007278.3	ENO1	IL18R1	MCCD1	PRRC2A	STMN3
AC116366.6	EP300	IL18RAP	MED1	PSMB9	SUOX
ACO2	EQTN	IL1RL1	MEI1	PSMD3	TAP2
ADAMTS4	ERBB2	IL1RL2	MFSD9	PTGES	TAPBP
ADORA1	ERBB3	IL2	MICA	PTPRK	TBKBP1
AGPAT1	ERMP1	IL21	MICB	PYHIN1	TBL1XR1
AIF1	ETS1	IL2RA	MIR5708	RAB5B	TBX21
AL022393.7	F11R	IL2RB	MOB3B	RAD50	TCP11
AP5B1	FADS1	IL33	MUC16	RAD51B	TDRKH
APITD1	FADS2	IL4	MUC2	RANBP6	TEF
APOA2	FAM105A	IL4R	MUC5B	RERE	TESPA1
ARFRP1	FAM9C	IL6R	MYBPH	RMI2	TFRC
B4GALT3	FASLG	IL7R	MYC	RORA	THEM4
BACH2	FBXO45	ILDR1	MYL6B	RORC	TLR1
BCL6	FCER1G	INO80	MYRF	RP11-132N15.1	TLR6
BCLAF1P1	FLG	INSM2	NAB2	RP11-132N15.2	TMEM258
C12orf43	FLOT1	IQCB1	NARG2	RP11-554D20.1	TMEM99
C4A	GATA3	IRF1	NCR3	RP11-770G2.5	TMSB4X
C5orf56	GJA10	ITGB8	NDFIP1	RP11-94L15.2	TNF
C9orf114	GLB1	ITPKA	NDUFS2	RPL21P124	TNFSF18
CAMK4	GNDPA1	ITPR3	NFKBIA	RPS26	TNFSF4
CAPSL	GNGT2	JAZF1	NOD2	RPTN	TOB2
CCR7	GPR183	JMJD1C	NOTCH4	RTEL1	TOMM40L
CD247	GPXS	KIAA1109	NUSAP1	RTF1	TPD52
CDHR3	GRM4	KIF1B	OASL	RUNX1	TRAM2
CEBPA	GSDMB	KIRREL3-AS3	OLIG3	RUNX3	TRIM27
CELF2	HCG23	KRT18P9	OR1M1	SCUBE3	TRK-TTT7-1
CEP19	HCP5	KRT24	ORMDL3	SDHAP1	TRR-CCG1-2

CHI3L1	HDAC3	LAYN	OVOL1	SEPT8	TSHZ2
CHIT1	HLA-B	LIME1	PA2G4	SIK2	TSLP
CLEC16A	HLA-C	LINC00299	PAQR8	SIPA1	UBAC2
COQ10A	HLA-DPA1	LINGO4	PDCD1	SLC22A4	UBE2E2
CRB1	HLA-DPB1	LITAF	PDE4D	SLC22A5	USF1
CSDC2	HLA-DQA1	LOC100130476	PEX14	SLC25A46	VPS13C
CTC-551A13.2	HLA-DQA2	LOC100287014	PGAP3	SLC30A8	WDR36
CXCR5	HLA-DQB1	LOC102724596	PHB	SLC44A4	WNT11
CYP21A1P	HLA-DQB2	LOC105376879	PHF5A	SLC7A10	ZBT10
D2HGDH	HLA-DRA	LOC105376993	PHOSPHO1	SMAD3	ZBTB10
DDX6	HLA-DRB1	LOC105377623	PMM1	SMAD6	ZBTB38
DEF6	HLA-DRB5	LPP	POLI	SMARCE1	ZFYVE19
DEXI	HLA-DRB6	LPP-AS2	POLR3H	SNX32	ZNF217
DFFA	HNF1A-AS1	LRP1	POU5F1B	SOCS1	ZNF365
DIAPH1	HRNR	LRRC32	PPFIA4	SPPL3	ZNF652
DNAJC17	HSD17B8	LRRC8A	PPOX	SPRY4	ZPBP2
EFEMP2	IER3	LST1	PPP1R3F	SPSB1	ZSCAN12
EFHC1	IKZF3	LTA	PPP2R1B	STARD3	ZSCAN23
					ZSCAN31

COPD-associated genes					
AAGAB	CDC123	FAM168A	LRP1	PTPA	SPATS2L
ABCB5	CDK12	FBXL20	LRRC37A4P	PYGB	SPPL2C
ABHD12	CDK2	FGD6	LRRC45	QSOX2	SSH2
ABLIM3	CDKAL1	FGF18	LTBP4	RAB5B	STN1
ADAM19	CENPW	FILIP1L	LTK	RAD51	SULT1A1
ADAMTSL3	CEP72	GDF5	LY86	RALGPS2	SULT1A2
ADCY5	CEPT1	GLIS3	LYPLAL1	RARB	SYN2
ADGRG6	CFDP1	GOPC	MAB21L4	RASEF	SYN3
ADORA2B	CHAC1	GSTCD	MAPT	RBMS3	TBX3
ADRB2	CHI3L2	GYPA	MAPT-AS1	RCAN1	TEAD1
AGER	CHP1	HDGFL1	MECOM	RFX3	TEKT5
AIMP1	CHRM3	HHIP	MED13L	RFX6	TEPP
AMZ1	CHRNA3	HIVEP2	MET	RHOBTB3	TESK2
ANGPTL1	CHRNB4	HLA-DQB1	MFAP2	RIN3	TET2
AP3B1	CITED2	HMGN2	MFHAS1	RMC1	TGFB2
ARHGEF17	CLEC16A	HSPA4	MICA	RNF220	THBS4
ARMC2	CLN3	HTR4	MICAL3	RNU6-71P	THRA
ARNTL	COL15A1	ID2	MIR548G	ROM1	THSD4
ASAP2	CPSF2	ID4	MIR588	RPAP1	TIGD2
ASB1	CRAT	IER3	MMP15	RPL23	TNFSF12
ASPSCR1	CRLF3	IER5L	MRPS21	RPRD2	TNFSF13
ASTN2	CSNK1A1	IL27	MTCL1	RREB1	TNPO1
ATAD2B	CYFIP2	INO80	MYPN	RRP15	TNS1

