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Regulatory B cells in seasonal allergic rhinitis and the influence of grass pollen immunotherapy

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Abstract

Introduction:

Interleukin (IL)-10-producing B cells (Bregs) regulate immune responses in autoimmune disease; however their role in allergy is unclear. Allergen exposure in predisposed atopic individuals results in the induction of IgE-secreting B cells, crucial in the immunopathophysiology of allergic rhinitis. Allergen-specific immunotherapy (AIT) is the only disease-modifying treatment for allergic rhinitis. AIT results in long-term clinical and immunological tolerance; however, whether Bregs contribute towards AIT-induced tolerance remains unclear.

Hypotheses:

1. *In vitro* induced IL-10-producing B cells regulate allergen-driven Th2 inflammation,

2. Bregs are present in fewer numbers in seasonal grass pollen allergic (SAR) individuals compared with healthy controls, which is restored during AIT.

Methods:

B cells were isolated and subjected to flow cytometry to detect surface markers and IL-10 capacity following CpG stimulation. FluoroSpot, ELISA or qPCR were used to confirm IL-10. Suppression of T cell proliferation (by CFSE) and cytokine production (by ELISA) were carried out in co-cultures. Regulatory B cells in SAR (n=14), AIT (n=18) and healthy (n=14) donors were compared. Nasal allergen challenge (NAC) was carried out, with blood taken pre and post challenge for flow cytometry.

Results:

CpG significantly enhanced proportions of Bregs, with enrichment particularly within $CD24^{hi}CD27^{+}$, $CD5^{hi}$, $PD\text{-}L1^{+}$ and $CD24^{hi}CD38^{hi}$ populations. Bregs suppressed both polyclonally- and grass pollen allergen-stimulated T cells. *Ex vivo*, proportions of $IL\text{-}10^{+}$ B cells from SAR and healthy donors matched, but were significantly greater amongst AIT donors (particularly sublingual immunotherapy - SLIT) compared to SAR. Following NAC, proportions of B cells within $CD24^{hi}CD38^{hi}$, $CD5^{hi}$, $CD24^{hi}CD27^{hi}$ and $CD25^{+}$ subsets were significantly increased amongst non-allergic and AIT groups, but not amongst SAR donors.

Conclusion:

Bregs are capable of suppressing allergen induced, Th2-driven inflammation *in vitro* and may be involved in the induction of tolerance during allergen immunotherapy *in vivo*, particularly following SLIT.

Declaration

I confirm that the contents of this thesis are my own work; all laboratory-based experiments were performed by me. Clinical and diagnostic procedures were carried out by the persons acknowledged overleaf, for which I am extremely grateful. Experimental assistance, laboratory guidance and supervision are also acknowledged overleaf. A full list of references is given, with citing throughout the text where evidential assertions have been made. This work has not been submitted in application for any other higher degree.

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Abstracts relating to this thesis

J.E.G. Charlesworth, S.M. Quli Khan, G.W. Scadding, D.J. Cousins, S.R. Durham and M.H. Shamji *IL-10-producing regulatory B cells suppress allergen-specific T cell proliferation and are elevated ex vivo following specific immunotherapy* - - Clinical and Experimental Allergy, 2012, vol.42(12):p1817

James E. G. Charlesworth, Andrea Goldstone, Moises A. Calderon, David Cousins, Stephen R. Durham, Mohamed H. Shamji *Grass Pollen Allergics Have Fewer IL-10-Producing B Cells Than Non-Atopic Controls* -— J. Allergy and Clinical Immunology, 2013, vol.131(2 supplemental):pAB204

J. Charlesworth, A. Goldstone, R. Yan, M.A. Calderon, D.J. Cousins, S.R. Durham and M.H.Shamji *Regulatory B cells are reduced amongst grass-pollen allergic patients compared with non-allergic controls* -— Clinical and Experimental Allergy, 2013, vol.43(12):p1429

J.E.G. Charlesworth, G.W. Scadding, A. Eifan, R. Yan, A.E. Goldstone, M.A. Calderon, S.R. Durham, M.H. Shamji *IL-10-Producing B Cells Are Increased After Grass Pollen Immunotherapy Compared to Untreated Grass Pollen Allergic Controls: A Blinded Cross-Sectional Study* -— J. Allergy and Clinical Immunology, 2014, vol.133(2 supplemental):pAB53w

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List of abbreviations

7AAD – 7-aminoactinomycin D

Ab – Antibody

AID – Activation-induced cytidine deaminase

AIT – Allergen-specific immunotherapy

AP - Alkaline

APC - Antigen presenting cell

AR – Allergic rhinitis

B10 – IL-10-producing regulatory B cell

B10pro – IL-10-capable B cell (once induced)

BAT – Basophil activation test

BCR – B cell receptor

Breg – Regulatory B cell

BSA - Bovine serum albumin

CD – Cluster of designation

CFSE - carboxyfluorescein succinimidyl ester

CpG – CpG-containing single stranded DNA

CSR – Class switch recombination

CTLA – Cytotoxic T lymphocyte antigen

DC – Dendritic cell

Dex – Dexamethasone

DMSO - Dimethyl sulfoxide

EAE – Experimental autoimmune encephalitis

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-Linked ImmunoSorbant Assay

Fc – Fraction crystallising (of antibody)

FcεR – Fc epsilon receptor

FEV – Forced expiatory volume

FoxP3 – Forkhead box protein 3

GC – Germinal Centre

IFN - Interferon

Ig – Immunoglobulin

IL – Interleukin

IL-10R – Interleukin 10 receptor

KU/L – Kilo units per litre (arbitrary)

LPS – Lipopolysaccharide

LTRA – Leukotriene receptor antagonist

mAb – Monoclonal antibody

MHC – Major histocompatibility complex

mRNA – Messenger ribonucleic acid

mRQLQ - mini Rhinoconjunctivitis Quality of Life Questionnaire

MS – multiple sclerosis

NAC – Nasal allergen challenge

NHS – Normal human (AB) serum

NFκB – Nuclear factor κB

OVA - ovalbumin

PBMC – Peripheral blood mononuclear cell

PBS - Phosphate buffered saline

PC – Plasma cell

PCR - Polymerase chain reaction

PD-L – Programmed death ligand

Phl p - Phleum *pratense*

PLA – Phospholipase A

PMA - phorbol 12-myristate 13-acetate

PPD – Purified protein derivative

PRR – Pattern recognition receptor

QC - Quality control

qPCR – Quantitative polymerase chain reaction

RA – Rheumatoid arthritis

SAR – Seasonal allergic rhinitis

SCIT – Subcutaneous immunotherapy

SHM – Somatic hypermutation

SLE – Systemic lupus erythematosus

SLIT – Sublingual immunotherapy

SNP – Single nucleotide polymorphism

SPT – Skin prick test

SQ-U – Standardised quantity units (of allergen)

SS – primary Sjögren's syndrome

STAT – Signal transducer and activator of transcription

TCM – Tissue culture medium

TCR – T cell receptor

Tfh – T follicular helper cell

TGF – Tissue growth factor

Th – T helper cell

TIM – T cell immunoglobulin and mucin domain

TLR – Toll like receptor

TMB – Tetramethylbenzidine

TNF – Tissue necrosis factor

Tr1 – IL-10-producing regulatory T cell

Treg – Regulatory T cell

TT – Tetanus toxoid

1. Introduction

1.1. Allergy

Allergic responses (type I hypersensitivity reactions) are immunoglobulin (Ig) E-mediated and directed at exogenous and innocuous antigens. Sensitisation can occur anywhere the external environment comes into contact with the immune system, such as broken skin, the respiratory and nasal mucosa, the eyes and the entire digestive tract.

Repeated allergen exposure is required for allergen sensitisation, without which, antigen-specific IgE cannot be generated. Sensitisation can be shown by the detection of specific IgE in serum or by demonstrating a wheal and flare response following a skin prick test (SPT) with an allergen solution on the skin. Whilst allergic sensitisation is typically systemic, allergic diseases are typically localised to the site of exposure to the sensitised allergen. For example, allergic sensitisation to airborne allergens can lead to allergic rhinitis (AR), asthma or both in the upper, lower or combined airway. Skin reactivity with allergen can result in atopic eczema, whilst food allergens consumed orally (given that these can also be inhaled) typically cause gastrointestinal reactions.

The exception to these localised allergic diseases is anaphylaxis, which is typically IgE-mediated (although can be non-allergen/IgE-mediated, driving systemic mast cell degranulation) and can be fatal if rapid treatment is not provided. This multi-organ disease can be fatal due to its rapid onset (minutes to hours), systemic nature and the impairment of both respiratory and cardiac systems.

Whilst there has been suggested to be around 60% heritability for atopic disease, this does not account for the increasing prevalence of allergic sensitisation in recent decades (Custovic et al., 2012). The environment therefore not only accounts for the initiation of allergy amongst genetically pre-disposed individuals, but also an altered environment must account for increasing prevalence.

The increasing prevalence of allergy has been associated with a rise in sanitisation in the Western world and increasing urbanisation, reviewed in (Von Hertzen and Haahtela, 2004). The hygiene hypothesis predicts that reduced exposure to the infectious agents amongst which humans have evolved, has led to a loss of peripheral tolerance to immune challenge. This in turn is suggested to result in a reduced capacity to resolve inflammation once triggered, which especially relates to helminth immunity which is called on infrequently in the Western world (Rook, 2009). The hygiene hypothesis is supported by studies showing greater incidence of atopy and AR amongst urban children

as compared to rural children (Cooper et al., 2014, Majkowska-Wojciechowska et al., 2007). Children growing up on farms show a greater protection from atopy, beyond those in rural settings alone, and have been extensively studied in Europe. These studies have shown that the consumption of unpasteurised milk and greater exposure to endotoxin are the major contributing factors in protection from allergy (Perkin and Strachan, 2006, Loss et al., 2011). Single nucleotide polymorphisms (SNPs) affecting *CD14* expression can confer either protection or increased risk of allergic sensitisation or asthma depending on both genotype and relative level of endotoxin exposure (Simpson et al., 2006, Bieli et al., 2007). This protective farm-living effect can be conferred *in utero* by maternal exposure to farms (Ege et al., 2008). Maternal farmyard exposure has been shown to induce toll like receptor (TLR) 2 and 4 and CD14 mRNA induction, which favour T helper type 1 (Th1) rather than T helper type 2 (Th2) priming of the immune system (Ege et al., 2006). A similar impact on immunological phenotype was observed amongst children growing up on farms, compared to matched non-farm controls, who showed increased proportions of regulatory T cells, especially in response to endotoxin stimulation (Lluis et al., 2014).

1.2.Allergic Rhinitis

1.2.1. Clinical features of allergic rhinitis

AR, a chronic disease of the upper airway, typically presents with either perennial or seasonal symptoms which are increased or induced on allergen exposure. Nasal allergic symptoms were shown to have a prevalence of 27% amongst UK adults, occurring at least once in the 12 months preceding the study, accounting for a mean of 25 per 100,000 GP consultations (Gupta et al., 2004). Patients suffer with a combination of upper respiratory symptoms which include rhinorrhoea or nasal congestion, nasal itching, sneezing and ocular symptoms of allergic conjunctivitis such as chemosis and periorbital oedema, resulting in painful, itchy and watery eyes (Greiner et al., 2011). These symptoms can also present with a systemic response, which is unsurprising given the typically elevated levels of serum-detectable allergen-specific IgE which can result in urticaria on allergen contact with the skin.

Diagnosis of AR is led by a history of symptoms upon exposure to allergen, if this can be recalled accurately, which may be a persistent allergen such as house dust mite, or a seasonal allergen, such as grass pollen. A SPT is routinely employed as a cheap and accurate method of confirming sensitisation to a trigger (Bousquet et al., 2012), taking just 20 minutes to develop. In a similar vein to SPT, intradermal allergen challenge can also show an allergen-specific sensitisation, with titration of allergen concentration in this case used to discriminate patients by magnitude of response.

Intradermal testing is rarely used for the diagnosis of AR, but may be used in research to evaluate suppression of allergic response over time. Further *in vivo* testing is rarely carried out outside of research for AR, but can include nasal allergen challenge (NAC) (Scadding et al., 2012) and allergen challenge chamber (ACC) (Devillier et al., 2011). Both of these techniques aim to replicate natural allergen exposure and will call on patients to score symptoms with the potential to additionally measure nasal secretions or peripheral blood and serum *in vitro* throughout challenge.

The typical definition of an AR patient as systemically sensitised has been complicated by the identification of local AR (Rondon et al., 2012), which presents with identical symptoms but an absence of systemic allergen-specific IgE. These patients show positive clinical symptoms on nasal allergen provocation and have detectable secreted nasal IgE, suggesting a localised allergic response.

1.2.2. Epidemiology of allergic rhinitis

AR in the UK impacts on quality of life for the most severe sufferers and given peak incidence is amongst 15-19-year olds, this can seriously affect seasonal allergic rhinitis (SAR) sufferers during the pollen, coincidently also the exam, season (Ghouri et al., 2008). This study identified an increase in the prevalence of GP-diagnosed AR in just 5 years between 2001 and 2005 from 4.6-6.6%, although this underestimates the disease incidence of AR. A large self-report study conducted across Europe found allergic nasal symptoms in 21.9-31% of responders across 4 centres in the UK (Burney and ECRHS., 1996).

AR was documented in 17% of asthmatics in a large UK-based study of general practice-treated asthmatics (Price et al., 2005), which showed a significantly increased severity (likelihood of hospitalisation) of asthma in concomitant AR. This likely underestimates the concomitance of these diseases as shown by a comprehensive literature review examining the prevalence of AR amongst asthmatics in the US and Europe, and a single study in China (Gaugris et al., 2006), which found a cross-sectional prevalence of AR in asthma was 60%-94% amongst the European and US studies. Lifetime prevalence of any AR was greater still, which was typically >67.2%, up to 100% in a Danish study of 734 patients. Asthma amongst AR patients was shown to be present in 7.6-22.6% of cases in a large multi-centred European study (Leynaert et al., 2004).

1.2.2.1. Grass pollen allergic rhinitis

Commonly referred to as hay fever, grass pollen SAR occurs between May and July in the UK, during the peak grass pollen season. Sensitisation to pollen is extremely common (Scala et al., 2010), however

given the disparity of allergenic pollens detected within Europe, and the further complexity of divergent pollen seasons for the same pollen (Smith et al., 2014), a lack of clarity with respect to allergen trigger exposures has been shown amongst patients who miss-diagnose themselves (positively or negatively) in 30% of cases of grass or tree pollinosis (Smith et al., 2009). Structured history and SPT allows patients to make lifestyle changes and allergen avoidance measures in order to address symptoms. Although, surprisingly, grass pollen counts by regions across Europe showed an inverse correlation with the prevalence of AR (Burr et al., 2003), adding weight to the suggestion that low dose allergen is required to drive Th2 responses (discussed further below, section 1.3.3).

Grasses belong to the Poaceae family which contains 9,000 species (Andersson and Lidholm, 2003), few of which are allergenic. Efforts to limit the number of pollens needed to screen to confirm grass pollen allergy has identified reactivity to Phleum *pratense* (Phl p) as highly concordant with at least 12 other grass commonly allergenic species (Andersson and Lidholm, 2003). Indeed, specific IgE towards recombinant Phl p 1, 2 and 5 proteins identifies the majority of Phleum-sensitised individuals, along with a large proportion of grass-allergic patients (Niederberger et al., 1998, Laffer et al., 1996). Whilst Phl p peptides 1, 2 and 5 are still acknowledged as immunodominant, a recent study shows further complexity with identification of an additional 52 Phl P Th2 peptides (Schulten et al., 2013). Additionally, allergen-specific immunotherapy (AIT) towards Phl p is also successful at inducing protective tolerance towards other grasses (Hejl et al., 2009).

1.2.3. Immunological basis of allergic rhinitis

AR is initiated and maintained by a Th2-dominated response, resulting in the induction of allergen-specific IgE. IgE is bound to high affinity Fcε receptors (FcεRI) on the surface of mast cells (predominantly) and basophils (Baraniuk, 2001, Stone et al., 2010). Tissue-resident mast cells of the nasal mucosa are activated by the crosslinking of FcεR1 following engagement of allergen with surface-bound IgE. Upon activation, the early phase cascade of allergic responses, is triggered within seconds or minutes of allergen inhalation. Mast cells release preformed granules which contain histamine, leukotrienes, prostaglandins, tryptase and bradykinin, the key mediators of the early phase allergic responses interacting with nerves, blood vessels and mucus-producing cells of the nasal mucosa (Parikh et al., 2003).

Following these immediate responses, mast cells are also able to produce cytokines which propagate late phase and Th2 responses over the following hours, reinforcing sensitisation and allergen recognition. The late phase response may be a second peak of rhinitic symptoms, or maintenance of symptoms above baseline for up to 8 hours following exposure (Scadding, 2014, Scadding et al., 2012).

TNFα, GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-13 can all be produced by mast cells (Parikh et al., 2003, Stone et al., 2010), and can recruit and promote eosinophils and basophils (GM-CSF, IL-3 and IL-5), Th2 cells (IL-4 and IL-13) and IgE-switching in B cells (IL-4 and IL-13). Basophils share many properties of mast cells but are not tissue resident and have a much shorter half-life. Basophils will traffic to the mucosal tissue and produce much greater levels of IL-4 and IL-13. Eosinophils may reside in the nasal mucosa, where they are thought to traffic to from the bone marrow in response to IL-5, contributing to the early phase response (Barnes, 2011, Stone et al., 2010, Eliashar and Levi-Schaffer, 2005). Eosinophils have a very short half-life (hours) in the circulation and traffic rapidly to the tissue. Upon activation by IL-3, IL-4, IL-5, eotaxin and GM-CSF, eosinophils will degranulate, releasing toxic proteins from lytic granules as well as synthesising and releasing cytokines and leukotrienes into the local inflammatory environment (Bystrom et al., 2011). The discovery of a previously unrecognised innate lymphoid cell, ILC2, which produces copious quantities of IL-13 and IL-5 in response to the allergic-type epithelial cytokines IL-33 and IL-25 (Neill et al., 2010), suggests these cells are able to contribute further to the late phase response and may be critical in promoting Th2 cells (Walker and McKenzie, 2013).

The generation of Th2 cells and IgE-switched B cells is discussed in further detail elsewhere (section 1.3.3 and 1.5.2, respectively). Allergen-specific naïve T cells are driven towards Th2 in the local mucosa or draining lymph, these drive naïve antigen-specific B cells to switch towards IgE production by further producing IL-4, IL-13 and expressing CD154 in the germinal centre. IgE-switched B cells go on to become IgE-secreting plasma cells (PCs) or plasma blasts and may migrate to the local mucosa for the short term, or to the bone marrow and spleen for the longer term (Luger et al., 2009). Th2 memory T cells reside within the local mucosa and draining lymph, contributing IL-4 and IL-13 to the late phase response following activation both non-specifically by inflammatory cells *in situ* (through CD154, CD28 and IL-4R), and through antigen-specific T cell receptor (TCR) engagement.