ATP2A3	CYP2J2	INPP5E	NCOR1	RSRC1	TOP2B
ATP5PO	DDX1	INTS12	NDUFA10	SBK1	TRAF3IP1
ATXN2L	DENND2D	IQCH	NEXN	SCARF2	TRPM7
BCHE	DHDDS	ITGA1	NPIP87	SENP3	TSEN54
BMP6	DMWD	ITGAV	NPNT	SERP2	TSPAN14
BTBD1	DNLZ	ITGB8	NR4A2	SFTPD	TTC19
BTC	DOCK9	ITPK1	NUDT5	SGF29	TUFM
C1GALT1	DRAM2	ITPKA	P4HA2	SH2B1	TWIST2
C1orf54	DSP	JAZF1	P4HA2-AS1	SH2D7	TXLNB
C1orf87	DST	KCNE2	PABPC4	SHISA4	TXNDC17
C1QTNF5	DTWD1	KIAA0753	PAPPA	SLC22A5	TYRO3
C5orf15	ECM1	KRTCAP2	PARVA	SLC2A4RG	UBXN2A
C5orf56	EEF1G	LIME1	PICALM	SLC30A10	UQCC1
CACNA2D3	EEFSEC	LINGO1	PID1	SLMAP	VGLL4
CARD9	EFCAB5	LMOD1	PITPNM3	SMAD3	VTA1
CASKIN2	ELAVL2	LOC100996325	PKDCC	SNAPC4	ZBTB38
CASTOR3	EML3	LOC101929516	PPP1R3B	SNCA	ZFP14
CCDC69	EML4	LRMDA	PRL	SNRPF	ZFP82
CCDC91	FAM13A	LRP	PRSS23	SOX9	ZKSCAN1
				SPATA9	ZMIZ1

4.6.3 GSEA analyses



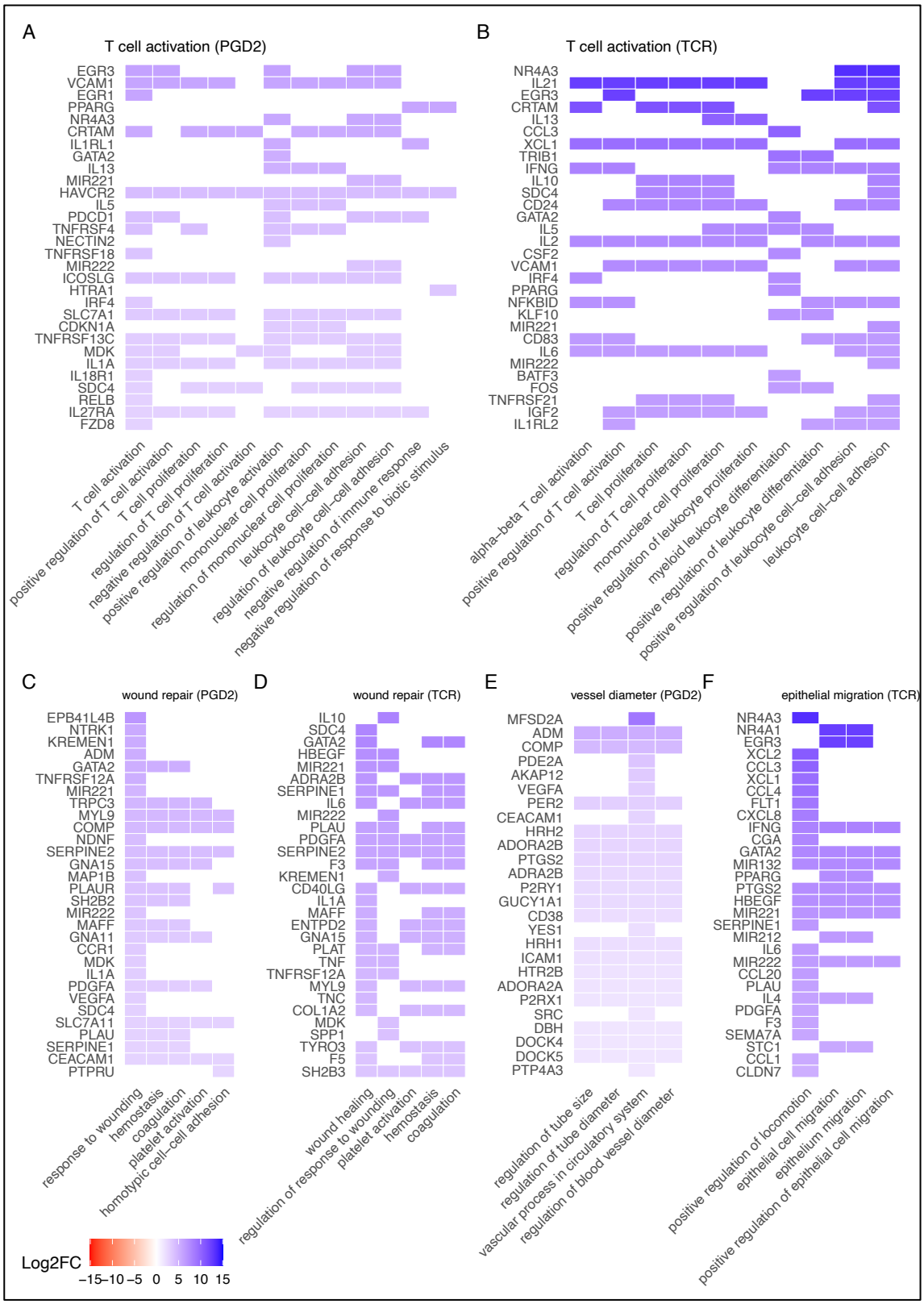
Supplementary Figure 40 Venn diagrams for GSEA analysis

Black writing indicates upregulated genes sets, red writing downregulated gene sets.