Despite the common description of an early and late phase response to allergens, *in vivo* these responses overlap, with early acute response (sneezing, rhinorrhoea and itching) giving way to an extended period of nasal blockage or rhinorrhoea following controlled single exposure (Scadding et al., 2012). It is also clear from the above description that the allergic nasal response requires allergen-specific sensitisation, as mentioned previously (section 1.1), and allergic priming to generate high affinity allergen-specific IgE bound to the surface of mast cells for the initiation and progression of AR, which may take one or more pollen seasons in the case of SAR.

1.2.3.1. TLRs in allergy

TLRs are proteins expressed across many lymphoid cells and detect molecular patterns which occur commonly amongst bacteria or viruses. Of the 10 TLRs, TLR3, 7, 8 and 9 are found on intracellular endosomes, while the remainder are found on the extracellular membrane (Liew et al., 2005, Takeda and Akira, 2007). TLRs may dimerise or act alone to signal though MyD88 (excluding TLR3). Recognition of the molecular patterns by TLRs, as shown in in Table 1, results in the induction of the nuclear factor κB (NFκB) and a range of cell- and TLR-specific responses.

TLR (+ indicates dimer)	Target organism	Molecular target
TLR1 + TLR2	Parasites	GPI-linked proteins
	Fungi	Zymosan
	Bacteria	Lipoproteins
TLR3	Viruses	dsRNA
	Self	mRNA
TLR4 + TLR4	Gram negative bacteria	Lipopolysaccharide
	Viruses (RSV)	F Protein
	Self	Heat shock protein 60, fibrinogen, β-defensin, hyaluronic-acid
TLR5	Bacteria	Flagellin
TLR6 + TLR2	Parasites	GPI-linked proteins
	Fungi	Zymosan
	Bacteria	Lipoproteins
TLR7 or TLR8	Viruses	ssRNA
TLR9	Viruses	Herpes-virus DNA
	Bacteria	Unmethylated CpG DNA motifs
TLR10	Unknown	Unknown

Table 1 – Description of human TLRs and their ligands

The above describes the TLRs expressed in humans and their molecular targets. Adapted from (Liew et al., 2005, Takeda and Akira, 2007, Tesse et al., 2011)

Innate pattern recognition is critical to the protective function of the mucosal barrier to detect potential infection, whilst trigging of innate pattern recognition receptors (PRRs) is also required for the initiation of allergy, to progress towards an IgE-mediated allergic recall response. This has been discussed above with respect to the epidemiological evidence of a protective effect of endotoxin exposure (section 1.1). SNPs in TLRs 1, 2, 4, 6, 7, 8, 9 and 10 have been variously associated with risk of developing, or protecting from, asthma (as reviewed in (Tesse et al., 2011)), whilst fewer studies have identified TLR SNPs associated with AR. However, SNPs in TLRs 2, 4, 6, 7, 8 and 10 have all been shown to be associated with increased or decreased risk of AR (reviewed in (Gao et al., 2010)). Some allergens have been shown to bind directly to TLR4, such as major dust mite allergens and cat dander allergens, and others are also able to bind LPS and therefore activate TLR4 indirectly. Pollens may be

carriers for gram negative bacteria, driving low level TLR1, 2 or 4 activation (Deifl and Bohle, 2011, Heydenreich et al., 2012).

Low level immune activation, partly through TLR stimulation in places, is thought to drive Th2 responses. Conversely, TLR agonists have been thought to be able to drive Th1 responses when offered as adjuvants to AIT (high dose allergen with higher concentration TLR stimuli), or indeed in isolation. TLR4 agonists have been successfully used in clinical trials as adjuvants for grass pollen immunotherapy, showing significant induction of regulatory T cells, specific IgG4 and leukotrienes, however neither study examined clinical suppression of AR symptoms (Rosewich et al., 2010, Mothes et al., 2003). Of relevance to this thesis, the use of the TLR9 agonist, CpG, as adjuvants to ragweed pollen AIT have shown successful suppression of Th2 responses and clinical symptoms against placebo throughout the pollen season in a number of studies (Creticos et al., 2006, Simons et al., 2004, Tighe et al., 2000, Tulic et al., 2004). In mouse models of OVA-induced allergic airway inflammation, successful tolerance and reversal of allergy was shown with CpG alone and, to a greater extent, as an adjuvant (Fonseca et al., 2011), leading to the suggestion that CpG alone may be used in humans, potentially by intranasal administration, as pollen-free therapy. Interestingly, a study of TLR9 protein across allergic rhinitics found expression across Th2 cells in the periphery, mucosa and bone marrow, but no difference compared to controls (Fransson et al., 2007), suggesting these patients will be equally receptive to CpG.

1.2.3.2. Immunoregulation in allergic disease

Whilst the immunoregulatory balance of both T and B cells in autoimmunity has been extensively studied, a similarly defective regulatory compartment has been demonstrated in allergic inflammation (Akdis, 2009) (section 1.3.5). Allergic donors are differentiated from non-allergics by their production of Th2 cytokines, IL-4, IL-5 and IL-13, in allergen-driven PBMC cultures, which is not detected in cultures of non-allergic PBMCs (Akdis et al., 2004). A comparable IL-10 response between allergic and non-allergic donors suggests that the capacity to produce an allergen-specific IL-10 response is not defective (Bullens et al., 2004, Domdey et al., 2010, Nouri-Aria et al., 2004); the excessive Th2 cytokine response is the feature of the immunological dysregulation amongst allergics. Grass pollen-stimulated peripheral blood mononuclear cells (PBMCs) show increased IL-10 during the grass pollen season amongst grass allergic individuals (Singh et al., 2011), which may be a product of Th2 or Tr1 cells, but is insufficient to prevent Th2 cytokine production or clinically relevant pathology. Alternatively, blockade of allergen-induced IL-10 in PBMC cultures exacerbates allergen-driven proliferation and cytokine responses in allergic donors only, showing the IL-10 produced is successful, to a degree, in Th2 suppression (Domdey et al., 2010). Th1-biased responses to polyclonal T cell stimulation, tetanus

toxoid (TT) or purified-protein derivative (PPD) of *Mycobacterium tuberculosis* amongst allergic individuals is similar to non-allergic controls, demonstrating an allergen-specific, Th2 biased, response (Domdey et al., 2010, Bullens et al., 2004, Till et al., 1997a, Till et al., 1997b).

Beyond measures of IL-10, there is no consensus of evidence suggesting quantitative differences in CD25hiFoxP3^{+} Tregs amongst CD4^{+} T cells in allergic patients (revisited below, 1.3.5). Studies comparing proportions of Tregs show equivalent (Provoost et al., 2009, Han et al., 2010), decreased (Stelmaszczyk-Emmel et al., 2013) or increased proportions (Ito et al., 2009) in allergic disease compared with controls. Interestingly, Tregs derived from grass or birch pollen allergic individuals have been shown incapable of controlling allergen-stimulated effector T cell proliferation *in vitro* compared with matched non-allergic controls (Thunberg et al., 2007, Bellinghausen et al., 2003, Ling et al., 2004). Tregs of grass pollen allergic patients showed a further loss of suppressive activity during the pollen season (Ling et al., 2004). A recent study has also shown greater methylation of the FoxP3 locus amongst the isolated memory (CD45RO^{+}) Tregs from allergics, compared to non-allergics, which was directly related to reduced FOXP3 transcript in these patients compared to controls (Swamy et al., 2012).

1.2.4. Treatment of allergic rhinitis (focusing on SAR)

The patient experience of allergic rhinitic symptoms varies greatly, with little predictive capacity of severity using current diagnostic techniques. Approach to AR treatment in the UK is typically stepwise (Figure 1), with more advanced (and expensive) treatment offered in addition to and following first line treatment, if these alone are unable to provide satisfactory relief (Scadding et al., 2008, Angier et al., 2010).

Following accurate diagnosis, AR patients are offered disease education with respect to likely triggers (based on clinical history, SPT and specific IgE), likely disease progression (development of asthma and increased susceptibility to viral respiratory infections (Cirillo et al., 2007, Mahesh et al., 2009)) and advice on allergen avoidance, where possible. Allergen avoidance is likely to be accompanied by second generation (non-sedating) oral anti-histamines, which provide a cost effective benefit to patients, especially when used prophylactically (Demoly et al., 2014). Nasal corticosteroids are also one of the most effective treatments available for AR, with greater symptom relief experienced than anti-histamines alone and limited penetration of the drug into the systemic circulation this is a widely popular option (Weiner et al., 1998). In practice, both anti-histamines and nasal corticosteroids are both advised as first line treatments and are often used concurrently (Figure 1) (Scadding et al., 2008). Second line treatment may be offered in addition to first line treatments for use as required if the

patient feels adequate relief is not offered from first line treatments alone, these are also relatively inexpensive but may address specific needs, such as nasal rinse for reducing the experience of nasal symptoms in pollen SAR (Figure 1).

Systemic treatments such as oral leukotriene receptor antagonists (LTRA) are effective but less predictably so than antihistamines or nasal steroids (Wilson et al., 2004). There has been a suggestion that LTRAs may be of use for asthma complicated by AR as opposed to AR alone (Nishimura et al., 2011), but are not routinely recommended for AR (Scadding et al., 2008). Oral corticosteroids are rarely used in SAR patients, other than for short-term use when judged appropriate for moderate/severe or severe uncontrolled AR.

Omalizumab, an anti-IgE monoclonal antibody (mAb) therapy, has shown efficacy in poorly controlled allergic asthma (Holgate et al., 2009). This results in down-regulation of FcεR1 on the surface of mast cells and basophils due to a reduction in cell-free IgE, whereas anti-IgE does not result in FcεR1 cross-linking or binding to surface-bound IgE, thereby avoiding the risk of anaphylaxis. There is evidence that Omalizumab is effective in reducing symptom scores and increasing quality of life in poorly controlled AR, when compared with placebo (Tsabouri et al., 2014). When used in combination with subcutaneous immunotherapy (SCIT), Omalizumab for 18 weeks, beginning 10 weeks prior to the pollen season, there was an additional 36% reduction in symptom severity during the pollen season (Kopp et al., 2013). However, this was not maintained in subsequent pollen seasons in which Omalizumab was not used in addition to AIT. This effective treatment is more expensive than AIT and does not provide a persistent benefit following withdrawal; as such it is unlikely to be adopted widely.

1.2.4.1. Allergen Specific Immunotherapy

AIT as an immunomodulatory therapy for severe AR and asthma has been well described since it was first demonstrated over 100 years ago (Durham and Leung, 2011, Akdis and Akdis, 2011, Frew, 2010). AIT is the only treatment for allergic disease which is disease-modifying and shows efficacy following withdrawal of treatment (Durham et al., 1999, Eifan et al., 2011, Marogna et al., 2010). AIT is indicated in patients with severe AR, poorly controlled by pharmacotherapy and where allergen avoidance is not possible (Figure 1) (Walker et al., 2011). AIT is expensive and time consuming, with poor adherence, only 18% completed the full 3 year course in a retrospective audit of AIT outpatients (Kiel et al., 2013). Therefore patients are screened carefully before commencing treatment. Efficacy of both SCIT and sub-lingual immunotherapy (SLIT) has been shown by meta-analysis of double-blinded placebo-controlled studies showing significant reduction of symptoms and use of medication (Calderon et al., 2010, Calderon et al., 2007, Radulovic et al., 2010). In grass pollen immunotherapy,

significant reductions in symptom and medication scores have been clearly demonstrated by both routes, which extends at least 2 years following cessation of the 3 year standard protocol (Durham et al., 1999, Durham et al., 2012).

The protocols and dosing for the only two forms of grass AIT licensed for use in the UK (Pollinex® – SCIT and Grazax® – SLIT) differ dramatically (Joint-Formulary-Committee, 2014). SCIT, as licenced in the UK, is a solution of 13 grass allergoids with no adjuvant. SCIT requires an up-dosing regimen, typically over 4-6 months, before achieving a maximum allergen dose (100,000 standard quantity units (SQ-U), which equates to approximately 22.5μg of major allergen). Up-dosing is patient-specific, and depends on their ability to tolerate injections, with serious local reactions or systemic reactions likely to lead to repeated or lower dosing before continuing to increase. Maintenance dosing is then continued, ideally every 4 weeks. In comparison, SLIT tablets contain 75,000 SQ-U of lyophilised timothy grass pollen, also with no adjuvant. The SLIT tablet is taken first in the clinic to ensure no severe local or systemic reactions take place, which is extremely rare, after which they are taken daily at home. It is placed under the tongue and swallowing is avoided for 1 minute, and food and drink avoided for 5 minutes, with allergen uptake expected to take place within 2 minutes in the sublingual mucosa.

Grass pollen SCIT has greater risk associated with treatment, with systemic reactions more common by this route but also a slightly greater mean improvement in quality of life and reduction in symptoms and medication amongst this group compared to SLIT (Calderon et al., 2010), although no head to head studies of SLIT and SCIT have been sufficiently powered to detect differences between the two. Adherence is also greater with SCIT, although this is likely due to the requirement to attend outpatient appointments to receive treatment (Kiel et al., 2013). In a large population-based analysis of SCIT treatment in Denmark (Linneberg et al., 2012), SCIT was shown to be protective against developing autoimmune disease and myocardial infarction, and, surprisingly, all-cause mortality.

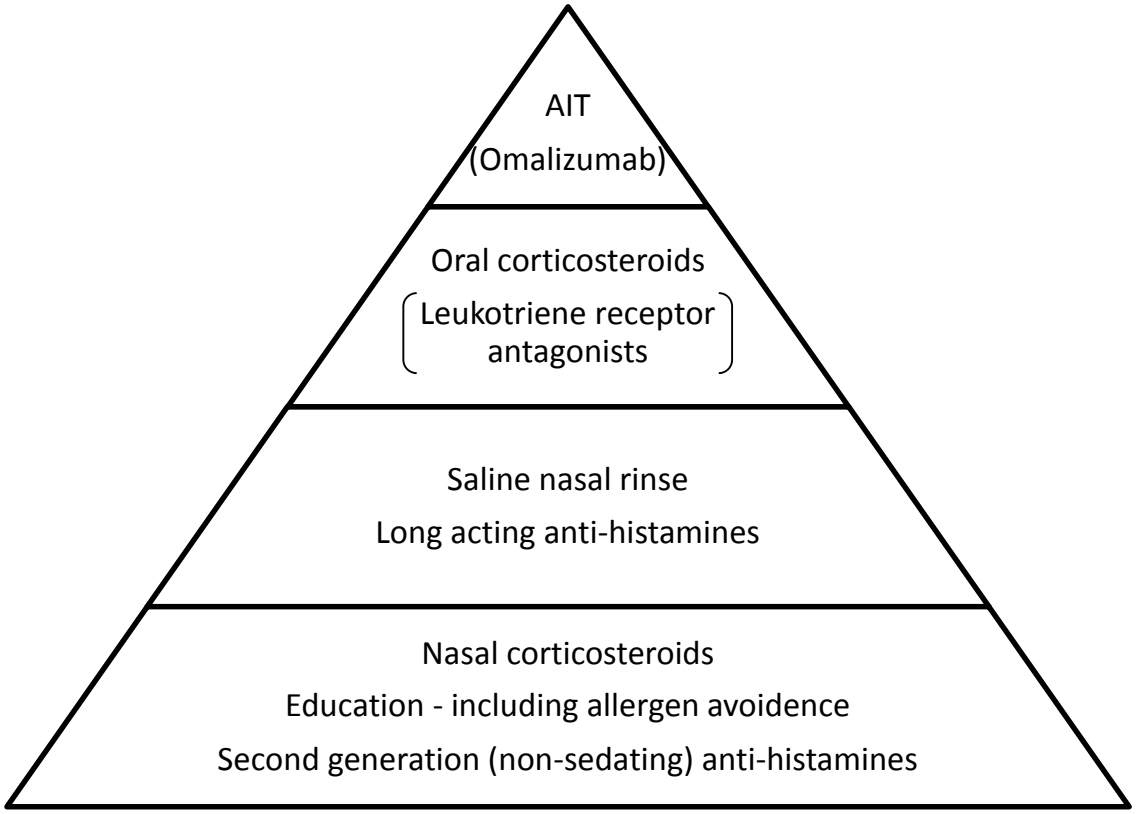


Figure 1 – Hierarchy of treatments for AR.

Hierarchy of suggested treatment regimens which should be offered for AR (adapted from (Greiner et al., 2011, Scadding et al., 2008)) is shown above, in order of severity of impact on quality of life (from bottom, least, to top, most). Those in brackets may be used but are not recommended or are recommended in limited circumstances.

1.2.4.2. Immunological features of AIT

Both SCIT and SLIT result in high cumulative allergen-dosing and immune deviation towards Th1 and Tr1 as a result (see sections 1.3.4 and 1.3.5). The mechanisms of induced immunological tolerance have been extensively reported to involve the induction of suppressive cytokines such as IL-10 and TGFβ (Akdis et al., 1998, Bohle et al., 2007, Ciprandi et al., 2009, Maggi et al., 2012, Pilette et al., 2007, Nouri-Aria et al., 2004), Tregs (Scadding et al., 2010, Radulovic et al., 2008, Francis et al., 2003) and the de-novo generation of allergen-specific IgG4 and IgA2 (James et al., 2011b, Pilette et al., 2007, Francis et al., 2008). Clearly the T and B cell responses of AIT are interrelated and the time course of induction is critical to understand the dominant immunological responses driving allergen tolerance in this model.

Early induction of allergen-specific IL-10 was first shown in bee venom AIT (Akdis et al., 1998), detectable at day 7 following a rapid single day up-dosing regimen. Interestingly, allergen-induced IL-

10 was principally derived from T cells at day 7, followed by monocytes and B cells by day 28. Further studies have confirmed the early induction of allergen-specific IL-10 responses in PBMCs during birch and grass pollen immunotherapy, compared with placebo-treated patients (Francis et al., 2008, Möbs et al., 2012). A prospective study of birch AIT followed IL-10 responses through 3 years of AIT maintenance dosing and 2 years of withdrawal (Möbs et al., 2012). The increase in IL-10 responses observed at 6 months returned to and remained at baseline throughout the remainder of the study. Conversely, the grass study showed a very early increase in peripheral IL-10 production at 2-4 weeks achieved at low cumulative allergen doses and these increases in IL-10 were sustained throughout the 12 months of follow up. (Francis et al., 2008).