Supplementary Table 33 GO Enrichment analysis – top results##

Abbreviations: NES, normalised enrichment score; *padj,* adjusted p-value. Only the 15 most enriched sets for each condition are shown. Full results available at ORA (Hoyle & Xue, 2021).

PGD_2			
ID	**Description**	**NES**	***padj***
GO:0014015	positive regulation of gliogenesis	2.25	1.88E-05
GO:0042267	natural killer cell mediated cytotoxicity	2.11	4.06E-04
GO:1903037	regulation of leukocyte cell-cell adhesion	2.06	4.14E-08
GO:0002228	natural killer cell mediated immunity	2.04	6.12E-04
GO:0002696	positive regulation of leukocyte activation	2.03	7.49E-08
GO:1903039	positive regulation of leukocyte cell-cell adhesion	2.02	1.47E-06
GO:0050863	regulation of T cell activation	2.01	1.14E-07
GO:0070373	negative regulation of ERK1 and ERK2 cascade	2.00	1.17E-03
GO:0050870	positive regulation of T cell activation	2.00	1.06E-05
GO:0001936	regulation of endothelial cell proliferation	1.98	4.06E-04
GO:0002573	myeloid leukocyte differentiation	1.98	4.60E-05
GO:0070372	regulation of ERK1 and ERK2 cascade	1.98	1.71E-05
GO:0050867	positive regulation of cell activation	1.97	1.24E-07
GO:0022407	regulation of cell-cell adhesion	1.97	1.24E-07
GO:0022408	negative regulation of cell-cell adhesion	1.96	7.40E-05
TCR			
ID	**Description**	**NES**	***padj***
GO:1904894	positive regulation of receptor signaling pathway via…	2.47	5.80E-13
GO:0046427	positive regulation of receptor signaling pathway via…	2.47	1.58E-12
GO:1904892	regulation of receptor signaling pathway via STAT	2.42	1.30E-13
GO:0046425	regulation of receptor signaling pathway via JAK-STAT	2.42	1.25E-13
GO:0007260	tyrosine phosphorylation of STAT protein	2.41	2.00E-11
GO:0042509	regulation of tyrosine phosphorylation of STAT protein	2.39	4.90E-11
GO:0042531	positive regulation of tyrosine phosphorylation of…	2.36	6.28E-10
GO:1903039	positive regulation of leukocyte cell-cell adhesion	2.34	6.06E-18
GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	2.33	4.12E-14
GO:1903037	regulation of leukocyte cell-cell adhesion	2.33	1.84E-21
GO:0050870	positive regulation of T cell activation	2.33	2.34E-16
GO:0007259	receptor signaling pathway via JAK-STAT	2.32	8.35E-12
GO:0097696	receptor signaling pathway via STAT	2.31	4.42E-12
GO:0022409	positive regulation of cell-cell adhesion	2.30	6.06E-18
GO:0042129	regulation of T cell proliferation	2.30	2.17E-13



Supplementary Figure 41 Heatmaps of top core genes – GO (BP) terms

On the x-axis are gene sets within the module. On the y-axis are the top core genes across all sets in the module, listed by fold-change in expression.

Supplementary Table 34 KEGG pathway enrichment analysis – top results

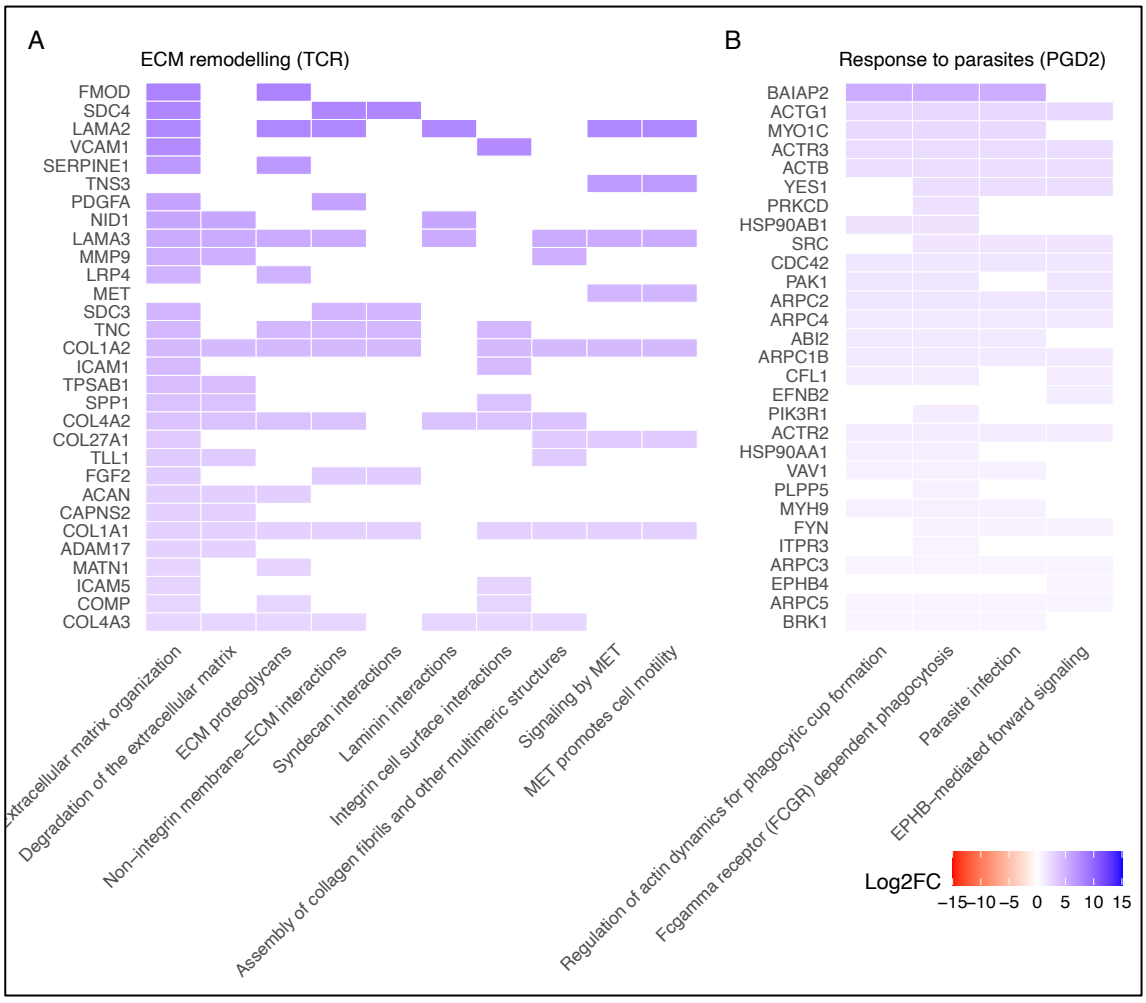
NES = normalised enrichment score. '*padj*' = adjusted p-value. Only the 15 most enriched sets for each condition are shown. Full results are available at ORA (Hoyle & Xue, 2021).

TCR			
ID	**Description**	**NES**	***padj***
hsa04060	Cytokine-cytokine receptor interaction	2.52	2.06E-27
hsa05321	Inflammatory bowel disease	2.39	1.61E-11
hsa04630	JAK-STAT signaling pathway	2.38	4.61E-15
hsa04061	Viral protein interaction with cytokine and cytokine…	2.30	1.11E-09
hsa05323	Rheumatoid arthritis	2.30	4.91E-10
hsa04657	IL-17 signaling pathway	2.25	3.75E-09
hsa04659	Th17 cell differentiation	2.25	3.31E-10
hsa04064	NF-kappa B signaling pathway	2.23	5.89E-10
hsa04380	Osteoclast differentiation	2.13	8.85E-08
hsa04668	TNF signaling pathway	2.12	2.54E-08
hsa05330	Allograft rejection	2.09	8.61E-06
hsa05144	Malaria	2.08	9.45E-06
hsa04658	Th1 and Th2 cell differentiation	2.08	4.61E-07
hsa04625	C-type lectin receptor signaling pathway	2.07	3.59E-07
hsa05202	Transcriptional misregulation in cancer	2.05	8.49E-09
PGD_2			
ID	**Description**	**NES**	***padj***
hsa05321	Inflammatory bowel disease	2.41	4.42E-07
hsa04658	Th1 and Th2 cell differentiation	2.17	1.08E-05
hsa04657	IL-17 signaling pathway	2.16	6.99E-05
hsa05330	Allograft rejection	2.15	2.62E-04
hsa04060	Cytokine-cytokine receptor interaction	2.14	1.37E-07
hsa05323	Rheumatoid arthritis	2.12	1.57E-04
hsa05202	Transcriptional misregulation in cancer	2.12	1.03E-05
hsa04940	Type I diabetes mellitus	2.10	9.15E-04
hsa05332	Graft-versus-host disease	2.10	6.40E-04
hsa05320	Autoimmune thyroid disease	2.07	1.45E-03
hsa04659	Th17 cell differentiation	2.04	5.75E-05
hsa03050	Proteasome	2.03	7.81E-04
hsa05310	Asthma	2.03	2.75E-03
hsa05140	Leishmaniasis	1.98	7.81E-04
hsa04064	NF-kappa B signaling pathway	1.96	5.43E-04

Supplementary Table 35 Reactome pathway enrichment analysis – top results

NES = normalised enrichment score. '*padj*' = adjusted p-value. Only the 15 most enriched sets for each condition are shown. Full results are available at ORA (Hoyle & Xue, 2021).

TCR			
ID	**Description**	**NES**	**padj**
R-HSA-9031628	NGF-stimulated transcription	2.39	8.13E-09
R-HSA-6783783	Interleukin-10 signaling	2.34	4.41E-08
R-HSA-198725	Nuclear Events (kinase and transcription factor…	2.31	3.43E-08
R-HSA-6785807	Interleukin-4 and Interleukin-13 signaling	2.28	5.03E-10
R-HSA-187037	Signaling by NTRK1 (TRKA)	2.23	2.00E-09
R-HSA-5669034	TNFs bind their physiological receptors	2.18	1.64E-05
R-HSA-166520	Signaling by NTRKs	2.16	1.14E-08
R-HSA-3000171	Non-integrin membrane-ECM interactions	2.14	1.46E-05
R-HSA-449147	Signaling by Interleukins	2.10	2.57E-19
R-HSA-451927	Interleukin-2 family signaling	2.10	8.02E-05
R-HSA-3000170	Syndecan interactions	2.03	4.49E-04
R-HSA-6790901	rRNA modification in the nucleus and cytosol	2.00	3.23E-04
R-HSA-3000178	ECM proteoglycans	2.00	6.73E-04
R-HSA-380108	Chemokine receptors bind chemokines	1.99	2.29E-03
R-HSA-112409	RAF-independent MAPK1/3 activation	1.96	2.76E-03
PGD_2			
ID	**Description**	**NES**	**padj**
R-HSA-9031628	NGF-stimulated transcription	2.51	1.42E-07
R-HSA-198725	Nuclear Events (kinase and transcription factor…	2.38	4.96E-07
R-HSA-187037	Signaling by NTRK1 (TRKA)	2.29	1.42E-07
R-HSA-6783783	Interleukin-10 signaling	2.24	3.42E-05
R-HSA-6785807	Interleukin-4 and Interleukin-13 signaling	2.18	1.30E-05
R-HSA-5668541	TNFR2 non-canonical NF-kB pathway	2.14	1.43E-05
R-HSA-72163	mRNA Splicing - Major Pathway	2.13	2.09E-07
R-HSA-166520	Signaling by NTRKs	2.12	4.35E-06
R-HSA-72172	mRNA Splicing	2.07	4.62E-07
R-HSA-72203	Processing of Capped Intron-Containing Pre…	2.05	3.43E-07
R-HSA-8868773	rRNA processing in the nucleus and cytosol	2.03	2.88E-06
R-HSA-1169091	Activation of NF-kappaB in B cells	2.03	6.11E-04
R-HSA-6791226	Major pathway of rRNA processing in the…	2.02	2.81E-06
R-HSA-8941858	Regulation of RUNX3 expression and activity	2.02	7.71E-04
R-HSA-1168372	Downstream signaling events of B Cell Receptor…	2.01	3.51E-04



Supplementary Figure 42 heatmaps - Reactome modules

On the x-axis are gene sets within the module. On the y-axis are the top core genes across all sets in the module, listed by fold-change in expression.