1.2.4.3. Allergen-specific IgG4 and AIT

Induction of allergen-specific IgG4 antibodies, capable of blocking allergen-IgE interactions, is critical to the clinical efficacy of AIT (James et al., 2011b, Shamji et al., 2012). Switching towards IgG4 production requires IL-10 (Akdis et al., 1998, Aalberse et al., 2009, Satoguina et al., 2005), which is thought to be derived from Tr1 (Satoguina et al., 2008, Satoguina et al., 2005). Allergen-specific IgG4 is significantly induced after 6 months of AIT (Francis et al., 2008). Although clinical efficacy of AIT is maintained following withdrawal of AIT (Marogna et al., 2010, Calderon et al., 2007, Durham et al., 1999), allergen-specific IgG4 declines toward, but does not return to, baseline levels (Möbs et al., 2012, James et al., 2011a). The persistence of clinical efficacy post withdrawal is due to retention of functional IgG4 capable of blocking allergen-IgE interactions *in vitro* to the same degree observed during AIT maintenance (James et al., 2011a). The first study of IgG4 in relation to Bregs was able to sort bee venom- (phospholipase A2; PLA) specific B cells in bee-allergic, bee venom AIT-treated and bee venom tolerised beekeepers (van de Veen et al., 2013). PLA-specific B cells had increased IL-10 and IgG4 mRNA directly *ex vivo*, whilst IL-10-producing B cells were enriched within the PLA^+ but not PLA- B cells following 3 days of CpG stimulation. Additionally, CpG-induced IL-10-expressing B cells showed expression of IgG4 mRNA and protein when further stimulated with CpG whereas blockade of IL-10 receptor prevented IgG4 induction. The authors finally show that CD27- (naïve) $IL\text{-}10^+$ B cells produce IgG4 following further CpG stimulation, which is not observed amongst IL-10- and $CD27^+$ B cells. Previous data has drawn correlation between the induction of allergen-specific IgG4 and regulatory T cells (both $IL\text{-}10^+$ and $FoxP3^+$ Tregs) (Pereira-Santos et al., 2008).

1.3. T cells

T cells are critical for effective adaptive and sterile immunity. T cells exist in many forms in the periphery; the two numerically dominant divisions of T cells are $CD4^+$ and $CD8^+$ T cells, which act as 'helper' cells (to macrophages and B cells) and cytotoxic cells, respectively. Although $CD8^+$ T cells will not be the focus of this introduction, they are critical in effective anti-viral immunity and for tackling intracellular bacteria. Additionally, invariant natural killer T cells (iNK T) and γδ T cells will not be the focus of this introduction. Although iNK T cells will be briefly introduced later in this section due to their recognition of CD1d which can be found on B cells (Blair et al., 2010).

$CD4^+$ and $CD8^+$ T cells recognise protein antigens held in the context of either class II or class I major histocompatibility complex (MHC), respectively (Sercarz and Maverakis, 2003). TCR engagement in order to promote T cell activation requires a threshold of affinity (strength of recognition) and avidity (number of interactions) to be met (Brownlie and Zamoyska, 2013), in addition to co-stimulatory and cytokine signals. The expression of antigen in MHC requires the processing of proteins for insertion into MHC and as such, T cells do not recognise folded protein structures (unlike B cells) but will respond to a processed linear peptide sequence, typically 8-10 amino acids for MHC class I or 13-22 amino acids in MHC class II (Sercarz and Maverakis, 2003), expressed by self-MHC. Peptides presented in MHC class I are fragmented from cytosolic proteins in the proteasome and are loaded into MHC class I by the loading complex in the endoplasmic reticulum. MHC class II-presented antigens are obtained by phagocytosis of extracellular material, which may be antibody-mediated. Class II MHC is expressed on professional APCs, such as dendritic cells (DCs), B cells and macrophages, as well as activated T cells at a low level. As such $CD4^+$ T cells are directed at extracellular epitopes whilst $CD8^+$ T cells detect and kill cells with a burden of non-self, intracellular antigens.

1.3.1. $CD4^+$ T cell development and selection

All T cells mature in the thymus, having migrated from the bone marrow as thymocytes without the expression of T cell receptors (TCRs). TCR diversity is generated by rearrangement of genes encoding V, D and J segments of the α and β chains which form the vast majority of T cells that exit the thymus in adults (Carpenter and Bosselut, 2010). Complete expression of an αβ TCR on CD4 and CD8 double positive T cells leads to selection of the appropriate co-receptor based on successful engagement of MHC class II or MHC class I, respectively. T cells are selected against self-reactivity by a process of positive and negative selection within the thymus to ensure selection *for* T cells that can recognise self-MHC but *against* those that engage with self-peptide in this context with too great an affinity (Brownlie and Zamoyska, 2013, Morris and Allen, 2012). This process is an imperfect balance between

generating T cells with broad antigen recognition and preventing autoimmunity; despite this, all healthy individuals have T cell clones which can react to self. Thymically generated 'natural' Tregs, which express the transcription factor FoxP3 on egress from the thymus and recognise self-antigens with moderate affinity, are selected to assist with self-tolerance in the periphery (Hsieh et al., 2012). Peripheral tolerance resists T cell self-recognition by controlling the context of T cell activation, driving peripherally-generated Tregs or anergy where T cells are activated in non-inflammatory settings.

1.3.2. T cell activation

Mature, naïve, $CD4^+$ T cells migrate from the thymus to circulate through the secondary lymphoid organs via the peripheral blood. Naïve $CD4^+$ T cells require constant interaction with self-MHC through the TCR/CD3/CD4 complex in order to survive (Morris and Allen, 2012). T cells are therefore seeking constant contacts with DCs and B cells expressing MHC class II whilst recirculating through the secondary lymph. They are carried through the lymph by a chemotactic gradient of CCL21, which engages with CCR7 on the surface of naïve T cells (Gatto and Brink, 2010). Engagement with non-self-peptide in the context of MHC class II in sufficient concentration to trigger TCR signalling alone is not sufficient for T cell activation and requires co-stimulation (signal 2) and cytokine stimuli (signal 3), which also direct the differentiation of the T helper subset. Co-stimulatory molecules including CD80/86 and CD40, which bind CD28 and CD40L on T cells, respectively, are able to stabilise the activatory signals of the TCR and are not expressed on APCs in the absence of inflammation. Inflammatory cytokines in the microenvironment and engagement of pattern recognition receptors (PRRs) (such as TLRs, described previously, section 1.2.3.1) lead to the expression of co-stimulatory molecules and cytokine production by APCs. T cell activation leads to expression of CD25, the high affinity IL-2 receptor α chain, along with production of IL-2 which maintains survival and promotes proliferation. The cytokine production of APCs, local cells or even more peripheral cells is largely thought to influence T helper cell subset fate, which is ultimately determined by specific transcription factors.

1.3.3. Th2 cells

The key T cells orchestrating allergic responses and parasitic defences are Th2 T cells, which are able to drive the production of IgE amongst B cells as well as promoting mast cell, basophil and eosinophil responses towards multi-cellular organisms. IL-4 is crucial to the induction of Th2 cells *in vivo* (Kopf et al., 1993, Seder et al., 1992), IL-4, accompanied by TCR recognition of antigen, leads to an induction of the transcription factor GATA3 amongst mature T cells, binding the Th2 locus which encodes IL-4, -5 and -13, driving and maintaining the Th2 phenotype and cytokines (IL-4, IL-5 and IL-13) (Zheng and Flavell, 1997, Wan, 2014, Zeng, 2013, Cook and Miller, 2010). The source of IL-4 that drives Th2

development is not APCs, although an absence of IL-12 or IFNγ may allow Th2 cytokines to become more dominant. IL-4 may be produced by other Th2 cells, as well as T follicular helper cells (Tfh), mast cells, basophils and iNK T cells (Zeng, 2013), to drive Th2 expansion in the microenvironment.

Given that T cells are the dominant source of IL-4, the initiation of Th2 responses requires an IL-4-independent polarising factor. Mature naïve T cells express GATA3, although function is antagonised in the absence of TCR signalling (Kurata et al., 2002). However, weak TCR engagement (poor affinity or avidity) drives Th2-differentiation in the absence of IL-4, as GATA3 repression has a low threshold for reversal (Milner et al., 2010, Tao et al., 1997). This is a critical step towards priming a Th2 immune response, given the early absence of IL-4. The grass pollen proteins Phl p 1, 3 and 5 are the dominant Th2 antigens in grass allergic individuals (Oseroff et al., 2010), which are small, rapidly soluble, proteins (Vrtala et al., 1993). Low-dose grass pollen, present in very low abundance, with weak triggering of PRRs, is incapable of driving a Th1 response but achieves a threshold for Th2 priming.

1.3.4. Th1, Th17 & Tfh subsets

A typical, non-pathological immune response does not include one T cell subset alone and will show a balance of helper subsets in order to target a variety of effector mechanisms, although depending on the triggering agent some helper subsets will dominate.

Th1 responses drive immunity against intracellular pathogens, specifically arming macrophages towards an inflammatory phenotype, promoting intercellular killing, as well as driving the production of opsonising IgG antibody from B cells (Zhou et al., 2009a, Jutel and Akdis, 2011). Th1 differentiation is driven by IL-12 produced by APCs under the transcriptional control of T-bet, and assisted by high affinity and avidity of MHC class II-peptide-TCR complex signalling along with efficient co-stimulation (induced on APCs by PAMPs) (Milner et al., 2010, Zhou et al., 2009a). Expression of IL-12Rβ or IL-4R and subsequent signalling reinforces the expression of T-bet and GATA3, respectively, and transcriptionally suppress Th2 or Th1 cytokines and differentiation, respectively. IL-2 and IFNγ production is core to Th1 functionality, promoting T cell survival and proliferation, whilst driving the activation and differentiation of inflammatory macrophages, respectively (Mills, 2012). Th1 and Tr1 cells have been suggested to be critical in the induction of immunological tolerance by AIT (Wisniewski et al., 2013, Soyer et al., 2013). Administration of chronic high dose allergen is likely to drive this Th1 phenotype, rather than low dose allergen which induces Th2 responses amongst allergics. Furthermore, chronic antigen stimulation induces IL-10 amongst Th1 cells, a Tr1 phenotype thought to mediate induced allergen tolerance (Saraiva et al., 2009, O'Garra and Vieira, 2007, Thunberg et al., 2007, Soyer et al., 2013). A recent study of the grass pollen-specific T cell repertoire showed T cells

the major allergens Phl p 1 and 5, were predominantly Th2 cells (at least 75%) amongst allergics. T cells recognising the same peptide allergens in non-allergics were significantly fewer and, of those, were predominantly Th1/Tr1 (at least 75%) amongst AIT-treated and non-allergic individuals (Wambre et al., 2014). Although Th2 cells are able to assume a Th1 phenotype in a Th1 microenvironment *in vitro* or *in vivo* (Smits et al., 2001, Krawczyk et al., 2007), it appears that AIT leads to the selective deletion of allergen-specific Th2 cells in favour of Th1/Tr1 (Wambre et al., 2014).

Whilst the balance of Th1 and Th2 responses have been the focus of T cell research in AR, the Th17 subset, which shows plasticity between FoxP3-expressing Tregs, may also contribute to the pathogenesis of AR (Liu et al., 2014c). IL-17A (commonly called IL-17), IFNγ, TNFα and IL-17E (commonly referred to as IL-25) are produced by Th17 cells under the transcriptional control of RORγt in mice, or RORC in humans (Kimura and Kishimoto, 2010). Whilst these cytokine products are predominantly pro-inflammatory, IL-25 has been associated with Th2 priming, predominantly produced by the epithelium along with IL-33. Studies examining the Th17 subset in AR have shown increased frequencies of Th17 cells in humans (Tsvetkova-Vicheva et al., 2014), whilst Th17 cells in a murine model of AR support Th2-driven inflammation (Quan et al., 2012). In mice, Tregs and Th17 cells show plasticity between one another, with IL-6 and TGFβ able to induce Th17 cells from naïve T cells, instead of FoxP3$^+$ Tregs induced by TGFβ alone (Kimura and Kishimoto, 2010, Liu et al., 2014c). In humans, IL-6 remains critical for the development of a Th17 phenotype, however, the addition of IL-1β is required rather than TGFβ (Tuomela et al., 2012, Wilson et al., 2007). TGFβ in humans may drive a subset of IL-21-secreting T cells (Tfh), derived from Th17 or Th1 cells (Liu et al., 2013b). The unstable and plastic Th17 phenotype has shown these cells are capable of switching to Th1 or Th2 cytokine producers in the context of Th1 and Th2 cytokines, respectively, in spite of 3 weeks of *in vitro* polarisation towards a Th17 phenotype (Lexberg et al., 2008), with plasticity demonstrated towards Th1 in models of Th1 disease *in vivo* (Lee et al., 2009, Zhou et al., 2009a).

Tfh are critical in the formation of the germinal centre (GC) (Barr and Gray, 2012, Kemeny, 2012), and secrete the cytokine IL-21. The classical phenotype of these cells is thought to be CD4$^+$ CXCR5$^+$ CD40L$^+$ ICOS$^+$ PD-1$^+$. They are dependent on ICOS signalling for survival and differentiate in the presence of IL-6 and IL-21. The GC transcription factor BCL-6, a feature of B cells within the GC, is also expressed by Tfh. The subtypes of Tfh cells appear to mirror the T helper subsets in the periphery, co-opting the cytokines IL-4, IFNγ, IL-10 or IL-17 with IL-21 production depending on the subset. Loss of IL-21 in humans leads to a dramatic loss of class-switched antibody and PCs (Salzer et al., 2014), demonstrating a critical role for this cytokine and the Tfh cellular subset in regulation of GCs.

1.3.5. Regulatory T cells

Tregs have been explored in great depth in the last 2 decades; research has shown these cells as a critical regulator of peripheral tolerance. This is apparent by selective murine *FOX3* knockouts, such as the scurfy mouse, or the loss of functional FoxP3 in humans, leading to the development of IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which are characterised by spontaneous allergy, autoimmunity and inflammatory bowel disease (Sakaguchi et al., 2008, Liston and Gray, 2014, Zhou et al., 2009b). The transcription factor, FoxP3, induced by TGFβ and maintained by IL-2, drives and maintains the regulatory phenotype both as a function of centrally induced (nTreg) and peripherally induced (iTreg) Tregs. The intracellular identification of FoxP3, as well as the expression of the high affinity IL-2 receptor chain, CD25, has been crucial to identification of these cells *ex vivo*. However, plasticity between Treg and Th17, Th1 or Tfh, and the instability of FoxP3 in the absence of high IL-2 make these cells a heterogeneous and dynamic population, dependent on the local cytokine milieu. However, in relation to Th2 immunity, the expression of IL-4 and GATA3 are inversely related to FoxP3, suggesting iTreg are unlikely to be able to differentiate in a Th2-biased microenvironment (Mantel et al., 2007). Tregs produce immunosuppressive cytokines such as IL-10, TGFβ and IL-35. Of which, IL-10 and TGFβ have been shown to be up regulated following AIT and capable of inducing B cell IgG4 and IgA production (Ciprandi et al., 2009, Satoguina et al., 2008), respectively and cooperatively. Tregs act additionally through cell surface markers such as CTLA-4, CD39 and CD73 (Tang and Bluestone, 2008).

The role of Tregs in AR has been described briefly above (section 1.2.4.2), with a focus on IL-10. Although it is clear that FoxP3-expressing regulatory T cells produce suppressive IL-10, it is unclear to what extent a reduction in $FoxP3^+$ Tregs might be relevant to loss of allergen tolerance in AR. There is no clear data of a numerical deficiency of Tregs from dust mite allergic individuals were shown to be equally as functional as non allergics (Maggi et al., 2007). However, a larger study of grass pollen allergics showed a functional deficit of Tregs in suppressing grass pollen-driven proliferation (Ling et al., 2004), which was amplified during the pollen season. Although Tregs have been shown to be increased both locally and peripherally following AIT (Scadding et al., 2010, Radulovic et al., 2008, Pereira-Santos et al., 2008), conflicting data suggests that the peripheral detection of $FoxP3^+$ Tregs may be either transient or matched by placebo (Mobs et al., 2010, Bonvalet et al., 2012). It remains unclear whether the Tregs detected post AIT are allergen-specific or induced by bystander TGFβ or IL-10 production, but these studies have shown they are certainly capable of suppressing allergen-driven T cell responses.

1.4. Immunobiology of IL-10

IL-10 is a 35kDa homodimer encoded on chromosome 1, across 5 exons, which was first described as a Th2 cytokine (Sabat et al., 2010). *In vitro* it is possible to show IL-10 production from all subsets of human $CD4^+$ T cells and $CD8^+$ T cells as well as B cells, monocytes, macrophages, dendritic cells, mast cells, natural killer cells, eosinophils and neutrophils (Saraiva and O'Garra, 2010, Sabat et al., 2010), implicating this cytokine in many aspects of immune function, both adaptive and innate. Given the broadly anti-inflammatory role of IL-10, it is clear that this cytokine is a critical part of the regulation and resolution of inflammation (Ng et al., 2013). Loss of IL-10 in mice is associated with chronic inflammatory bowel disease, possibly due to an inability to suppress inflammatory responses to commensal gut bacteria (Kuhn et al., 1993). A mouse model of allergic airway disease showed IL-10-deficient mice developed less airway remodelling and hyper-responsiveness, whereas the inflammatory infiltrate and resulting inflammation was exacerbated (Makela et al., 2000). A separate study has shown that transfer of Tregs into wild-type mice with an OVA-induced allergic airway disease was able to resolve Th2 inflammation through an IL-10-dependet mechanism (Kearley et al., 2005). Whilst IL-10 appears to be crucial to the mechanism of action of AIT (reviewed above, section 1.2.4.2), allergen-induced IL-10 production does not appear to be dysregulated amongst a subset of grass AR patients compared to controls (also reviewed above, section 1.2.3.2).

The action of IL-10 is broad but well understood. In order to respond to IL-10, cells express the IL-10 receptor (IL-10R), of which two chains exist, IL-10R1 and IL-10R2 (also referred to as IL-10Rα and IL-10Rβ, respectively). IL-10R1 is specific for IL-10, which cannot bind IL-10R2, however, once bound to IL-10R1, IL-10R2 is able to associate and increase the affinity of the IL-10/receptor interaction by up to 10x (Sabat et al., 2010). Expression of IL-10R1 determines the cellular responsiveness to human IL-10 (Ding et al., 2001). Signalling through IL-10R1 results in the phosphorylation and the nuclear translocation of STAT3, with STAT1 under some conditions, (Finbloom and Winestock, 1995) which leads to cell-specific gene promotion. Monocytes and macrophages are key innate targets of IL-10 which drives a regulatory macrophage phenotype, inhibiting pro-inflammatory cytokine production, inducing the up-regulation of scavenger receptors and the down-regulation of cell surface MHC class II and co-stimulatory molecules (Nguyen et al., 2012, Mosser and Edwards, 2008, de Waal Malefyt et al., 1991). IL-10, acting on APCs, is able to suppress T cell activation and T cell polarisation indirectly whilst also acting directly on T cells, predominantly activated or memory T cells. T cell inflammation is suppressed by the targeted suppression of Th1 cytokines (such as IFNγ and TNFα), Th2 cytokines (IL-4, IL-5 and IL-13) and the suppression of Th17 activation (Hawrylowicz and O'Garra, 2005, O'Garra and Vieira, 2007, Grunig et al., 1997). However, relevant to data in this thesis is the suggestion that IL-17A,

produced by Th17 cells, is not suppressed by IL-10 (Naundorf et al., 2009). IL-10 is able to suppress the responsiveness of eosinophils and mast cells, with mast cell antigen-specific responses suppressed by IL-10 along with reduction of the IgE receptor on the surface (Royer et al., 2001, Takanaski et al., 1994).