Chapter 5

General Discussion and Conclusion

5.1 What are CRTH2$^+$ CD8$^+$ T cells?

When CRTH2$^+$ T cells were discovered, important observations about their biology were made (Nagata, Tanaka, et al., 1999). The authors showed that CRTH2 is a seven-transmembrane GPCR enriched on Th2 populations generated *in vitro*, and they developed the BM16 clone detection antibody that remains in widespread use. CRTH2 expression could be induced or suppressed *in vitro* by addition of IL-4 or IL-12 respectively. Cultured or *ex vivo* sorted CRTH2$^+$ CD4$^+$ T cells produced type 2 cytokines but not type 1. CRTH2 was present on the surface of most Th2 cells, but a small population of CRTH2$^-$ Th2 cells was also observed. However, CRTH2$^+$ Th2 cells produced more type 2 cytokine than CRTH2$^-$ Th2 cells. The authors suggested CRTH2$^-$ Th2 cells may be artificially induced by the strong PMA/ionomycin stimulation, but the existence of CRTH2$^-$ Th2 cells was since confirmed without the need for artificial stimulation (Bangert et al., 2021). The original CRTH2 paper noted that single clones often had higher CRTH2$^+$ purity than polyclonal Th2 lines, and cultured cells appeared to produce slightly more cytokine than *ex vivo* cells.

The work presented in this thesis largely agrees with the findings of that initial report (Nagata, Tanaka, et al., 1999), but includes some further observations. Instead of showing flow cytometry plots from a single donor (Cosmi et al., 2000; Nagata, Tanaka, et al., 1999), I have shown averages from eight donors, capturing the biological variation between individuals. Indeed, one difference between our studies is that the proportion of CRTH2$^+$ Tc cells in my data seems to be higher (11.7 % vs 3.5 %) and was variable. The ability of CRTH2 to predict type 2 identity was quantified in terms of common test indicators. The sensitivity of CRTH2 as a marker of Tc2 was estimated at around 80 %, whereas for Th2 the figure was lower at approximately 50 %. This confirms the existence of a population of CRTH2$^-$ Th2 cells as observed in some previous studies (Bangert et al., 2021; Nagata,

Tanaka, et al., 1999) but missed by others (Cosmi et al., 2000). These CRTH2$^-$ Th2 cells could be analogous to CRTH2$^-$ ILC2s ('nonconventional' ILC2) reported elsewhere (S. Liu et al., 2020). Another difference is that due to improvements in fluorescence chemistry I was able to measure three cytokines simultaneously rather than separately. This showed that IL-4, IL-5, and IL-13 production is largely but not completely overlapping. Whether CRTH2$^+$ T cells can be considered synonymous with type 2 cytokine producers probably depends on context. For example, in specific discussions of Th2 or Tc2 subsets, it is clearly necessary to specify the precise markers expressed by a cell (e.g., CRTH2$^+$ IL-5$^+$). However, in more general discussions about the immune system or the human body as a whole, it may be reasonable to consider CRTH2$^+$ T cells and type 2 T cells synonymously.

William E. Paul recognised the important distinction between type 2 lymphocytes differentiated *in vivo* vs *in vitro* (J. Zhu et al., 2010). To my knowledge, this is the first instance in which CRTH2$^+$ CD8$^+$ T cells have been massively expanded from an *ex vivo* source, rather than using cells differentiated *in vitro*. The experiments show that small populations can be cloned out and expanded for study, whilst retaining their fundamental properties of type 2 cytokine production and migration towards PGD_2. I hope that this method of FACS-sorting and rapid expansion will become considered as an alternative *in vitro* differentiation of type 2 T cells, and future study may compare the two resulting cell populations. The principle can also be applied to the study of other rare T cell subsets.

Using bulk RNA sequencing, data was generated that extends our knowledge of the activation of CRTH2$^+$ CD8$^+$ T cells. The cells transcribed high levels of type 2 cytokines, other type 2 mediators (e.g., amphiregulin), and cytotoxic effector molecules. This suggests they have the capacity to contribute to type 2 inflammation and its associated functions (antihelminth immunity and tissue repair). Indeed, Tc2 cells were expanded in patients

affected by helminth infections, and fell after deworming, but to a lesser extent than Th2 (de Ruiter et al., 2020). It was clear that there are different pathways to CRTH2$^+$ CD8$^+$ T cell activation and that the outcome depends on the input stimulus. The response to PGD_2 and TCR triggering was substantially overlapping, suggesting that strong TCR engagement is not essential for activation, perhaps going against the paradigm that only unconventional T cells are activated by innate signals and alarmins (Mayassi et al., 2021). Overall, the data showed that CRTH2$^+$ CD8$^+$ T cells have the capability to contribute to type 2 inflammation through multiple routes of stimulation. To what extent this actually happens depends on future observations of *in vivo* type 2 inflammatory environments in humans.