As well as being producers of IL-10 (discussed further in section 1.6), B cells are crucial targets of IL-10 in order to direct their development and antibody-producing role. Survival of germinal centre B cells is dependent on IL-10 (Levy and Brouet, 1994), as is the proliferation and development of B cells into long-lived plasma cells (Rousset et al., 1992). Switching of B cells is controlled by the cytokine milieu of activated and anti-CD40-stimulated B cells, of which IL-10 drives switching towards IgG (1-4) classes and can inhibit switching towards IgE in preference of IgG4 (Milovanovic et al., 2009, Jeannin et al., 1998). This is of particular interest in AIT where allergen-specific IgG4 is generated in an environment rich in IL-10-secreting cells. The source of IL-10 driving switching and plasmablast development has been associated with B cell derived IL-10, which may have an autocrine function (Gary-Gouy et al., 2002, Heine et al., 2014, van de Veen et al., 2013, Maseda et al., 2012) and which may be positively regulated by Tfh cells and their cytokine, IL-21, in secondary lymphoid organs (Yoshizaki et al., 2012, Yang et al., 2013).

1.5. B cells

B cells, so named due to their production from the bone marrow, develop from a common lymphoid progenitor and share many features in common with their T cell cousins, such as the development of receptor diversity. Immature B cells develop in the bone marrow and are selected against auto-reactivity by stromal cell expression of self-proteins. As the B cell receptor (BCR) does not rely on peptide presentation in the context of MHC, B cells do not require a process of positive selection (Pieper et al., 2013). B cell somatic gene rearrangement drives a diverse repertoire of surface immunoglobulin which acts as the B cell receptor and, following further affinity maturation in the periphery, results in the secretory arm of the humoral immune system. B cells are known for their ability to secrete antibody, however recent years have seen an expansion of research exploring their non-antibody-dependent roles. B cells are potent APCs (matching that of DCs), phagocytic, capable of producing a variety of inflammatory and non-inflammatory cytokines, as well as directing the development and activation of T cells (Barr et al., 2012, Mauri and Bosma, 2012, Fillatreau, 2012). A full understanding of the complex and diverse roles of B cells *in vivo* is especially important given the current ever-increasing use of monoclonal antibodies (mAbs) to deplete B cells in the treatment of autoimmunity and cancer.

1.5.1. Development

Within the bone marrow niche B cells, supported by stromal cells, differentiate into naïve immature B cells which ultimately enter peripheral circulation (Maddaly et al., 2010, Pieper et al., 2013). The pro-B cell undergoes rearrangements of the immunoglobulin heavy chain with random use of the V, D and J segments of the heavy chain, similar to the β chain rearrangements of the TCR. The pre-B cell, consisting of 2 arranged heavy chains with a μ constant region, is then expressed alongside two surrogate light chains and Igα/Igβ to permit BCR signalling to form a functional early BCR. If the rearranged heavy chain recognises self-proteins on stromal cells, resulting in BCR signalling, heavy chain rearrangement will be attempted on the other chromosome. If neither chromosomal rearrangement is successful, self-reactive BCR signalling results in clonal deletion by apoptosis. Much like T cell development, this is an imperfect process which may allow Ig which show weak affinity to self to progress. Healthy humans can be shown to have self-reactive BCRs (Tiller et al., 2007, Wardemann et al., 2003, Koelsch et al., 2007), and further mechanisms of peripheral tolerance are necessary to select against auto reactivity. Following successful heavy chain expression, the light chain will undergo a similar process, in which V and J genes are randomly rearranged. There are two possible light chains, with κ rearranging on both chromosomes (if self-reactive in the first instance) before the attempted rearrangement of the λ light chain on each chromosome. If a rearrangement is successful,

and does not produce BCR signalling, maturation will progress. If all of the light chain rearrangements contribute to a self-reactive BCR then the cell is selected against. The final immature B cell will express 2 identical heavy chains, which include the constant region, determining antibody isotype, alongside 2 identical light chains. Each heavy and light chain pair constitutes a fragment antigen binding or F(ab) region, allowing a single antibody to bind 2 antigens.

Immature naïve B cells leave the bone marrow as a 'transitional' (T) subset in blood (Marie-Cardine et al., 2008, Sims et al., 2005), expressing the rearranged BCR in the form of both IgM and IgD isotypes by alternative splicing (Chen and Cerutti, 2011). These cells are $CD24^{hi}CD38^{hi}$ and may make up two distinct populations (T1 and T2; with decreasing staining intensity for both CD24 and CD38 between T1 and T2), similar to those described in mice, in which T1-3 exist (Vossenkamper et al., 2012). Human $CD24^{hi}CD38^{hi}$ B cells make up a small proportion of circulating $CD19^{+}$ B cells, around 5-7% in adults, but are up to 50% of the peripheral blood B cell pool at birth, decreasing rapidly until age 6, reaching adult proportions around age 13 (Marie-Cardine et al., 2008). These cells have been associated with regulatory capacity (Blair et al., 2010), as discussed further below (section 1.6.1.4), but also were first reported to express CD5 (Sims et al., 2005), which is associated with IL-10 capacity in B cells (Mageed et al., 2012, Garaud et al., 2009, Gary-Gouy et al., 2002). Transitional B cells appear to exist as a checkpoint for maturation into follicular or marginal zone B cells in the spleen. They are highly prone to death and require stromal cells or IL-4 and CD40 signalling for survival and cell cycle progression (Sims et al., 2005), BCR signalling acts as a regulator of autoreactivity by promoting cell death.

1.5.2. Germinal centre interactions

If not selected against, transitional B cells become follicular or marginal zone (MZ) B cells, losing the expression of CD10 and also losing the intensity of CD24 and CD38 expression (Sims et al., 2005). Follicular B cells circulate through the B cell zones of the spleen and other secondary lymphoid organs, whilst MZ B cells in humans occupy the margin of the red and white pulp of the spleen, analogous to the marginal sinus in mice (Weill et al., 2009). MZ B cells respond to T-independent antigens, supported by a network of neutrophils driven towards a B helper phenotype (Puga et al., 2012), as well as DCs and macrophages which are able to present antigen (Vossenkamper et al., 2012). Marginal zone B cells can produce rapid responses to pathogens in the absence of T cell help, becoming short lived IgM-producing plasma cells (Weill et al., 2009). These cells are high expressers of surface IgM (compared to IgD), express CD27 and have undergone somatic hypermutation (SHM) in >80% of cases (Garraud et al., 2012).

Follicular B cells circulate through the secondary lymphoid follicles responding to BCR/antigen cognate interactions (Gonzalez et al., 2011, Shlomchik and Weisel, 2012). B cells express CXCR5 which directs them to interact with follicular DCs (FDCs) in response to a gradient of CXCL13, produced by stromal cells (Cyster, 2010). FDCs are non-proliferative, specialised presenting cells of the lymphoid organs which express high levels of the low affinity FcγRs and complement receptors in the absence of MHC, trapping antigen complexes in their conformational form for presentation to B cells. Successful BCR signalling leads initially to B cells migrating to the follicular boundary with T cells, driven by expression of CCR7, to seek T cell help (Gatto and Brink, 2010). B cells at this stage require the presentation of CD40L by activated T cells. Activated follicular B cells commit to either becoming plasma cells, secreting high affinity, somatically hypermutated, class-switched antibody, or may become extra-follicular plasmablasts early on during GC formation, leaving the follicle to produce low affinity IgM, similar to marginal zone B cells.

GC formation, under the control of the transcription factor BCL-6, is the result of very few clones, potentially even one, which retain expression of CXCR5 (Shlomchik and Weisel, 2012, Gatto and Brink, 2010). These cells rapidly undergo proliferation and seed the GC. The structure of GCs has been well established and consists of a 'light' and 'dark' zone. Within the light zone B cells have contact with T cells and FDCs and are subjected to antigenic selection, whilst in the dark zone SHM and class switch recombination (CSR) take place. B cell migration within the GC is driven by the expression of CXCR4, the ligand for, CXCL12, is highly expressed in the dark zone. Whereas CXCL13 is highly expressed in the light zone, allowing the retained expression of CXCR5 to direct $CXCR4^{lo}$ B cell centrocytes to the light zone, whilst $CXCR4^{high}$ centroblasts are driven to the dark zone. SHM is the random targeted mutagenesis of the variable region of the heavy and light chains of Ig in order to alter affinity for antigen. CSR involves the pairing of the rearranged and mutated V, (D) and J sections with a differing constant region, allowing expression of IgG1-4, IgA1, IgA2 or IgE, depending on the T cell-produced cytokine milieu which direct switching. Pertinent to the pathogenesis of AR and AIT-induced tolerance is the knowledge that switching towards IgE and IgG1, in which switching to IgG1 may be a precursor to IgE switching, can be driven by IL-4 and inhibited by IFNγ (Davies et al., 2013). IgG4 is driven by IL-10, whilst IL-21 is able to further drive IgG4 and inhibit the switching of IgE (Aalberse et al., 2009).

Following SHM, the BCR may have improved affinity for antigen or, given the randomness of the mutations, may have weaker affinity for antigen in the light zone (Shlomchik and Weisel, 2012). The BCR, post-SHM, is tested in the light zone by BCR engagement with antigen, strong signalling may lead to internalisation of antigen and presentation to Tfh through MHC class II and increased co-stimulatory molecules which drive proliferation. Some of these positively selected cells may go on to become

memory B cells or terminally differentiated plasma cells, whilst others may undergo further proliferation or return to the light zone for further SHM. Weak affinity or no BCR/antigen interactions, with limited co-stimulation, leads to death by neglect. Some B cells with BCRs showing moderate affinity for antigen may be able to return to the light zone to attempt further SHM in order to improve affinity.

GC B cells can differentiate into plasma cells, which are long lived antibody secreting cells that reside predominantly in the bone marrow, or memory B cells, which recirculate through the secondary lymphoid organs and may populate the marginal zone and allow antigen recall. Plasma cell differentiation, driven by the transcription factor BLIMP-1, has been reported to be directed on the basis of high affinity BCR signalling in addition to cytokine signals such as IL-21 and is defined by the expression of CD138 in the absence of CD19, CD20 and CD21 (Shlomchik and Weisel, 2012, Gatto and Brink, 2010, Eibel et al., 2014). Memory B cells have BCRs with lower affinity than plasma cells, suggesting they left the GC reaction earlier on. Memory B cells are predominantly $CD27^{+}IgD^{-}$ although there is also evidence of a small population of $CD27^{-}$ memory B cells (Giesecke et al., 2014).

1.5.2.1. Ectopic germinal centres

Whilst GCs typically form in the secondary lymph, supported by a network of stromal cells and FDCs. GCs, or GC-like reactions such as SHM and CSR, indicated by the ectopic expression of the necessary molecular machinery, activation-induced cytidine deaminase (AID), in local tissue have been shown in both autoimmune disease and allergy (Gatto and Brink, 2010, Coker et al., 2003, Takhar et al., 2005). The nasal mucosa may be more receptive to IgE switching. In mice, IgE switching is inhibited by the component GC cytokine, IL-21 (Suto et al., 2002). It is unclear what the role the cytokine IL-21 plays in human B cells switching towards IgE; there is support for IL-21 inhibition of IgE switching and promoting IgG4 in mixed PBMC cultures (Wood et al., 2004). However, IL-21 may be able to promote IgE switching (Avery et al., 2008), particularly from $IgG1^{+}$ cells, in the absence of BCL-6 expression, as has been modelled in mice (Kobayashi et al., 2009). IL-21 as an inhibitor of IgE in humans is supported by decreasing levels of T cell-produced IL-21 in atopic dermatitis of increasing severity, with corresponding increased IgE (Lin et al., 2011). AID expression in the nasal mucosa in humans, along with IgA and IgG transcripts has led to the suggestion that IgE switching takes place in the local mucosa, due to the GC being more restrictive to IgE switching due to the expression of BCL-6 and IL-21 (Coker et al., 2003, Takhar et al., 2005, Gatto and Brink, 2010, Davies et al., 2013).

1.5.3. B cells and TLRs

B cells in humans express TLRs 1, 6, 7, 8, 9 and 10 (Hornung et al., 2002, Bourke et al., 2003). Whilst expression is detected in the absence of stimulation, expression is rapidly upregulated following a range of stimuli, including TLR, BCR, CD40 and non-specific mitogens. The ligation of TLRs by B cells has been shown to boost the generation of class switched B cells under GC conditions and drive the differentiation of plasma cells (Pone et al., 2012, Jiang et al., 2011, Eckl-Dorna and Batista, 2009, Huggins et al., 2007). It is likely that TLRs on B cells contribute to the GC response by providing further positive feedback, similar to that generated by co-stimulatory molecules, whilst also inducing co-stimulatory molecule expression on B cells (Agrawal and Gupta, 2011). TLR signalling may also contribute to the switching decisions of B cells. In PBMC cultures, TLR 3, 7 and 9 agonists have been shown to drive IgG, IgA and IgE switching in the presence of IL-4 and CD40L (Sackesen et al., 2013). This study showed some bias of TLR9 stimulation to select for switching toward IgG or IgA over IgE, compared to TLR3 or 7 agonists. In addition, patients with common variable immunodeficiency (CVID), who have a deficiency in circulating IgA, IgG and/or IgM, showed a lack of B cell responsiveness to TLR7 or 9 stimulation, along with a loss of IgD-$CD27^+$ memory B cells (Yu et al., 2009). The action of TLRs in inducing B cell switching may be contributed to, or indeed driven, by cytokines released by B cells, or other PBMCs, following TLR stimulation.

1.5.4. Cytokine-producing B cells

Pertinent to this thesis, B cells have been shown to produce IL-10, IL-6, TNFα, IP10 (CXCL10), IL-8, IL-1α/β and IL-13 following TLR1, 7 or 9 stimulation (Agrawal and Gupta, 2011). This study showed IL-10, IL-6 and TNFα were produced by all three agonists, whilst the Th2-associated cytokine, IL-13, was restricted to TLR1 stimulation. The induction of IL-10 secretion will be discussed further in the next section, however the detection of IL-10 appears to be accompanied by detection of both IL-6 and TNFα in B cells (Ziegler et al., 2014, Thibult et al., 2013, Iwata et al., 2012). The production of immunoregulatory cytokines by B cells is discussed in the next section (1.6). A plethora of cytokines have been suggested to be produced by murine B cells, although little of this data has been repeated in humans or expanded further in mice (Bao and Cao, 2014). Human B cells have been shown to produce IL-4 both spontaneously *in vitro*, enhanced on additional IL-4R stimulation, and *in vivo*, in the dark zone of the GC response (Johansson-Lindbom and Borrebaeck, 2002). Additionally, IL-12 was shown to be induced by human B cells, following CD40 and TLR9 stimulation (Wagner et al., 2004) and capable of inducing IFNγ amongst Th1 T cells. However, a more recent study was unable to replicate B cell IL-12 production using TLR9 stimulation alone (Agrawal and Gupta, 2011). The detection of IL4 and IL-12-producing B cells, at least amongst humans, remains unrepeated and requires further work

to explore the mechanisms underlying these findings. However, the data referred to in this section suggests that B cells may contribute more to the cytokine signals and switch decisions in immune responses than T and B cell dogma would predict, this is at least in part through TLR engagement, although similar B cell cytokine responses have been reported by CD40 or BCR stimulation.

1.6. Regulatory B cells

The concept of suppressor B cells originated in the mid-1970s following a series of publications reporting that adoptive transfer of B cells in guinea pigs resulted in suppression of ovalbumin (OVA)-induced contact hypersensitivity responses (Katz et al., 1974, Neta and Salvin, 1974). These studies were not able to demonstrate antigen-specific suppression or the B cell subset(s) responsible for the observed suppressive activity. Increased interest in suppressor B cells took place during the 1990s. Wolf and colleagues showed that B cell-deficient mice failed to resolve experimental autoimmune encephalomyelitis (EAE) (Wolf et al., 1996). Additionally, in a T cell-deficient murine model of colitis, further loss of B cells lead to exacerbated disease severity on subsequent histological examination (Mizoguchi et al., 1997). Adoptive transfer of B cells was able to show recovery of colitis in this model, commensurate with the numbers of B cells transferred. IL-10 has been associated with B cells since the early 1990s (O'Garra et al., 1990). At this time it was known that CD5-expressing B cells were the dominant IL-10 producers (O'Garra et al., 1992, Ishida et al., 1992). Murine models have been used to explore the relevance of IL-10-producing B cells in autoimmune disease. Loss of IL-10 restricted to B cells is sufficient to exacerbate disease scores for EAE, collagen-induced arthritis and colitis (Fillatreau et al., 2002, Mauri et al., 2003, Carter et al., 2011, Mizoguchi et al., 2002). In mice antigen-specific suppression by IL-10 producing B cells has been demonstrated in the resolution of inflammatory models (Yanaba et al., 2008, Yoshizaki et al., 2012). In a recent study, adoptive transfer of IL-10-competent B cells (Bregs) from naïve or EAE mice into EAE mice showed *in vivo* suppression of IFNγ or IL-17-producing T cells (Yoshizaki et al., 2012). Suppression was achieved using both B cell donors, but was not nearly as great in mice receiving Bregs from naïve mice compared to antigen-experienced Bregs. However, antigen-specific B cell mediated suppression has not been demonstrated in humans.

1.6.1. Phenotypes of human IL-10-producing B cells

IL-10-producing B cells have been most extensively studied for their regulatory potential in man and mice. However there is no consensus, as yet, of cell surface markers to discriminate IL-10-competent B cells from those incapable of IL-10 production. Stimulation to directly detect IL-10^{+} B cells by flow

cytometry has produced a number of well-characterised subsets, within which the IL-10-producing B cells were enriched. This may in part be due to the variety of stimuli used to detect IL-10-competent B cells, which in man and mouse include BCR and antigen stimuli, CD40 stimulation, TLR ligands and helminthic infections (Iwata et al., 2011, Milovanovic et al., 2009, Blair et al., 2010, Bouaziz et al., 2010, Iwata et al., 2012, Correale et al., 2008). Recent work in mice has suggested that IL-21 is able to induce and sustain IL-10-producing B cells in mice *in vitro* and *in vivo* (Yang et al., 2013, Yoshizaki et al., 2012). The most comprehensive of these studies showed IL-21 capable of maintaining and expanding IL-10-producing B cells for 9 days in culture (Yoshizaki et al., 2012). This has been disputed by Holan et al, who show that IL-21 or IL-10 inhibited murine B cell IL-10 production *in vitro* (Holan et al., 2013). Little of this work has focused on human B cells. Studies by the Jahrsdörfer group have shown that IL-21 and BCR stimulation can induce a $CD5^+$ $CD25^+$ granzyme B^+ (GZB) and IL-10-producing phenotype, able to supress T cell activation and proliferation by GZB-dependent killing (Lindner et al., 2013, Hagn et al., 2012, Hagn et al., 2009). This area is well worth further study, especially in human B cells to determine the potential for IL-21 to influence Bregs, especially in light of evidence, relevant to AIT, that both IL-21 and IL-10 enhance TLR-mediated IgG production in human B cells (Liu et al., 2013a). To date, IL-10^+ B cells have been reported to reside within $CD1d^{hi}CD5^+$, $CD24^{hi}CD27^+$, $CD25^+CD71^+CD73^-$ and $CD24^{hi}CD38^{hi}$ B cell subsets.

Despite the Breg subset being predominantly immature, CD27 expression has been previously associated with regulatory B cells (Iwata et al., 2011), although it is clear that IL-10^+ B cells are not exclusively $CD27^+$ and may express IgD and IgM (Bouaziz et al., 2010, Khoder et al., 2014). Although TLR9 stimulation appears artificial, Gray et al., have shown apoptotic cells induce B cell IL-10 responses (Gray et al., 2007), which in man and mouse were attributed to TLR-stimulating DNA exposed by the apoptotic cells (Miles et al., 2012). TLR9-deficient mice showed exacerbated disease scores for EAE, which were alleviated by supplementation with TLR9-competent B cells *in vivo*.

1.6.1.1. $CD1d^{hi}CD5^+$ B cells

TLR 4 and TLR 9 ligands, LPS and type B CpG-containing oligonucleotides, respectively, have been the mainstay of B cell IL-10 induction in mice (Mauri and Ehrenstein, 2008). This has led to the well-established $CD1d^{hi}CD5^+$ Breg subset in mice and man, within which the majority of IL-10-producing B cells reside (Yanaba et al., 2008, Blair et al., 2010). CD5 has a long history of association with IL-10-producing B cells in mice (Ishida et al., 1992, O'Garra et al., 1992). A direct relationship suggesting that CD5 is sufficient for the induction of IL-10 has been shown *in vitro* in humans (Garaud et al., 2009, Garaud et al., 2011, Gary-Gouy et al., 2002).

1.6.1.2. CD24hiCD27^{+} B cells

Two phenotypes of IL-10-producing B cells have been demonstrated following the use of TLR9 ligands to identify IL-10-producing B cells. Of these the CD24^{hi}CD27^{+} B cells were shown to be IL-10-dominant following both 5 hours of TLR stimulation (described by the authors as B10 cells – approximately 0.8% of peripheral blood B cells) and following 48 hours of stimulation (described as a combination of B10 and B10-pro B cells – approximately 4% following TLR9 stimulation) (Iwata et al., 2011). These cells did not act directly on T cells, but on monocytes to suppress TNFα production.

1.6.1.3. CD25^{+}CD71^{+}CD73^{-} B cells

van de Veen and colleagues have recently identified a new subset of IL-10 producing CD25^{+}CD71^{+}CD73^{-} B cells following mRNA microarray analysis of IL-10^{+} and IL-10- B cells following CpG stimulation, followed by flow cytometry. This subset of Breg was reported as expressing higher levels of PD-L1 amongst IL-10-expressing B cells (van de Veen et al., 2013). CD25^{+}CD71^{+}CD73^{-} B cells were competent at suppressing PPD-driven CD4^{+} T cell proliferation within peripheral blood mononuclear cell (PBMC) cultures in an IL-10-dependent manner.

1.6.1.4. CD24hiCD38hi B cells

In addition to TLR9-derived IL-10 phenotypes, Mauri and colleagues have shown that T2 transitional B cells are the dominant IL-10 secretors in comparison with other B cell subsets in mice (Evans et al., 2007). These CD21^{hi}CD23^{hi} T2 B cells in mice are represented by the human phenotype CD24^{hi}CD38^{hi} (Blair et al., 2010) and were shown to be the dominant IL-10 producers following CD40 stimulation in humans. Additionally, CD1dhiCD5^{+} B cells were shown to fall within this transitional B cell population. CD24^{hi}CD38^{hi} B cells are capable of controlling T cell Th1 cytokine responses *in vitro*, mediated partly by IL-10 but also CD80 and CD86 co-receptor interactions (Blair et al., 2010).

1.6.1.5. IL-10-producing plasma cells

Evidence in mice has shown IL-10-producing B cells become class-switched plasma cells (Maseda et al., 2012, Neves et al., 2010), particularly switching towards IgM- and IgG-secreting plasma cells. Recent human evidence supports this murine data, showing human IL-10-producing B cells become IgM- and IgG-secreting B cells, with a sustained capacity to produce IL-10 amongst CD138^{+} B cells (Heine et al., 2014, Shen et al., 2014, van de Veen et al., 2013). This recent concept has shown that the life cycle of the Breg has not been fully explored, that these cells populate the plasma cell pool and may continue to produce IL-10, which is of clear interest to the induction of allergen tolerance by AIT.

1.6.1.6. Beyond IL-10 amongst regulatory B cells

Although IL-10-producing B cells have been the most extensively studied, other phenotypes of regulatory B cells have been demonstrated in mice and man. Data published in the last year has shown an IL-35-producing B cell subset in mice (Wang et al., 2014). This study showed that IL-35$^+$ and IL-10$^+$ B cell populations coincide within the CD5$^+$ pool when B cells are treated with IL-35. IL-35 drove B cells towards an IL-10-producing phenotype in mice and humans, and in mice these cells were able to control autoimmune uveitis and inflammatory T cell responses. An earlier study showed that IL-35- or IL-10-producing B cells could be detected amongst murine plasma cells, which increased following salmonella infection, and, in conflict with the previously cited study, IL-35 or IL-10 was not co-expressed in these cells (Shen et al., 2014). Loss of IL-35 amongst B cells showed exacerbated EAE disease severity, which does not resolve, unlike in IL-35 wild type mice, along with exacerbated T cell inflammatory responses. There is limited evidence of TGFβ-producing B cells in mice and man (Lee et al., 2011, Lee et al., 2014), which may be due to the challenge of detecting this cytokine. If these cells exist, they may act much like TGFβ-producing T cells in promoting Tregs.

As well as secretory mechanisms of suppression, there is growing evidence that regulatory B cells may act by cell-cell contact, such as through PD-L1 (Bodhankar et al., 2013, van de Veen et al., 2013), which may explain the lack of maximal reversal of suppression observed with blockade of IL-10 or IL-10 receptor (van de Veen et al., 2013). Additionally, cytotoxic B cells have been proposed as potential regulators of inflammation (Klinker and Lundy, 2012), with evidence of CD5$^+$ B cells also capable of expressing FASL (CD95L) (Lundy, 2002, Lundy and Fox, 2009). Granzyme B-expressing B cells, with cytotoxic capacity, have also been shown to be present within the CD5$^+$ population (Hagn et al., 2010, Hagn et al., 2009, Hagn et al., 2012), with granzyme B expression induced by IL-21 (Hagn et al., 2009, Lindner et al., 2013).

An emerging marker of IL-10-expressing B cells in mice is TIM-1 (T cell immunoglobulin and mucin domain 1) (Ding et al., 2011, Lee et al., 2012, Xiao et al., 2012). In murine models, TIM-1-deficient mice had reduced proportions of IL-10-producing B cells, a discrepancy which was exaggerated by age and accompanied by generalised autoimmunity (Xiao et al., 2012). Although mouse data for allergic airway disease paints a similar picture (Curtiss et al., 2012), with systemic loss of TIM-1 resulting in exacerbated Th2 responses, a recent study of TIM-1 in human allergen immunotherapy has shown a negative relationship with TIM-1 and allergen tolerance (Lin et al., 2013). Sublingual immunotherapy in children for house dust mite allergy was clinically effective, whereas an inverse correlation was observed between TIM-1 expression and IL-10 mRNA expression by PBMCs sampled before and at 6

months during immunotherapy. IL-5 mRNA positively correlated with TIM-1 mRNA expression. This study did not however explore the cell-source of TIM-1 expression, beyond PBMC isolation.

1.6.2. Regulatory B cells in autoimmune disease

The role of regulatory B cells has been much explored in human autoimmune disease; given that a loss of self-tolerance is required for the onset of autoimmune disease, Bregs are a likely target. Most of these studies show decreased proportions or a functional loss of capacity in the Breg compartment relative to healthy controls. This is true for systemic lupus erythematosus (SLE) (Blair et al., 2010, Lemoine et al., 2011), Graves' disease (Zha et al., 2012), multiple sclerosis (MS) (Knippenberg et al., 2011, Correale et al., 2008) and rheumatoid arthritis (RA) (Ma et al., 2013, Flores-Borja et al., 2013). However, Iwata and colleagues examined IL-10^+ B cells in moderately sized cohorts of RA, SLE, primary Sjögren's syndrome (SS), autoimmune vesiculobullous skin disease and MS patients, demonstrating a large spread in B cell IL-10 capacity, but a tendency towards increased proportions of IL-10^+ B cells in disease compared with controls (Iwata et al., 2011). This was in agreement with Lemoine et al., who also showed no changes in functionally suppressive B cells in RA or SS, but a dramatic loss of T cell suppression by CpG and anti-CD40-primed B cells amongst SLE donors compared with healthy controls (Lemoine et al., 2011). Very little work has shown the direct result of antigen stimulation on IL-10-producing B cells. One such study compared B cell IL-10 responses to the self-antigen, thyroglobulin and against tetanus toxoid (TT) in healthy control subjects (Langkjaer et al., 2012). Thyroglobulin produced higher IL-10 and IL-6 responses in B cells compared with TT stimulation, suggesting these B cells capable of autoregulation.

1.6.3. Regulatory B cells in infectious disease

Regulatory B cells in autoimmunity are a clear cell of interest, due to the loss of tolerance. Whilst no loss of self-tolerance has taken place in infectious disease, chronic disease persists due to an ability to evade or suppress immune responses; the latter may involve regulatory B cells. Evidence has been emerging of this in humans, especially with respect to viral infection. Total IL-10-producing B cells, detected by CpG stimulation, were increased in chronic Hepatitis B (HBV)-infected individuals compared to controls (Das et al., 2012). Whilst these patients also had IL-10 responsiveness to HBV stimulation, both total and HBV-induced IL-10^+ B cells correlated with viral flares. Additionally, a series of papers from different groups have recently shown similar evidence amongst HIV-1-infected individuals (Jiao et al., 2014, Liu et al., 2014b, Siewe et al., 2013). Increased proportions of IL-10^+ B cells were detected amongst HIV-1^+ donors compared to controls, which related to viral load in all studies, the latter two studies also showed depletion of IL-10^+ B cells restored CD8 responses towards the virus. Liu et al., were further able to show that the greatest proportions of Bregs were within

untreated or early disease donors, compared to those on treatment or described as 'long-term non-progressors', with IL-10$^+$ B cells predominantly CD10$^-$CD27$^+$, suggesting a memory phenotype which also expressed TIM-1.

1.6.4. Helminth infection, allergy and regulatory B cells

It has been proposed that Tregs are induced during helminth infection, deviating a Th2 response towards a Treg response (Nausch et al., 2011, van der Vlugt et al., 2012). Additionally IL-10$^+$ B cells have recently been shown to be associated with helminth burden in mice and man (Correale et al., 2008, van der Vlugt et al., 2012, Hussaarts et al., 2011). B cells derived from patients with MS were unable to suppress myelin-driven responses of a peptide-specific T cell line in co-cultures, which was fully suppressed by B cells derived from healthy controls (Correale et al., 2008). The capacity to suppress myelin-specific T cell responses was recovered in B cells derived from helminth-infected MS patients.

Helminth infection has been proposed to have therapeutic potential in allergic disease (Pritchard et al., 2012). In mouse models helminth infection has been used to demonstrate suppression of allergic airway inflammation. Two studies have independently shown that OVA-allergic models of disease can be suppressed and resolved by adoptive transfer of CD1dhi IL-10-producing regulatory B cells (Amu et al., 2010, van der Vlugt et al., 2012). Amu and colleagues were able to further demonstrate that established allergic airway inflammation was reversed by adoptive transfer of IL-10-producing CD1dhi B cells induced by Schistosome infection (Amu et al., 2010), which resolved OVA-allergic airway inflammation by the IL-10-mediated induction of regulatory T cells.

1.7. Regulatory B cells in allergic disease

Dissection of the Breg contribution to allergic inflammation has been lacking. In a recent publication, van der Vlugt and colleagues examined the characterised IL-10-producing regulatory B cell phenotypes in response to LPS and CpG (van der Vlugt et al., 2013). LPS-stimulated (but not CpG-stimulated) B cells demonstrated reduced IL-10-responses amongst allergic asthmatics compared to controls. The reduced B cell IL-10 capacity was restricted to the CD24hiCD27$^+$ subset, not CD24hiCD38 or CD1dhi B cells. The authors also demonstrate a quantitative decrease in CD24hiCD27$^+$ B cells amongst allergic asthmatics. Allergen-specific triggers have rarely been used to illicit B cell IL-10 responses. A series of studies in cow milk allergic children has suggested that B cells expressing either IL-10 or TGFβ, which compared allergic patients against sensitised, but non-allergic, subjects (Lee et al., 2011, Noh et al.,

2010). The studies of milk-allergic versus milk-sensitised children used the cow milk allergen, casein, to stimulate PBMCs *in vitro* (Noh et al., 2010). Aeroallergens from dog, grass and house dust mite have also been examined for B cell IL-10-inducing capacity (Milovanovic et al., 2009). The authors found astounding proportions of B cells producing IL-10 in response to both allergen (7.5%) and CpG (42.9%), these proportions of IL-10-specific B cells have not been repeated elsewhere under either stimulatory condition (2-5% producing IL-10 is typically reported of CpG-stimulated B cells (Iwata et al., 2011, Bouaziz et al., 2010)), but presents the enticing prospect of allergen-induced B cell IL-10.

1.7.1. Regulatory B cells and AIT

CpG is commonly used to drive IL-10 responses of B cells *in vitro* and *in vivo* (Miles et al., 2012, de Brito et al., 2010), which has led to the suggestion that CpG may be of use clinically as an adjuvant to drive both Th1 and B10 responses (Fonseca and Kline, 2009, Vollmer and Krieg, 2009). Tempting data in mice has further shown that subcutaneous administration of CpG in OVA-allergic mice is capable of suppressing and reversing allergic airway inflammation (Fonseca et al., 2011, Ashino et al., 2008). In humans, a series of ragweed immunotherapy clinical trials explored the role of CpG adjuvants. In two independent trials, the major allergen, Amb a1, conjugated to CpG showed clinical improvement in SAR and a parallel local induction of Th1 responses within the nasal mucosa (Creticos et al., 2006, Tulic et al., 2004). Another study used weekly nebulised CpG as an immunotherapeutic intervention in asthma and failed to show clinical suppression of allergen-specific asthmatic or Th2 responses (Gauvreau et al., 2006). However this approach was successful in restoring clinical measures of allergic airway disease in horses, to the level observed in healthy animals (Klier et al., 2012), suggesting further work may be needed to optimise delivery or clinical results in humans. The latter study was the only to examine IL-10, showing significantly increased production from bronchoalveolar lavage-derived cells after just 3 inhalations, compared to baseline.

It is clear that prospective studies of immunological changes during allergen-immunotherapy and following withdrawal is worthy of further study to determine the time course of induction for IL-10 and/or FoxP3-expressing regulatory T cells, IL-10-producing regulatory B cells and allergen-specific IgG4. No study has specifically examined the induction or duration of B cell derived IL-10 following the course of AIT.

Based on the available data, allergen-specific IL-10-producing T cells appear prior to the induction of allergen-specific IgG4 and/or IL-10-producing B cells (Francis et al., 2008, Möbs et al., 2012, van de Veen et al., 2013, Akdis et al., 1998). It is unclear whether regulatory T cells induce regulatory B cells, or if the reverse is true. Indeed IL-10-producing B cells correlate with $CD39^{+}$ Tregs (Wilde et al., 2013)

and further studies have suggested Bregs are able to induce both FoxP3- and CLTA-4- (cytotoxic T lymphocyte associated antigen-4) expressing Tregs in humans and mice (Kessel et al., 2012, Ray et al., 2012, Olkhanud et al., 2011, Lemoine et al., 2011, Carter et al., 2011). There is little evidence thus far of regulatory B cells induced following interaction with regulatory T cells.

2. Methods

2.1. Ethical statement

Samples obtained for this thesis followed good clinical practice and received ethical approval and local approval from the Royal Brompton and Harefield Hospital NHS trust. Informed and written consent was taken from volunteers prior to phlebotomy or biopsy.

2.2. Blood donor selection

Volunteers were self-selected in response to advertisements for grass pollen allergic, non-allergic or allergen-specific immunotherapy (AIT)-treated blood donors. AIT-treated patients were obtained routine NHS outpatient clinical visits at the Royal Brompton Hospital. All volunteers were characterised as to their allergic status. Skin prick tests (SPT) were performed on the volar aspect of the forearm using an extract of timothy grass (Phleum pratense, Phl p) pollen (Aquagen SQ, ALK, Abelló, Denmark). Histamine (10 mg/mL histamine dihydrochloride, ALK) was used as positive control and saline diluent as negative control. A sterile lancet was used to pierce the skin where allergen drops had been applied. After 20 minutes, the presence of erythema and wheal size was recorded by encircling the wheal using a fine, fibre-tipped pen and transferring to a record sheet with adhesive tape. A wheal with diameter >3mm was taken as positive. Positive SPT, timothy grass-specific IgE (>0.35 KU/L) and a clinical history of grass pollen allergies lead to patients being classified as allergic. Non-atopic patients underwent the same classification and had a negative SPT to grass and other common aeroallergens, allergen-specific IgE and clinical history. Patients who received AIT were screened within the NHS and met a clinician-diagnosed need for either sublingual (SLIT) or subcutaneous (SCIT) immunotherapy.

2.3. Cell isolation and culture

2.3.1. Phlebotomy and PBMC isolation

Venous blood was collected in sodium-heparinised vacutainer tubes (BD Biosciences, Oxford, UK) and peripheral blood mononuclear cell (PBMC) isolation was begun within 2 hours of phlebotomy. Tubes were centrifuged at 1500rpm and plasma layer removed. Blood was diluted in equal volumes of RPMI 1650 medium (Gibco, Life Technologies, Paisley, UK). Diluted blood was layered over ficoll (GE Healthcare, Buckinghamshire, UK) and centrifuged at 2200 rpm for 25 minutes without braking.