There are unanswered questions about CRTH2$^+$ CD8$^+$ T cells that might be addressed in future projects. First, the transcriptomic data here suggests that these cells should be responsive to mediators not tested in this report, namely IL-4, IL-9, NMU, and possibly TGF-β, which is known to activate ILC2 cells (Rodriguez-Rodriguez et al., 2021). A similar stimulation experiment could be conducted with these mediators to determine if more distinct activation programmes exist than were described here. Secondly, CRTH2$^+$ CD8$^+$ T cells are likely to differ between tissue sites. These differences could be investigated either through low-input techniques such as single cell technologies, or by taking advantage of the rapid expansion protocol to amplify small numbers of tissue T cells. Thirdly, I have not compared the biology of CRTH2$^+$ CD8$^+$ T cells with other lymphocyte subsets in any real depth. Again, similar experiments could be conducted to compare, for example, the responses of CRTH2$^+$ Th, Tc and ILC populations, or to compare CRTH2$^+$ and CRTH2$^-$ Tc cells, or CRTH2$^+$ Tc2 and CRTH2$^-$ Tc2 cells. One possibility is that CRTH2$^-$ type 2 T cells are Tfh-like cells, because Tfh can produce IL-4 in the context of both Th1 and Th2 inflammation (Ruterbusch et al., 2020). Although CRTH2$^+$ CD8$^+$ T cells appeared to have

a stable identity *in vitro*, tracking their stability and plasticity *in vivo* would require animal models. Finally, the heterogeneity of CRTH2$^+$ CD8$^+$ T cells, if they are a heterogeneous population, will be revealed as more single-cell multi-omics data becomes available.

5.2 What role do CRTH2$^+$ CD8$^+$ T cells play in severe asthma?

The data presented in this thesis does not support the use of circulating CRTH2$^+$ T cell counts in the diagnosis, classification, or management of asthma. In some individuals, up to a third of CD8$^+$ T cells expressed surface CRTH2, but this was not reliably linked to any classification or measurement variable of asthma, despite the larger sample size than previous studies. However, a weak link between CRTH2$^+$ Th cell counts and allergy was reproduced. Secondly, a link between CRTH2$^+$ Tc counts and smoking was reproduced, in addition to links between CRTH2$^+$ T cells and related factors such as COPD, PYH, CVD, age, and maleness. A future study may seek to decipher whether CRTH2$^+$ Tc cells are really driven by smoking itself, or one of these related factors. Two potential explanations are I) blood CRTH2$^+$ Tc cells may expand in response to damage to blood vessels or II) Tc2 differentiation is enhanced by hypoxia (Ning et al., 2019), which is characteristic of COPD. Treating asthma with steroids or anti-IL-5 therapy did not significantly affect CRTH2$^+$ T cell counts in this study. This seems to support the observations of two large recent studies which both found that systemic steroids did not affect gene expression of type 2 cytokines in sputum (Fricker et al., 2021; M. C. Peters, Kerr, et al., 2019). It therefore seems unlikely that steroids operate by suppressing circulating (or indeed airway) type 2 T cells. This data does not rule out the possibility that circulating CRTH2$^+$ T cells are somehow changed (e.g., activated) in some endotypes of asthma, but this would require further study looking at more surface markers and/or gene expression.

The gene expression analysis of stimulated CRTH2$^+$ CD8$^+$ T cells revealed changes that are of plausible relevance to asthma and COPD. For example, the increase in perforin and type 2 cytokines may be in line with that seen in the airways in asthma death (O'Sullivan et al., 2001). Of particular interest was the link between alarmin stimulation and genes related to antiviral response and the androgen receptor, both of which are involved in asthma. However, only the gene for IL-25 receptor was expressed at rest, and not for TSLP or IL-33 receptors. Further work on proteins may need to be done to confirm whether receptors for all three alarmins are really expressed on Tc2 cells. In the ILC2 field this question is still open – the standard view is that ILC2 respond to all alarmins (Rodriguez-Rodriguez et al., 2021), but others have not been able to observe IL-33 or TSLP receptors in humans (Bal et al., 2016). This is an important question because both IL-33 and TSLP pathways are at advanced stages of clinical trials. PGD_2 stimulation induced many genes related to cell locomotion as expected, but was also linked to the lung-associated chemokine receptor CXCR6 (A. J. Morgan et al., 2005; Wein et al., 2019), and a number of asthma-associated genes including PGE_2 synthesis enzymes. As an aside, CXCR6 expression on Tc cells promotes their survival and effector function in tumours (Di Pilato et al., 2021), and this is a plausible explanation for the protective effects of PGD_2 against some cancers (Alves et al., 2019). TCR triggering specifically induced genes related to the Warburg effect of massive clonal expansion, and several of these are associated with asthma. There were also several genes uniquely altered by TCR triggering that are known to modulate the TCR signalling pathway, and are associated with COPD. It is not clear why these associations exist between components of the TCR signalling pathway and chronic airways disease, but it is possible that disease-associated gene forms promote hyper– or hypo –responsiveness of T cells. Future *in vitro* studies may look into how disease-associated SNP's affect TCR signalling and responsiveness to stimulation.

The results of two phase III trials (Christopher E. Brightling et al., 2021; Castro et al., 2021) showed insufficient benefit of systemic CRTH2 antagonism with fevipiprant, despite patient stratification by blood eosinophils, and frequency of exacerbation as the primary outcome. These failures may be due to the use of blood eosinophils as a proxy for airway $CRTH2^+$ cells. There are no large scale trials addressing whether fevipiprant prevents migration of $CRTH2^+$ cells to the tissues, or activation of tissue resident $CRTH2^+$ cells by PGD_2. Perhaps the trial outcome would be different if the stratification variables more specifically matched the drug mechanism. Secondly, we do not yet know whether PGD_2-CRTH2 mediated migration could have roles in specific contexts, such as the initial establishment of type 2 lymphocyte populations at early stage disease, or during an exacerbation when large quantities of PGD_2 could be released from mast cells (Murray et al., 1986). Therefore, as others have noted (Kerstjens & Gosens, 2021), whilst CRTH2 antagonism is clearly not a panacea, it may not yet be time to abandon the strategy. Indeed, there are still at least two CRTH2 antagonists (e.g., timapiprant) in ongoing clinical trials.