Following centrifugation the buffy coat layer was extracted and washed 3 times in RPMI 1650. PBMC viability was assessed by Trypan blue exclusion, with viable cells counted by haemocytometer.

2.3.2. Cell subset isolation

$CD19^+$ B cells or $CD4^+$ T cells were isolated by negative selection using EasySep magnetic enrichment (Stem Cell Technologies, Manchester, UK) following manufacturer's instructions. Purity of $CD19^+$ or $CD4^+$ cells following isolation was established by single colour flow cytometry. Although purity of 95% was considered acceptable for experiments with single cells, >98% purity was routinely detected by this protocol, which was superior to purity observed by magnetic separation using MACS columns (Miltenyi Biotec, Oxford, UK).

2.3.3. Cell culture

B or T cells were reconstituted to $5x10^6$ cells/mL in tissue culture medium (TCM). TCM contained 10% FCS in RPMI 1650 containing L-glutamine and HEPES (Gibco). Routinely, $5x10^5$ cells/well were cultured in 200 µL of TCM, incubated at 37 °C in 5% CO_2 atmosphere in flat-based 96 well plates (Nunc, Thermo Scientific, UK), unless otherwise stated. Cell culture reagents were added at the following concentrations unless shown otherwise, CpG ODN 2006, a type B CpG, 1 µg/mL (5' –tcg ttt cgt ttt gtc gtt – 3') (InvivoGen, Toulouse, France), LPS, 1 µg/mL (Sigma-Aldrich, Poole, UK), recombinant CD40L, 0.01 µg/mL (R&D systems, Abingdon, UK) and anti-human CD40 antibody, 1 µg/mL (clone 5C3, BD Biosciences).

2.4. CFSE

T cells were labelled with intracellular CFSE (carboxyfluorescein succinimidyl ester) in order to determine the proportion of cells proliferating. CellTrace CFSE (Invitrogen, Life Technologies, UK) was reconstituted to 5 mM with DMSO as per manufacturer instructions. A known number of cells to be labelled were pelleted by centrifugation and resuspended in 37 °C pre-warmed PBS with CFSE at 10 µM to a total cell concentration of $10x10^6$ cells/mL. Cells were incubated for 10 minutes in the dark at 37 °C and 5% CO_2 whilst CFSE was internalised. Following the 10 minute culture, the reaction was quenched with an equal volume of cold (4 °C) FCS initially and additional cold TCM to bring it to a final volume 10x that of the PBS/CFSE incubated with the cells. Cells were incubated at 4 °C for 10 minutes before centrifugation and resuspending in warm TCM for addition to cell culture plates. CFSE-labelled cells were always analysed on the flow cytometer immediately after staining to ensure internalisation of the dye.

2.5. Co-culture

Cells for co-culture were concurrently isolated from the same donor; the outline for the protocol is shown in Figure 2. B cells were either cultured in TCM or TCM containing 1ug/mL CpG for 48 hours at $2.5x10^6$ cells/mL on a flat bottom 48 well plate (Nunc). $CD4^+$ T cells were isolated separately and cultured alone for 48 hours in TCM at $5x10^6$ cells/mL, usually on 12 well plates due to increased numbers of cells. Following pre-culture T cells were washed in TCM and counted, using Trypan blue for dead cell exclusion (although few were observed). B cells from both CpG and TCM cultures were resuspended from wells, with wells washed with TCM to ensure maximum recovery. Cells were washed twice before counting, with Trypan blue exclusion for dead cells (death was consistent between cultures, although cells from CpG culture were often larger), and suspension at the required concentration.

2.5.1. Polyclonal T cell co-culture

For polyclonal T cell stimulus, flat based 96 well plates had been coated with anti-CD3 and anti-CD28 antibodies (clones UCHT1 and 37407, respectively, both from R&D systems, Oxford, UK), each at 1 µg/mL, in PBS for 6 hours at 37 °C. Wells which remained non-coated (PBS alone) would be for unstimulated negative controls. Before culture, plates were washed with PBS, pipetted empty, and not allowed to dry before the addition of cells or TCM. T cells were stained with CFSE (as described above) before addition to culture wells. Unstimulated and stimulated wells with T cells alone were used as negative and positive controls for proliferation, respectively. These wells received the same initial number of T cells and total volume of TCM in the absence of B cells. Co-culture took place in antibody coated wells only. Initially only equal numbers of T and B cells were added to each well, such that 100,000 cells of each were in a 200 µl final volume in 96 well flat bottom plates. Where cells were co-cultured at ratios to one another, T cell numbers remained the same with decreasing B cells used, such that 2:1 of T:B cells would indicate 100,000 T cells to 50,000 B cells. B cell numbers were reduced by adding decreasing volumes of stock, so 100,000 cells in 100 µl would mean the addition of 50 µl of cells for 50,000 cells. This way dilution was representative of the 1:1 starting point and minor counting errors were not compounded.

2.5.2. Allergen-driven T cell co-culture

Allergen-driven co-cultures required the presence of irradiated PBMCs, which were combined with T cells in equal numbers, to present allergen to T cells. Non-$CD4^+$ PBMCs were preserved for this purpose in TCM for 48 hours (following $CD4^+$ T cell negative selection), before being irradiated at 50 grey, washed in TCM and counted for co-culture. Irradiated PBMC (referred to in this protocol as antigen

presenting cells, APC) and T cells were combined in equal numbers and stained with CFSE (as described above) together. T cells and APC were cultured alone, stimulated and unstimulated as positive and negative controls respectively. Allergen-stimulated wells, which included all B and T co-culture wells, received 5µg/mL of Phl p. All wells had a final volume of 200 µl of cells in TCM with or without Phl p. The maximum number of cells were 100,000 T cells, 100,00 APC and 100,000 B cells. Ratios of decreasing B cells relative to T cells were achieved as described previously for polyclonal T cell stimulation. During this co-culture, inhibitory (blocking) antibodies were added to cultures at the start (day 0) or throughout (day 0, 3 and 6). Blocking antibodies listed in Table 2 were added at 5 µg/mL on each occasion they were added.

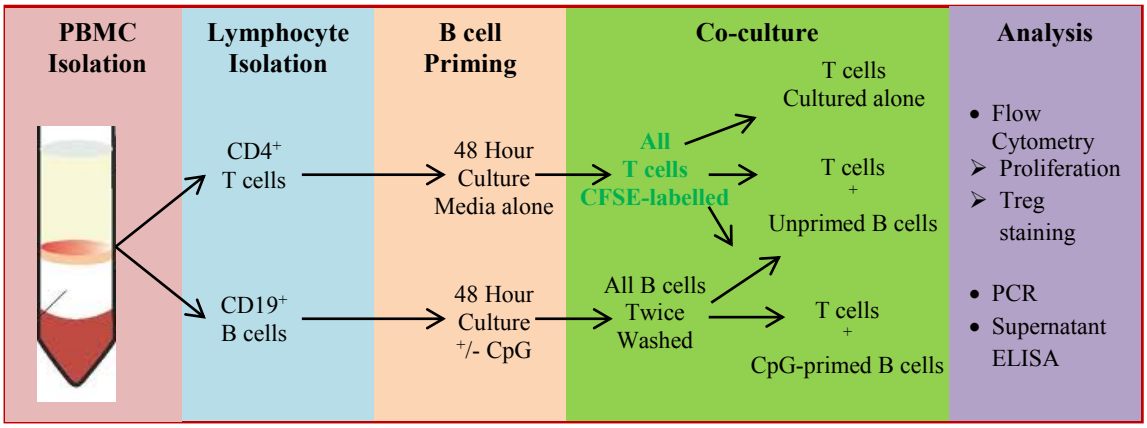


Figure 2 – Diagram of B and T cell co-cultures.

This illustration demonstrates the protocol for co-culture of T and B cells following PBMC isolation. B and T cells are separately isolated and cultured for 48 hours in medium or CpG as shown. Following this, primed or un-primed B cells are twice washed before co-culture with CFSE-labelled T cells. T cells were stimulated with anti-CD3 and CD28 or allergen in the presence of irradiated non-$CD4^+$ T cell PBMCs. Following co-culture, analysis was carried out by flow cytometry of proliferation or regulatory T cell generation. Cells were also lysed for qPCR, with supernatants retained for ELISA.

Target	Clone	Isotype	Purchased from
CD274 (PD-L1)	B7-H1	LEAF purified Mouse IgG_{2b}, κ	BioLegend
IL-10	25209	Mouse IgG_{2b}, κ	R&D
IL-10Ra	37607	Mouse IgG_1, κ	R&D
TGF-$\beta_{1,2\&3}$	1D11	Mouse IgG_1, κ	R&D
CD80	37711	Mouse IgG_1, κ	R&D
CD86	37301	Mouse IgG_1, κ	R&D
Isotype control	MPC-11	LEAF purified Mouse IgG_{2b}, κ	BioLegend

Table 2 – Blocking antibodies used in co-culture.

Above lists the antibody targets, clone, isotype and manufacturer of commercially available antibodies used in T and B cell co-cultures to block cell-cell or paracrine suppression of T cell responses.

2.6. Flow Cytometry

2.6.1. Staining Panels

Cells were stained with fluorochrome-coupled monoclonal antibodies (Table 3), at half the volume described by the manufacturer, which produced a clear signal in combination staining. Staining of 8 (or fewer) fluorochrome-conjugated antibodies were carried out using the following panel design:

1) FITC or Alexa Flour 488

2) PE

3) PerCP or PerCP-Cy5.5 or PerCP-eFlour 710

4) PE-Cy7

5) APC or Alex Flour 647

6) APC-Cy7 or APC-H7

7) Pacific Blue or BD Horizion V450

8) AmCyan or BD Horizion V500.

2.6.2. Compensation

Panels were compensated in advance of use in the main experiment. Initially, antibodies coupled to the same fluorochromes in the panels, but directed at common PBMC surface antigens (e.g. CD3, CD4) were used. Single stained and unstained tubes were analysed on the flow cytometer, with automatic software compensation (BD FACS Diva, BD Biosciences). The degree of compensation was subsequently adjusted by eye once both compensation and experimental panels had been acquired, if required. Preference for compensation was given to the experimental panel. However, it was common that less compensation was required with this protocol, as stronger signals could be produced from the antibodies used to compensate.

Later experiments used compensation beads (BioLegend), with which the experimental antibodies could be incubated in individual tubes. Following incubation the beads were washed and acquired as previously stated, with automated software compensation. The full panel was also run with the experimental antibodies (including permiablisation where relevant) on PBMCs to check compensation. If required, compensation was manually adjusted.

Compensation was unchanged within complete experiments.

2.6.3. Experimental surface staining

Cells were stained on U bottom 96 well plates or occasionally in 5 mL flow cytometry tubes. Cells for staining were washed in cell staining buffer (PBS, 1% BSA and 0.05% Sodium Azide), centrifuging at 200 g for 5 minutes between washing and vortexing to resuspended. Cells at $10x10^6$ cells/mL in staining buffer were incubated with 10 µL Fc receptor block (Miltenyi Biotec) for 10 minutes at room temperature. Pre-prepared (no more than 24 hours in advance) antibody aliquots, towards cell surface antigens, were added and fully distributed by pipetting, before incubation for 30 minutes at room temperature in the dark. Cells were hidden from light throughout the protocol from this point on. Following incubation cells were washed in staining buffer. If intracellular or intranuclear staining was carried out the protocol below was carried out. If surface staining alone was carried out cells were fixed in 1x cell fix solution (BD Biosciences) before running on FACS Canto II (BD Biosciences).

2.6.4. Experimental intracellular staining

To detect intracellular cytokines by flow cytometry, cells were re-stimulated for the final 5 hours of incubation with 20ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 µg/mL Ionomycin (Sigma-Aldrich) and 1 µL Golgi Plug (BD Biosciences) per well containing $5x10^5$ cells. This re-stimulation protocol was only relevant for detection of intracellular cytokines and was not used for intranuclear staining (FoxP3). Cell surface staining was carried out as detailed above, following which cells were resuspended in 1x ForxP3 perm/fix buffer (Biolegend, Cambridge, UK) and incubated for 20 minutes at room temperature. Cells were centrifuged again, washed in cell staining buffer. Cells were then washed and re-suspended in 1x perm/wash buffer (Biolegend) and incubated for 15 minutes at room temperature in the dark. Cells were centrifuged and resuspended in residual perm/wash buffer before the addition of antibodies towards intracellular and/or nuclear antigens. Cells and antibodies were incubated for 30 minutes at room temperature. Finally cells were washed and resuspended in cell staining buffer before running on FACS Canto II (BD Biosciences).

Analysis of flow cytometry data was carried out on FACS Diva (BD Biosciences) or FlowJo software (Tree Star, California, USA).

Target	Fluorochrome	Clone	Isotype	Purchased from
7AAD	PerCP	n/a	n/a	BD Biosciences
Annexin V	APC	n/a	n/a	BD Biosciences
CCR1	Alexa Flour 488	53504	Mouse IgG_{2b}, κ	R&D
CCR10	APC	6588-5	Hamster IgG_1	BioLegend
CCR2	PerCP-Cy5.5	TG5/CCR2	Mouse IgG_{2b}, κ	BioLegend
CCR3	Alexa Flour 647	5E8	Mouse IgG_{2b}, κ	BD Biosciences
CCR4	PE-Cy7	1G1	Mouse IgG_1, κ	BD Biosciences
CCR5	V450	2D7/CCR5	Mouse IgG_{2a}, κ	BD Biosciences
CCR6	PE-Cy7	11A9	Mouse IgG_1, κ	BD Biosciences
CCR7	V450	150503	Mouse IgG_{2a}, κ	BD Biosciences
CCR8	APC	191704	Rat IgG_{2b}, κ	R&D
CCR9	PerCP-Cy5.5	BL/CCR9	Mouse IgG_{2a}, κ	BioLegend
CD10	PerCP-Cy5.5	HI10a	Mouse IgG_1, κ	BioLegend
CD127	Alexa Flour 647	HIL-7R-M21	Mouse IgG_1, κ	BD Biosciences
CD19	PE-Cy5	HIB19	Mouse IgG_1, κ	BD Biosciences
CD19	V500	HIB19	Mouse IgG_1, κ	BD Biosciences
CD1d	PE	CD1d42	Mouse IgG_1, κ	BD Biosciences
CD24	APC	SN3 A5-2H10	Mouse IgG_1, κ	eBioscience
CD25	APC-Cy7	M-A251	Mouse IgG_1, κ	BD Biosciences
CD25	PerCP-Cy5.5	BC96	Mouse IgG_1, κ	BioLegend
CD27	APC-H7	M-T271	Mouse IgG_1, κ	BD Biosciences
CD274	FITC	MIH1	Mouse IgG_1, κ	BD Biosciences
CD3	PE-Cy7	SK7	Mouse IgG_1, κ	BD Biosciences
CD38	V450	HB7	Mouse IgG_1, κ	BD Biosciences
CD4	V450	RPA-T4	Mouse IgG_1, κ	BD Biosciences
CD5	PE-Cy7	L17F12	Mouse IgG_{2a}, κ	BD Biosciences
CD71	PE	M-A712	Mouse IgG_{2a}, κ	BD Biosciences
CD73	FITC	AD2	Mouse IgG_1, κ	BD Biosciences
CXCR1	FITC	5A12	Mouse IgG_{2b}, κ	BD Biosciences
CXCR2	FITC	6C6	Mouse IgG_1, κ	BD Biosciecnes
CXCR3	APC-Cy7	G025H7	Mouse IgG_1, κ	BioLegend
CXCR4	PE-Cy7	12G5	Mouse IgG_{2a}, κ	BD Biosciences
CXCR5	PerCP-Cy5.5	TG2/CXCR5	Mouse IgG_{2b}, κ	BioLegend
FoxP3	Alexa Flour 488	259D/C7	Mouse IgG_1, κ	BD Biosciences
IL-10	PE	JES3-19F1	Rat IgG_{2a}	BD Biosciences

Table 3 – Flow cytometry antibodies used.

This table lists the antibody targets, clone, isotype and manufacturer of commercially available antibodies used in flow cytometry both for staining cell surface markers and intracellular targets.

2.6.5. Flow cytometry of whole blood

Blood was collected as previously described and staining for flow cytometry began within 2 hours of collection. Antibody staining panels were designed as previously described, using antibodies in Table 3, with manufacturer described volumes used for each. Isotype matched control antibodies were used in the same concentrations as test antibodies. On collection, at least 200 µL of whole blood was reserved per tube (test or isotype), 20 µL Fc receptor block (Miltenyi Biotec) added per 200 µL of blood and incubated for 10 minutes at room temperature. Following incubation, 220 µL of this sample (so 200 µL of whole blood in total) was added to antibodies already prepared in 5mL flow cytometry tubes (BD Biosciences) combined into staining panels (test or isotype controls). Blood and antibodies were incubated for 30 minutes at room temperature in the dark before a further 10 minute incubation with 2mL of red blood cell lysis buffer (10x contains 90g NH_4Cl, 10g $KHCO_3$ and 370mg EDTA in 1L of dH_20 filtered through a 0.22 µm filter), and vortexed. Following lysis, tubes were centrifuged for 5 minutes at 200g at room temperature and washed twice (with vortexing) with 2mL cell staining buffer. Finally cells are re-suspended in 350 µL of cell fix (BD Biosciences). Immediately before acquisition 50 µL (therefore a known number, described on each sample) of counting beads (CountBright absolute count beads, Molecular Probes, Life Technologies, Paisley, UK) at room temperature are vortexed and added. Counted beads allowed for back calculation of cell numbers per µL of blood stained, as described by manufacturer. FSC and SSC voltages had been previously set to include both cell counting beads and whole blood cell subsets. Cells and beads were acquired on FACS Canto II (BD Biosciences). Analysis was carried out on FACS Diva (BD Biosciences) or FlowJo (Tree Star, California, USA) software.

2.6.6. Live/Dead staining

Following culture, cells were stained for stage of cell death. The dyes 7AAD and Annexin V (as described in Table 3), which bind exposed intranuclear DNA or phosphatidylserine on the inverted cell membrane, were used in combination to identify cells in the latter stages of death with permeable cell membranes, and cells with inverted cell membranes in the early stages of apoptosis, respectively. Live cells are double negative, pre-apoptotic cells stain for Annexin V only whilst apoptotic cells stain double positive for both 7AAD and Annexin V. All cells were kept on ice and washed twice in staining buffer before re-suspending cells in 100 µL 1x Annexin V binding buffer (BD Biosciences). 5 µL of 7AAD and Annexin V were each added to 90 µL of binding buffer per well required and vortexed before adding 100 µL to each well to be stained, pipetted to re-suspend cells and incubated for 15 minutes on ice. Cells were acquired on the flow cytometer within 60 minutes of 7AAD and Annexin V addition.

2.7. Enzyme-Linked ImmunoSorbant Assay (ELISA)

2.7.1. IL-10 Sandwich ELISA

IL-10 ELISAs (R&D Systems) were carried out following manufacturer's instructions, in 96 well maxisorb plates (Nunc, Fisher Scientific, Loughborough, UK). The concentration standard was diluted in serial dilutions from 2000-15.625 pg/mL and cell culture supernatants were diluted 1:2 with the recommended diluent buffer (PBS with 1% BSA – Merk Millipore, Watford, UK), 100 µL was added to duplicate wells. Plates were washed between steps, as recommended, 3x with 400 µL PBS with 0.05% Tween 20 (Sigma-Aldrich). The plate was developed with 100 µL per well of Tetramethylbenzidine (TMB)-containing buffer (20mL Phosphocitrate buffer (2.84 g Na_2HPO_4, 1.92 g anhydrous citric acid in 200 mL distilled water - pH 5), 200 µL TMB and 6 µL of 30% hydrogen peroxide solution – all reagents from Sigma-Aldrich). TMB-buffer was added for 30 minutes, or until significant colour change was observed in highest concentration controls, in the dark. Colour development was completed and arrested by adding 50 µL 1.8 M sulphuric acid (Sigma-Aldrich) per well. Plates were read at 450nm on a 96 well Emax plate reader (Molecular Probes, Oregon, USA) and analysis was carried out on Softmax Pro software (Molecular Devices Corporation, USA).

2.7.2. Multiplex ELISA

Multiplex ELISA (Merk Millipore) was carried out in experiments that called for multiple analytes to be assessed from limited samples. MILLIPLEX xMAP MAGPIX multiplex assays were used, both the 42 analyte (HCYTOMAG-60K-42plex) and 25 analyte (HT17MG-14K-PX25) ELISAs were used. Supernatants stored at -20 °C for no more than 3 months were fully defrosted at 4 °C and allowed to come to 20 °C before vortexing and addition where appropriate. The protocol was performed according to manufacturer's instructions. Briefly, all wells were washed with 200 µL of assay buffer and shaken for 10 minutes before decanting. 25 µL of sample was added to samples wells or 25 µL of TCM to standards, background and quality control (QC) wells. Assay buffer was added to all wells (containing cytokine standard dilutions or QCs where appropriate) followed by vortexed and sonicated magnetic cytokine detection beads. The plate was covered and incubated overnight at 4 °C continuously shaken. Magnetic beads were then twice washed with wash buffer (provided) and incubated with detection antibodies for 1 hour at room temperature. Following incubation, streptavidin-phycoerythrin was added and incubated for 30 minutes at room temperature. Magnetic beads were twice washed and before reconstituting in MAGPIX sheath fluid. The plate was then run on MAGPIX with xPONENT software (Merk Millipore) to determine MFI (of at least 50 beads) per well for each analyte being tested. The concentrations of analytes tested were determined based on the standard curve for known standards and confirmed by concentrations of 2 known QCs provided.

2.8. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Following or prior to culture, cells were pelleted and resuspended in 200 µL RLT lysis buffer (Qiagen, West Sussex, UK). RNA extraction used RNAeasy (Qiagen) spin columns and reagents, including the use of QIAshreader columns according to manufacturer's instructions. To generate cDNA, 11.5 µL of RNA was added to 4 µL of 5x RT buffer, 2 µL of dNTPs, 1 µL of random hexamere, 0.5 µL of RNAse inhibitor and 1 µL of reverse transcriptase enzyme per reaction tube (all from Fermentas, Thermo Fisher Scientific, Loughborough, UK). cDNA was transcribed in a PCR cycler (SensQuest, Applied Biosystems) for 10 minutes at 25 °C, 60 minutes at 42 °C and 10 minutes at 70 °C, cDNA samples were left at 4 °C for use within hours or at -80 °C for longer term storage. The relative expression of mRNA was determined using the log phase of amplification with specific primers (all obtained from Sigma Aldrich, Dorset, UK) directed at the cDNA for the gene of interest. Primers towards 18s were used in the same reverse transcribed samples, to control for DNA concentration variation such as cell numbers lysed. Primers were at 100 µmol in DNAse-free distilled water. Primer master mixes contained 230 µl of DNAse-free distilled water and 10 µL of forward and reverse primer. For relative real time PCR, 1.6 µL of primer master mix was added to 3.4 µL cDNA (diluted 1:10 with dH_2O) and 5 µL iTaq SYBR Green Supermix with ROX (Biorad, Hemel Hempstead, UK) using epMotion 5075 LH automated pipetting system (Eppendorf, Cambridge, UK) in triplicate for each sample and primer on 384-well plates (Applied Biosystems). For each primer, dH_2O was used in the place of cDNA to establish non-specific amplification. The plate was sealed and spun for 5 minutes at 500g to remove bubbles before running on 7900HT Fast Real-Time PCR System (Applied Biosystems) using the provided software (SDS, Applied Biosystems) relative delta-delta CT values between samples defined the relative change in IL-10 mRNA.

Target	Forward	Reverse
18s	CAG CCA CCC GAG ATT GAG CA	TAG TAG CGA CGG GGG GTG TG
IL-10	TGC CTA ACA TGC TTC GAG ATC TC	GTT GTC GAG CTG ATC CTT CAT TT
IL-4	AAA CGG CTC GAC AGG AAC CT	ACT CTG GTT GGC TTC CTT CAC A
IL-6	TGG CTG AAA AAG ATG GAT GCT	GAT GAT TTT CAC CAG GCA AGT
TGF-β	AAA TTG AGG GCT TTC GCC TTA	GGA CCC GTT GAT GTC CAC TTG
IL-13	AGA CCA GAC TCC CCT GTG CA	TGG GTC CTG TAG ATG GCA TTG
IFNγ	TCT CGG AAA CGA TGA AAT ATA CAA GTT	GTA ACA GCC AAG AGA ACC CAA AA
IL-17A	CCA TCC CCA GTT GAT TGG AA	CTC AGC AGT AGC AGT GAC A
FOXP3	GAA ACA GCA CAT TCC CAG AGT TC	ATG GCC CAG CGG ATG AG
GATA-3	GCG GGC TCT ATC ACA AAA TGA	GCT CTC CTG GCT GCA GAC AGC
T-BET	GAT GCG CCA GGA AGT TTC AT	GCA CAA TCA TCT GGG TCA CAT T
IL-10R2	CTG GAA AAA CGG TAC TGA AAA	CCA TGG CTC GAG CTT TCT GA

Table 4 – Primers used for qPCR of mRNA towards genes listed.

The forward and reverse primers used in this thesis are listed towards cDNA reverse transcribed from mRNA of the proteins listed.

2.9. IL-10 FluoroSpot

IL-10 FluoroSpot (Diaclone, Oxford, UK) was used to quantify proportions of IL-10-producing B cells. The protocol was followed as per manufacturer's instructions. Briefly, 96 well PVDF (polyvinylidene difluoride)-bottomed plates were washed and coated with IL-10 capture antibody overnight at 4 °C. The plate was washed and incubated with TCM for 2 hours at room temperature before washing and adding $5x10^5$ B cells in medium, along with the stimulatory conditions specified, totalling 200 µL/well. Where B cell numbers permitted, wells were repeated in triplicate and averages were taken of spot counts between these repeats. Wells A1-A4 always remained acellular with medium alone in order to determine assay background following development and IL-10 spot detection. The plate was incubated at 37 °C and 5% CO_2 for 42 hours, strictly motionless, to allow spots to form. Following incubation, wells were emptied and incubated with 100 µL wash buffer (PBS^+ 0.05% Tween 20) for 10 minutes at 4 °C before washing 3x. The plate was kept in the dark as much as possible from this point forward. FITC-conjugated anti-IL-10 detection antibody was made up in PBS with 1% BSA and added (100 µL per well) and incubated for 1.5 hours. Wells were washed 3x and incubated with anti-FITC green fluorescence conjugate (100 µL per well in PBS^+ 1% BSA) for 1 hour before washing again. Finally wells are incubated with 100 µL of 5% fluorescence buffer in PBS per well for 15 minutes before washing 3x as before and the membrane was washed in distilled water. The membrane was allowed to dry in the dark and a plate reader fitted with a UV light used to visualise IL-10 spots (green), automated image capture and counting of IL-10 spots was carried out on an AID iSpot FluoroSpot Reader System (Oxford Biosystems Cadama, Oxford, UK). Automated counting settings were adapted to count spots of all sizes, including the smallest, whilst sensitivity was adjusted to minimise counting of medium only wells. All plates were developed and analysed blind and counts of spots in acellular wells (mean of A1-A4) were deducted from the counts in every condition of that plate.

2.10. Cross-sectional study

Donors were recruited for a cross-sectional single-blinded (researchers and clinical staff) study of AIT. 14 SAR patients and 18 AIT-treated subjects were recruited from the Royal Brompton Hospital Allergy Clinic. 14 non-allergic healthy controls were recruited through advertisements for comparison. Of the immunotherapy treated participants, 8 had received SCIT and 6 SLIT, 4 subjects (2 on SCIT and 2 on SLIT had completed immunotherapy 12-48 months earlier, referred to as the 'immunotherapy withdrawal group'. Clinical administrators blinded all clinical staff and researchers to volunteer phenotype until analysis from the point at which volunteers were successfully included in the study, following clinical history and screening.

2.10.1. Inclusion and exclusion criteria

Volunteers for this study were screened and included on the basis of the previously described clinical criteria (section 2.2) before being admitted onto this study. Volunteer total and allergen specific (Phl p 5) IgE is also shown for these patients, but as before allergic donors had allergen-specific IgE > 0.35 IU/mL (quantified by technicians following manufacturer's instructions (ImmunoCAP, Phadia, Thermo Scientific) and in line with NHS routine diagnostic procedures). Donors were included based on meeting the minimal allergic or non-allergic criteria and were excluded if they experienced chronic or recurrent sinusitis, were currently smoking or had a smoking history of >5 pack years, had perennial asthma or FEV1 <70% of that predicted based on gender and age. This study was conducted outside of the grass pollen season in the UK.

2.10.2. Self-assessment questionnaires

Global evaluation of symptoms was a subjective symptom rating from 0 to 3 (labelled "none, mild, moderate and severe") for symptoms when they were at their most severe during the last grass pollen season (each of runny nose, blocked nose, sneezing, itchy nose, itchy eyes and watery eyes were rated), the maximum score of 18 could be achieved. This global evaluation is a long standing symptom score (Bousquet et al., 1987), which has recently also been validated and adapted within the department (Scadding et al., 2012). Change in pollen symptoms was a subjective assessment of how patients' hay fever this last year was, compared to previous years. Where 0 represented 'the same', +3 was labelled 'much better' and -3 was labelled 'much worse'. Patients receiving immunotherapy were asked how their hay fever this year compared with the years before they had started treatment. For those patients initially screened prior to their first pollen season, their initial data was excluded and change in pollen symptoms, global evaluation and pollen season mRQLQ (mini Rhinoconjunctivitis Quality of Life Questionnaire) were repeated following their first pollen season. mRQLQ testing recorded retrospective rhinoconjunctivitis quality of life outside of the grass pollen season and during the pollen season, both were retrospectively recorded. As mentioned, AIT patients who had not yet experienced a grass pollen season since commencement of AIT were followed up after their first season. mRQLQ reported scores from 0 (not troubled) to 6 (extremely troubled) for 3 discrete life impacts within each of the following sections; daily activities, nose symptoms, eye symptoms, other symptoms (fatigue, thirst and irritability) and 2 which impact practical problems. Scores were totalled and the mean was taken, with 6 being the greatest possible score.

2.10.3. Intradermal allergen challenge

Intradermal challenge was carried out on the extensor surface of the forearm, midway between the elbow and wrist, by injection of 0.02 mL of albumin-based diluent containing a total of 1BU of Phl p, equivalent to 33SQ-U or 670pg of major allergen Phl p 5. The resulting oedema (wheal) was measured at 15 minutes (early phase) and 8 hours (late phase) by palpating at 15 minutes and by tracing the skin with a pencil before resistance is felt. Ink pen was used to draw the outline and self-adhesive Scotch tape was used to transfer to patient's record. The mean diameter (in mm) was used for 'wheal size' reported here.

2.10.4. Nasal allergen challenge (NAC)

All volunteers included in the study took part in a nasal allergen challenge (NAC) which has been extensively validated by Dr. Scadding as a controlled analogue to environmental nasal allergen exposure (Scadding et al., 2012). Physiological measures of peak nasal inspiratory flow (PNIF) were undertaken using a mouth and nasal mask with Youlten peak nasal inspiratory flow meter attached. Clinicians oversaw PNIF measures with volunteers prior to challenge (baseline) and throughout the first hour of challenge (early phase response) and during the remaining 7 hours (late phase response). Data was recorded as change in PNIF (L/min) from baseline over time and reported as area under the curve during the early phase or late phase response, or the two combined equally weighted.

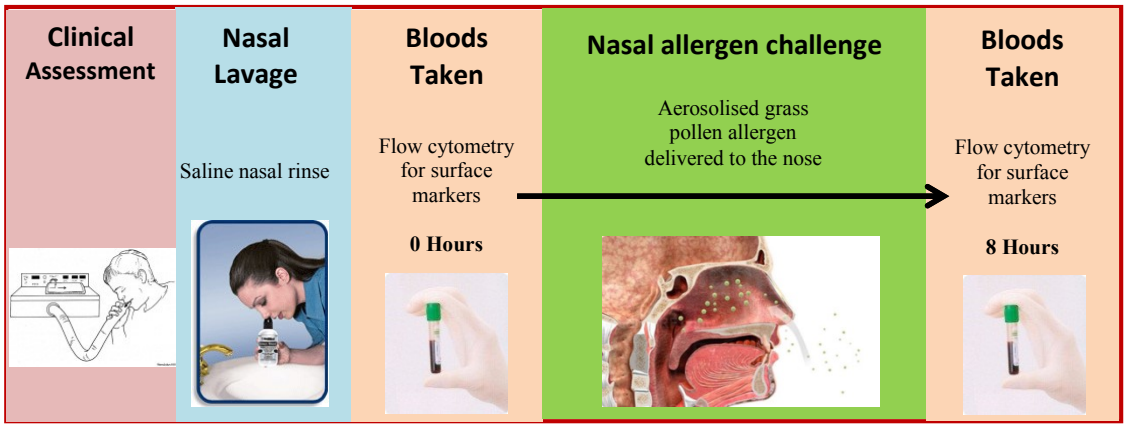


Figure 3 – Diagram of nasal allergen challenge (NAC) protocol.

Volunteers who were recruited for the cross-sectional study of allergen immunotherapy underwent a nasal allergen challenge, an *in vivo* allergen challenge able to replicate nasal allergic symptoms on allergen exposure in allergic individuals. Donors were initially screen clinically and had baseline clinical measures of respiratory function carried out before nasal lavage. Allergen challenge was preceded by phlebotomy for baseline blood characterisation. Allergen challenge was administered bilaterally and following 8 hours phlebotomy was repeated to examine post challenge cell subsets.

For the examination of B cells, 10 mL of blood was taken as previously described (section 2.3.1) before (0 hours) and after (8 hours) NAC in order to quantify B cell subsets by whole blood flow cytometry (described in section 2.6.5), as outlined in Figure 3. All volunteers underwent baseline observations of blood pressure, temperature, peak nasal inspiratory flow and spirometry. A bilateral saline nasal rinse (SinusRinse, Neilmed, USA) was carried out, following manufacturer's instructions. Following nasal rinse, 10,000 BU/mL of Phl p was administered in separate 100 µL sprays, one per nostril, by an applicator device (Aptar Pharma, Germany) aimed posterio-laterally.

2.11. Immunohistochemistry

Tonsil sections were received from routine tonsillectomy immersed in 5% paraformaldehyde (Sigma Aldrich) in PBS before storage. Sections of tonsil initially fixed in acetone had also been used, however although staining was successful, the morphology of the section was disrupted making it features unrecognisable in acetone fixed samples. Nasal sections were similarly treated, but acquired in the weeks prior to staining as part of routine validation of immunohistochemical staining from a pool of n=6 allergic donors (n=4 untreated allergic, n=2 SCIT). Nasal sections were taken from the inferior turbinate of either nostril. Biopsies were stored at -80 °C before cryostat sectioning to cut 3 sequential 4 µm sections per glass slide. Sections were allowed to dry onto the slides at 37 °C overnight before being wrapped in aluminium foil and stored at -80 °C until use.

2.11.1. Immunostaining

On the day of staining, sections were thawed at room temperature; slides were labelled with the staining they would receive in pencil, before a silicone ring was drawn around the section using an Immune Edge pen H4000 (Vector labs). Some tonsil sections had been stored unfixed, so were fixed on thawing in 4% paraformaldehyde in PBS for 5 minutes. All sections were then washed twice in PBS for 5 minutes with agitation and biotin block was used (Vector labs, Peterborough, UK). 1 drop of Avidin D solution was added to each section and incubated for 15 minutes, before washing for 5 minutes in PBS and again incubating for 15 minutes with 1 drop of biotin solution. Sections are washed once again for 5 minutes in PBS before being blocked with 60 µl per section of serum from the animal the secondary antibody was raised in. For donkey, 20% normal donkey serum (Stratech, Suffolk, UK), made up in PBS was used. Horse serum was used from Vectastain ABC-AP mouse IgG kit (Vector labs, Peterborough, UK), from which 3 drops were added to 10 mL of PBS. Where dual staining was carried out the IL-10-staining secondary antibody serum was prioritised (donkey serum). Blocking serum was incubated for 30 minutes at room temperature in a humid chamber with the lid on. Sections were not allowed to dry out once they had been immersed in PBS until the end of the protocol. Sections were incubated with primary antibody at the dilution indicated in Table 5 if not stated, for 1 hour at room

temperature. Dilutions of primary antibody were made up in 5% normal human male AB serum (NHS) (Sigma Aldrich) in PBS. Slides were washed 3x for 5 minutes in PBS with agitation before incubation in 60µl of secondary antibody as shown in Figure 4, to stain for detection by ABC-AP (Figure 4 A and B) or fluorescence microscopy (Figure 4 C). Secondary antibody, made up in 10% NHS, was incubated for 45 minutes at room temperature in a humidity chamber. The remaining staining is described below, for ABC-AP or fluorescence microscopy.