Another factor responsible for the failure of fevipiprant could be that the preclinical data underpinning them is not reproducible. For example, elevated PGD_2 levels in the airways of patients with asthma were detected in several small studies and this has been cited as evidence of the pathway's importance (Crea et al., 1992; Hilvering et al., 2018; M. C. Liu et al., 1990). However, a larger study could not reproduce this observation and PGD_2 was actually highest in the control group (Fricker et al., 2021). Association between circulating $CRTH2^+$ Th cells and asthma severity formed part of the justification of the phase III trials (Christopher E. Brightling et al., 2021), but the data presented here did not reproduce this finding. Problems with reproducing preclinical studies have been identified in other fields such as oncology, where the 'dismal' success of clinical trials was attributed in part to a

preclinical reproducibility rate as low as 11 % (Begley & Ellis, 2012). Poor reproducibility could explain why progress in drug discovery has been slower in airways disease than other areas of medicine (Pavord et al., 2018). Strategies for improving reproducibility include: increased sample sizes to better reflect the heterogeneity of airways disease; equal weighting of positive, null, and negative findings by publishers to mitigate publication bias; prioritisation of data quality over novelty; and preregistration of preclinical studies to mitigate positive results bias.

Nevertheless, the data presented here underscore the profound effects of PGD_2 on $CRTH2^+$ $CD8^+$ T cells, and support the notion that they could contribute to the type 2 inflammation and tissue remodelling characteristic of T2-high asthma. In the blood, future studies could investigate whether $CRTH2^+$ T cells are qualitatively different in asthma e.g., with epigenetics or large-panel cytometry. For example, one group found PD-1 elevated on $CRTH2^+$ Th cells in patients with allergic rhinitis (Shu-Hung Wang et al., 2021). A specific follow-up study could also seek to reproduce the relationship observed here between $CRTH2^+$ Tc cells and COPD (and associated variables). In the airways, current literature agrees on a general elevation of $CRTH2^+$ T cells in asthma (see 2.1). However, there is room for improving our understanding of what these cells are doing in the tissue, and whether they derive from circulating or resident T cells. Indeed, studies of the HDM mouse asthma model show that circulating and resident Th2 cells have distinct (Rahimi et al., 2020), so this is likely to be true of Tc2 cells. Ideally, paired multi-omics analyses of blood and lung T cells would be carried out on well-phenotyped patients, with CRTH2 being one of many molecular markers tested. We are beginning to see studies taking this approach, for example one study combined mass cytometry and bulk RNA sequencing of cryopreserved BAL cells from small groups of healthy control, MMA and severe asthma

patients (Camiolo et al., 2021) - but CRTH2 was not included. Similarly, multi-omics has been applied to related conditions in other tissues, such as atopic dermatitis (Bangert et al., 2021), where activated Tc2 cells were observed.

5.3 Sputum and the study of the human airways

The importance of paired blood and tissue sampling to understand chronic respiratory diseases cannot be overstated. In general, blood and tissue cells are not equivalent and it is in the tissue itself where immune responses are exerted (Farber, 2021). We already know from mouse models of asthma that resident Th cells have distinct roles from circulating T cells (Hondowicz et al., 2016; Rahimi et al., 2020), and this is likely true for Tc cells. It is also likely that Th2 and Tc2 cells become specialised based on their tissue of residence, as is known to be the case for ILC2s (Rodriguez-Rodriguez et al., 2021). In other fields, in-depth profiling of tissue cells has revealed mechanisms of chronic diseases of the gut (Martin et al., 2019), liver (Ramachandran et al., 2019), and skin (Bangert et al., 2021). Recently, paired sampling of the blood and oesophagus revealed clonally expanded, antigen specific Tpath2 cells are likely to be the cause of eosinophilic oesophagitis (D. M. Morgan et al., 2021). The acquisition of airway tissue samples is therefore a critical question for researchers. An ideal sampling method would be minimally invasive, low-cost, and repeatable for longitudinal study (e.g., of disease development or exacerbations). It would be scalable to large groups of patients so as to capture the heterogeneity of chronic respiratory diseases with no unified cause or mechanism (Pavord et al., 2018).

Sputum satisfies all of the desired criteria for a tissue sampling method. It is non-invasive, cheap, repeatable, and safe. However, the Achilles' heel of sputum sampling is poor reliability. In this study, patients in the OAS cohort had blood drawn and attempted

sputum induction at each visit. The success rate of slide generation was 42 % (53/127) and flow cytometry was successful in just 10 % (13/127) of visits, which was insufficient to investigate airway CRTH2$^+$ T cells in much detail. Looking at the literature, the range of success rates for sputum induction is wide. At the low end, slides were generated from just 37 % of patients after five visits each in a large (n = 1483) cohort of COPD patients. At the high end, success rates in excess of 95 % have been reported for medium-sized cohorts of healthy participants and asthma patients (Keatings et al., 1996; Koc-Günel et al., 2018). Sputum flow cytometry has been successfully performed across multiple visits (R. Chen et al., 2017), and the first single cell RNA-Seq experiments on healthy sputum was recently reported (Schupp et al., 2020). Why are some studies more successful than others? The sputum induction protocol used has not changed since the 1990s (Pin et al., 1992). One possible difference is that successful studies have used high-output ultrasonic nebulisers (Keatings et al., 1996; Koc-Günel et al., 2018), whereas in this study a handheld device was used, with lower air flow and nebulisation rate. However, successful induction has also been reported with Wright (R. Chen et al., 2017) and handheld (Davidson et al., 2014) devices. In my correspondence with other investigators, it has been suggested that other factors include: experience of the clinician, training and experience of the patient; and provision of a private place for the patient to expectorate. A second downside of sputum induction is that it poses an infection risk that has been deemed unacceptable since the outbreak of Covid-19. In summary, sputum induction has the potential to be a valuable sampling technique, but success rates vary widely between investigators and sites.

Sputum induction is just one of several methods available for sampling the airways. The most detailed lung data has come from large tissue resections (Travaglini et al., 2020) but these are only obtained in the context of cancer. Bronchoalveolar lavage (BAL) has been

called the 'gold-standard approach to obtain immune cells from the lungs' (Mould & Janssen, 2021). Tissue biopsies are arguably the gold standard for obtaining a mixture of immune and structural cells. The disadvantages of BAL and endobronchial biopsy/brushings are: that bronchoscopy is invasive; the cost is much higher than sputum; and a team of clinical staff is required. Bronchoscopy studies need to be carefully planned and require multiple clinicians and scientists to collaborate. It could be very challenging to perform these techniques on enough patients to fully capture the heterogeneity of asthma. However, the Covid-19 pandemic has proven that it is possible for a single centre to acquire BAL from large cohorts in a short space of time (Grant et al., 2021).

An alternative approach is to sample the upper airway which does not require endoscopy. Upper and lower airway inflammation is clearly related as evidenced by the association between asthma and allergic rhinitis (J. Bousquet et al., 2008) or CRSwNP (Langdon & Mullol, 2016), although recent direct comparison has questioned the 'one airway, one disease' concept (Giovannini-Chami et al., 2018). Nevertheless, upper airway samples such as nasal lavage (Altman et al., 2019), nasal curettage (Habibi et al., 2020), and nasal brushings (Pandey et al., 2018) have been successfully used to study asthma and related airway infections. Surgically removed nasal polyps can also be studied: recently, single cell analysis of Th cells showed populations of $CRTH2^+$ and $CRTH2^-$ Th2 cells, expressing *HPGDS,* and receptors for IL-9, IL-25, and IL-33 (Ma et al., 2021). It is possible that Tc2 cells also reside in these tissues, and perhaps also express type 2 mediators and receptors that were not detectable in my blood-derived, *in vitro* cultures.

Future studies should aim to obtain paired samples of the blood and airways, either in specific endotypes of patients (e.g., T2-high steroid resistant asthma), or in a large enough cohort of patients to capture disease heterogeneity. Ideally, researchers will take advantage

of current multi-omics data collection and analysis technologies, especially including single-cell RNA sequencing (Hewitt & Lloyd, 2021). These sorts of studies are beginning to be published in the asthma field (Camiolo et al., 2021; Vieira Braga et al., 2019), or looking at healthy volunteers (Deprez et al., 2020), but the number of patients in each study is small (often <10 per group). In contrast, studies of Covid-19 have shown it is possible to generate datasets with sample sizes in excess of one hundred individuals (Grant et al., 2021; Ren et al., 2021). Presumably an even greater sample size will be required for studies of chronic airways diseases, which have multiple causes, than Covid-19, which has a single cause. Secondly, longitudinal sampling would allow the study of critical periods such as lung development, onset of disease, and exacerbation of disease. Identified cells of interest from tissue can be sorted and massively expanded for further study, as shown in this thesis and in previous reports (Till et al., 1995). Sputum should not be abandoned yet, because clearly in some hands it is a reliable source of airway immune cells. However, there is room for research into the factors that determine reliable sputum acquisition.

5.4 Final conclusion

The aim of this work presented here is to improve our understanding of CRTH2$^+$ CD8$^+$ T cell biology and their role in airways disease. This thesis presents a modest increase in our understanding of CRTH2$^+$ CD8$^+$ T cells. With reference to the thesis aims outlined in 1.5, the following conclusions can be drawn:

1. Blood CRTH2$^+$ T cell counts are not meaningfully associated with asthma, nor with its severity or endotype. CRTH2$^+$ Th cells may be weakly associated with allergy. CRTH2$^+$ T cells in general were associated with COPD and CVD. CRTH2$^+$ Tc cells appear to be linked to smoking (and associated factors) and were higher in

men. Due to low success rates of sputum sampling, it was not possible to fully determine correlates of airway CRTH2$^+$ T cells, but CRTH2$^+$ Th cells were associated with airway eosinophilia. Blood CRTH2$^+$ T cell counts were unaffected by corticosteroid or anti-IL-5 therapies.

2. CRTH2 is a moderately sensitive marker of Th2 cells and a highly sensitive marker of Tc2 cells and triple-positive (IL-4, 5 and 13$^+$) type 2 T cells. CRTH2$^+$ T cells are much more likely to produce type 2 cytokines than CRTH2$^-$ T cells after stimulation. Although CRTH2$^-$ type 2 T cells do exist, they produce lower quantities of type 2 cytokines than CRTH2$^+$ type 2 T cells. The overlap between CRTH2 expression and type 2 cytokine production is significant but not great enough for the two measurements to be considered completely synonymous. CRTH2 expression and the type 2 identity of CRTH2$^+$ T cells remain stable after massive clonal expansion *in vitro*.

3. Cultured CRTH2$^+$ CD8$^+$ T cells can be activated through the TCR, through CRTH2 by PGD_2, or by alarmin cytokines (IL-25, IL-33, and TSLP). CRTH2$^+$ CD8$^+$ T cells respond to these activators with a mixture of shared and specific programmes. Common changes in gene expression include upregulation of type 2 cytokines and cytotoxic mediators. In addition to this, TCR triggering specifically induces the Warburg effect of clonal expansion. PGD_2 stimulation specifically induces genes related to cell locomotion and trafficking (including lung-associated *CXCR6*), and eicosanoid metabolism. Alarmin-mediated activation was associated with STAT5 signalling and genes related to host response to viruses. Multifunctionality of CRTH2$^+$ CD8$^+$ T cells is likely to reflect the specificity required of human immune responses.

Although the data here does not provide evidence for expanded circulating CRTH2$^+$ CD8$^+$ T cells in asthma, it still supports their potential to contribute to type 2 inflammation of the airways. Given the established link between type 2 inflammation and eosinophilic asthma and COPD, CRTH2$^+$ CD8$^+$ T cells should be considered in future studies of these diseases.

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