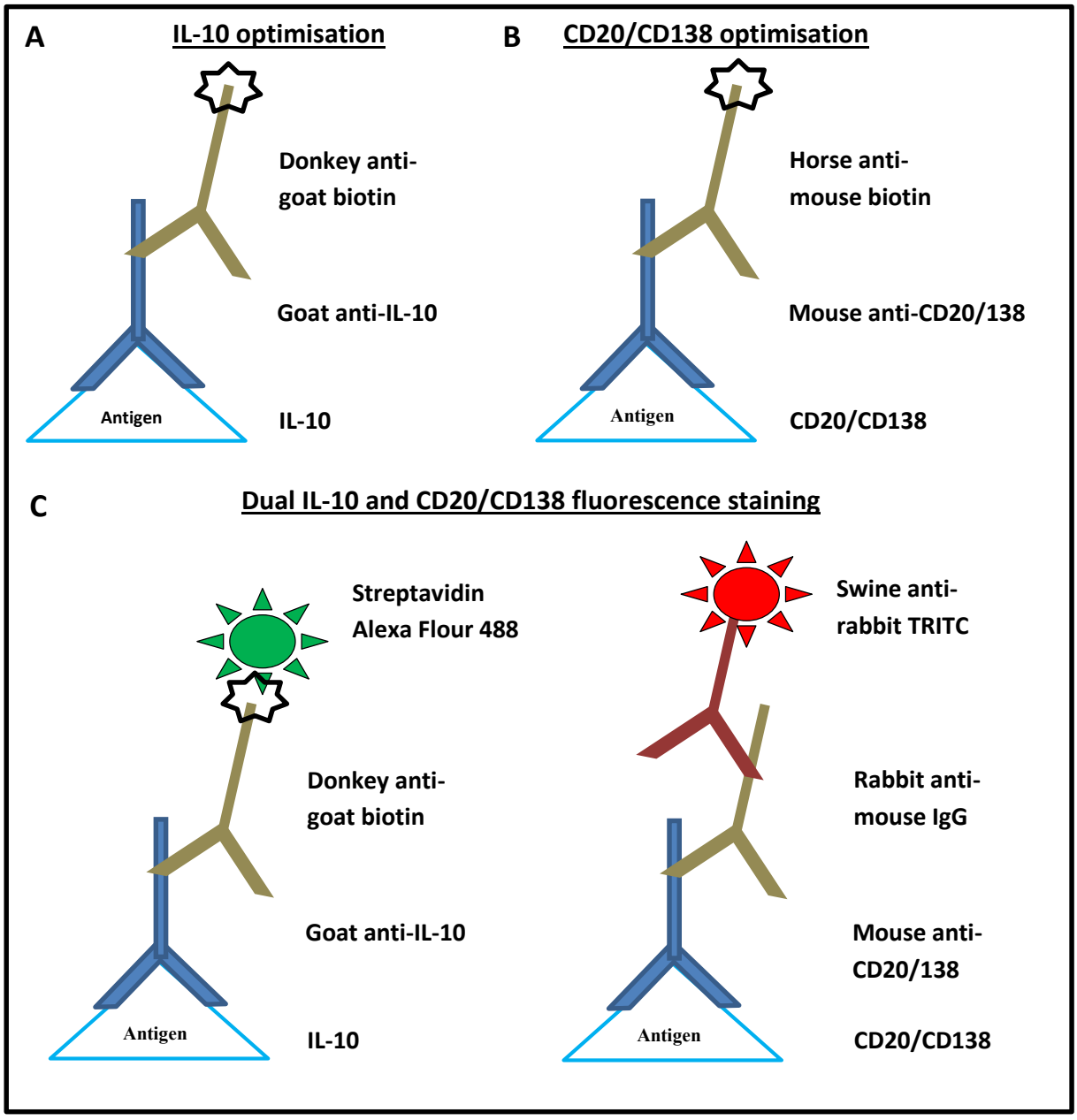


Figure 4 – Diagram of immunohistochemistry staining antibodies used.

Triple layer staining of IL-10, CD20 and CD138 was carried out to detect these antigens by light (**A** and **B**) and fluorescence (**C**) microscopy. Antibodies and substrates are listed, which were added in turn as described in the text.

Target	Clone	Species	Manufacturer	Dilution used
IL-10	Polyclonal	Goat	R&D	1/10 of 200 µg/mL
CD20	L26	Mouse	Dako	1/100 of 179 µg/mL
CD138	MI15	Mouse	Dako	1/50 of 54 µg/mL
CD3	UCHT1	Mouse	Biolegend	1/20 of 500 µg/mL
CD4	Polyclonal	Goat	R&D	1/20 of 100 µg/mL
Goat IgG	Polyclonal (biotin)	Donkey	Jackson Labs	1/170
Mouse IgG	Polyclonal	Rabbit	Dako	1/30
Rabbit IgG	Polyclonal (TRITC)	Swine	Dako	1/30
Alexa Fluor 488	(streptavidin)	n/a	Sigma Aldrich	1/400

Table 5 – Antibodies used for immunohistochemistry.

This table lists the antibody targets, clone, isotype (where relevant) and manufacturer of commercially available antibodies for the detection of antigens by immunohistochemistry.

2.11.1.1. Staining with fast red for light microscopy – ABC-AP

Biotin-conjugated secondary antibody was used from Table 5, as shown in Figure 4. For primary antibodies raised in mice the mouse ABC-AP kit (Vector labs) secondary antibody was used as described, with 1 drop of horse anti-mouse added in 10 mL of 10% NHS in PBS. During the 30 minute incubation the ABC-AP complex was mixed, with 2 drops of each A and B added to 10mLs of PBS whilst mixing, and left for 30 minutes at room temperature. Sections were washed 3x in PBS for 5 minutes with agitation, before adding one drop of the ABC-AP complex or more to cover each section. During the 30 minute incubation Fast Red (Sigma Aldrich) was prepared. Both tablets for the protocol are warmed to room temperature before use and one TRIS tablet is added to 10 mL of dH_20 and vortexed until dissolved. The red TR/Naphthol AS-MX tablet is then added and the solution was vortexed until dissolved. The solution was run through a 0.2 µm filter, before 2 drops of levemisol was added

(provided in the fast red staining kit, Sigma Aldrich, at 150 µg/mL) and the solution was stored in the dark before use. Following ABC-AP incubation, sections were washed 3x in PBS with agitation, at least 60 µL of fast red solution was then added to each section. Sections were incubated for 20 minutes at room temperature. During this incubation time hematoxylin (VWR, East Grinstead, UK) was filtered through filter paper. Following fast red incubation, the reaction was stopped in a cold tap water bath and agitated. To visualise cell nuclei, slides were added to a bath of the filtered hematoxylin for 5 seconds and washed in successive baths of fresh tap water, until the water was clear. Sections were dried briefly and a coverslip was fitted with faramount mounting medium (Dako), avoiding bubble. Slides were visualised by light microscopy using a Nikon Eclipse E400, with images captured at 20x unless stated using an attached camera and Lucia 4.8 software.

2.11.1.2. Staining with fluorochrome conjugates for UV microscopy

Fluorescence staining was carried out as shown in Figure 4 C, with the secondary antibodies shown and described in Table 5. Following the incubation of sections with secondary antibodies, as described above, slides were washed 3x in PBS with agitation for 5 minutes before the addition of the tertiary antibody or reagent. Initially, excess diluted streptavidin Alexa Fluor 488 (1/400 in PBS) in 1.5 mL micro centrifuge tubes was spun at >8000g for 3 minutes. The required quantity was pipetted from the top, leaving at least 50 µL, which prevented larger clumps of fluorescence appearing on the sections, swine anti-rabbit TRITC was added to the spun Alexa Fluor 488 to produce the final concentration of both shown in Table 5. At least 60 µL was incubated with sections for 30 minutes at room temperature in the dark in a humidity chamber. Slides were all washed 3x in PBS with agitation and sections were briefly dried, before adding DAPI-containing mountant and affixing coverslips, avoiding bubbles. Mountant, ProLong Gold (Cell Signalling Technologies, Invitrogen), was prepared following manufacturer's instructions and used at room temperature. Following mounting, slides were stored in slide trays with covers to protect from light, wrapped in aluminium foil, at 4 °C in the fridge. Sections were visualised under UV light, through appropriate filters, on a Nikon Eclipse E400. Images of sections, captured sequentially at 20x (unless indicated) using an attached camera and Lucia 4.8 software.

2.12. Statistical plan

Hypotheses were tested independently. Where multiple comparisons were performed, Kruskal-Wallis or Friedman tests (for unpaired or paired data, respectively) were carried out to determine overall significance. Thereafter, hierarchical testing was performed for the primary hypothesis prior to secondary or exploratory hypothesis testing. For paired data, Wilcoxon matched-pairs signed-rank test was used. For unpaired data, the Mann-Whitney U-test was employed.

If hierarchical hypothesis testing was not possible, for example where an optimal concentration of a reagent was being sought, then Dunn's test was used to correct for multiple comparisons.

Correlations were assessed by Spearman's rank correlation method. p values <0.15 were considered indicative of a trend, whilst p values <0.05 were considered significant.
Unless stated otherwise, data in figures and tables are shown as median and interquartile ranges.

3. Generation and function of human IL-10-producing B cells *in vitro*

This chapter aims to identify IL-10-producing B cell phenotypes (introduced in section 1) in peripheral blood following specific TLR or CD40 stimulation *in vitro*, in line with previous data demonstrating these methods of B cell IL-10 induction (Iwata et al., 2011, Blair et al., 2010). The functional capacity of IL-10-producing B cells to supress T cell responses will also be investigated.

Hypotheses

1. Peripheral blood B cells produce IL-10 following TLR, CD40 or BCR stimulation, that is enriched within distinct B cell subsets.

2. Peripheral B cells are capable of suppressing allergen-driven Th2 responses in an IL-10-dependent manner.

Aims

1. For hypothesis 1:

    a. Determine optimal *in vitro* stimulation for detecting IL-10-producing regulatory B cells.
    b. Determine whether PBMC or isolated B cell stimulation modifies the identification of IL-10-competent B cells.
    c. Assess the proportions of IL-10-producing B cells within $CD5^{hi}$, $CD5^{+}CD1d^{hi}$, $CD27^{+}$, $CD274^{+}$ (PD-$L1^{+}$), $CD27^{+}CD24^{hi}$ and $CD24^{hi}CD38^{hi}$ B cell subsets.
    d. Examine cell surface phenotype within IL-10^{+} and IL-10^{-} subtypes.
    e. Determine chemokine receptor expression on IL-10-producing B cells.

2. For hypothesis 2 :

    a. Assess whether T cell co-culture with IL-10-primed B cells suppress:
        i. Polyclonally-stimulated or
        ii. Allergen-stimulated T cell proliferation or inflammatory cytokine production.
    b. Assess IL-10, TGFβ or PD-L1 dependency of regulatory B cell suppression of T cell responses by blocking antibodies during co-culture.

3.1. Induction and detection of IL-10 producing B cells

3.1.1. Dose responses

PBMCs were stained for CD19 and IL-10 to discriminate B cells secreting IL-10, as shown in Figure 5 A. For all conditions, the median percentages of IL-10-producing B cells, at the lowest concentrations, were between 2 and 3.3%. Increasing concentrations of grass pollen allergen, Phl p (Figure 5 B), CD40 Ligand (Figure 5 C), anti-CD40 antibody (Figure 5 D) or lipopolysaccharide (LPS) (Figure 5 E) did not show any increase in the proportions of IL-10-producing B cells. CpG stimulation showed a significant increase in the proportions of IL-10-producing B cells at 1 and 3 µg/mL (Figure 5 F). From a median of 1.95% IL-10^{+} B cells when stimulated with 0.1 µg/mL CpG, to 8.2% and 9.55% when stimulated with 1 and 3 µg/mL, respectively.

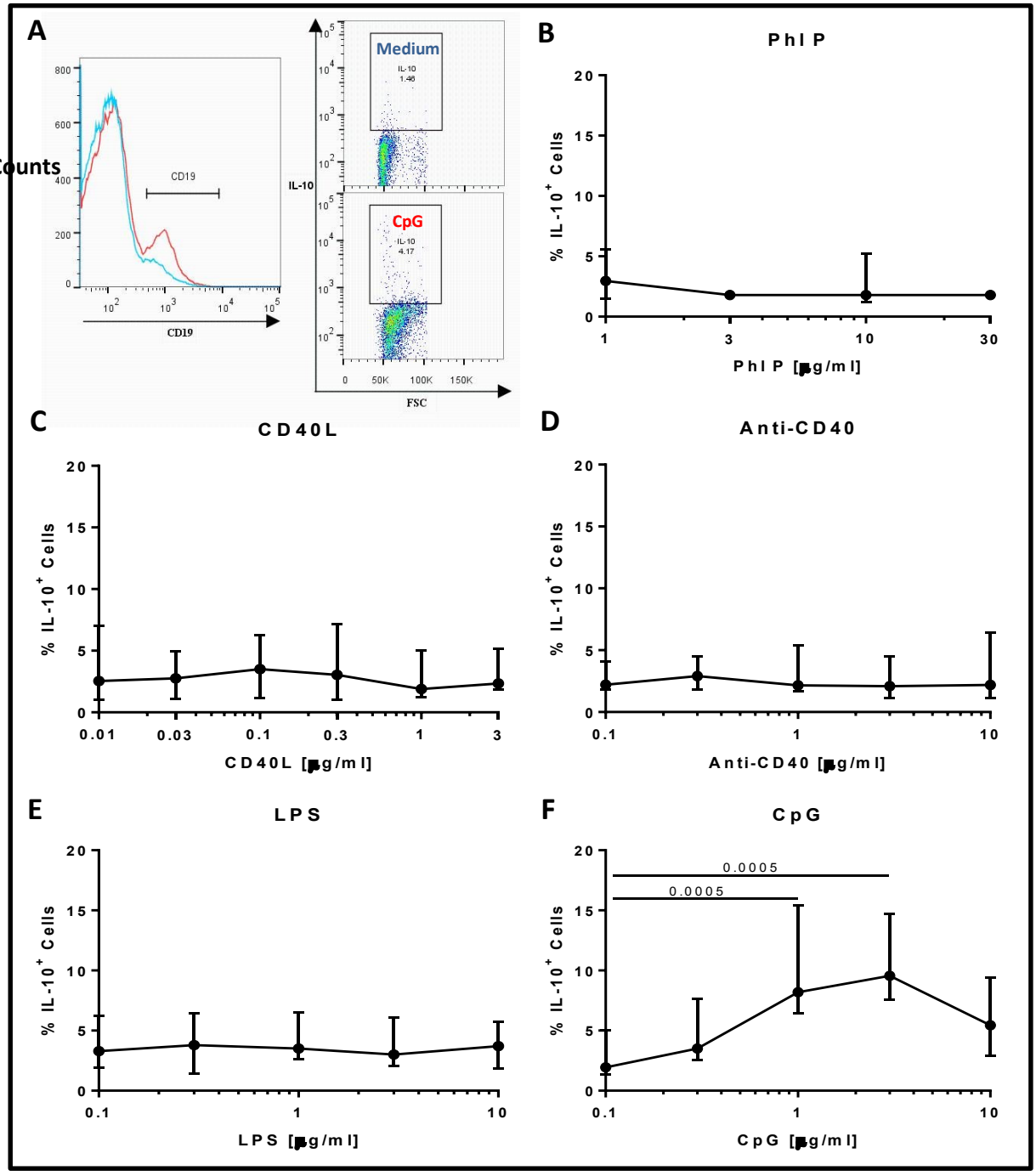


Figure 5 – Detection of IL-10-producing B cells.

PBMCs were stimulated for 48 hours in increasing concentrations (as shown) of stimuli. Cells stained for flow cytometry were gated as shown (**A**). Unstimulated (blue) and 1µg/mL CpG stimulated (red) B cells are shown for comparison of IL-10. Percentages of IL-10$^+$ B cells are shown following stimulation with grass pollen allergen (Phl p) (n=9, NA=5 AR=4) (**B**), CD40 ligand (CD154 – labelled CD40L) (n=5, NA=3 AR=2) (**C**), anti-CD40 antibodies (n=10, NA=6 AR=4) (**D**), LPS (n=10, NA=7 AR=3) (**E**) and CpG (n=11, NA=7 AR=4) (**F**). Data shown is median and interquartile range. P values are shown for Dunn's multiple comparisons following Friedman test.

3.1.2. Effect of CpG, CD40L or LPS stimulation on B cells amongst PBMC or isolated cultures

Stimuli previously used to stimulate PBMCs were combined, such as CpG in addition to CD40L. Furthermore, these singular or combined stimuli were compared for their ability to increase the proportions of IL-10-producing B cells when used in PBMC (Figure 6 A – left hand panel) or isolated B cell (Figure 6 A – right hand panel) cultures from the same donors. Median percentages of IL-10$^+$ B cells in medium and CpG were 3.9% and 7.7%, respectively, for B cells cultured amongst PBMCs, however median percentages amongst isolated B cells were 0.6% and 1.4%, respectively. LPS stimulation showed a trend towards an increase in IL-10-competent cells amongst PBMCs (p=0.07), despite no increase observed amongst isolated B cells following LPS stimulation. The addition of the steroid Dexamethasone also showed a dose response relationship, enhancing the percentage of IL-10-producing B cells amongst PBMC-cultured B cells (Appendix Figure 2). The median response with the highest concentration of Dexamethasone was not greater than CpG. Dexamethasone treatment was not repeated amongst isolated B cells due to limited cell numbers and has not been followed further. Within the main figure, the addition of CD40L or anti-CD40 to LPS amongst PBMCs resulted in significantly increased percentages of IL-10-secreting B cells compared to LPS or medium alone. CD40L with LPS in isolated B cell culture did not alter the proportions of IL-10$^+$ B cells compared to medium or LPS alone. Stimulation with CD40L alone in PBMC culture did not show a significantly greater proportion of IL-10$^+$ B cells compared to medium alone, surprisingly isolated B cells had significantly reduced proportions of IL-10-producing cells compared to medium alone. Anti-CD40 significantly enhanced percentages of IL-10-producing B cells amongst PBMCs compared to medium but could not be assessed within the isolated B cell condition with only 3 repeats. The greatest proportions of IL-10-producing B cells are again observed following CpG stimulation conditions for both PBMCs and isolated B cells. PBMC culture showed significantly greater proportions of IL-10$^+$ B cells than medium alone where CD40L or anti-CD40 was combined with CpG; however these are not significantly greater than CpG alone. Isolated B cells stimulated with anti-CD40 and CpG also showed significantly greater percentages of IL-10$^+$ B cells from medium, with significantly reduced proportions of IL-10$^+$ B cells compared to CpG alone. The proportions of IL-10-producing B cells detected in PBMC and isolated B cell cultures independently correlate significantly with cells in medium alone (Figure 6 B – left panel) (p=0.02). A weak trend is observed when comparing CpG stimulation of PBMC and isolated B cell cultures (Figure 6 B – centre panel) (p=0.20), no relationship is observed with LPS stimulation in PBMC conditions against isolated culture (Figure 6 B – right panel) (p=0.83).

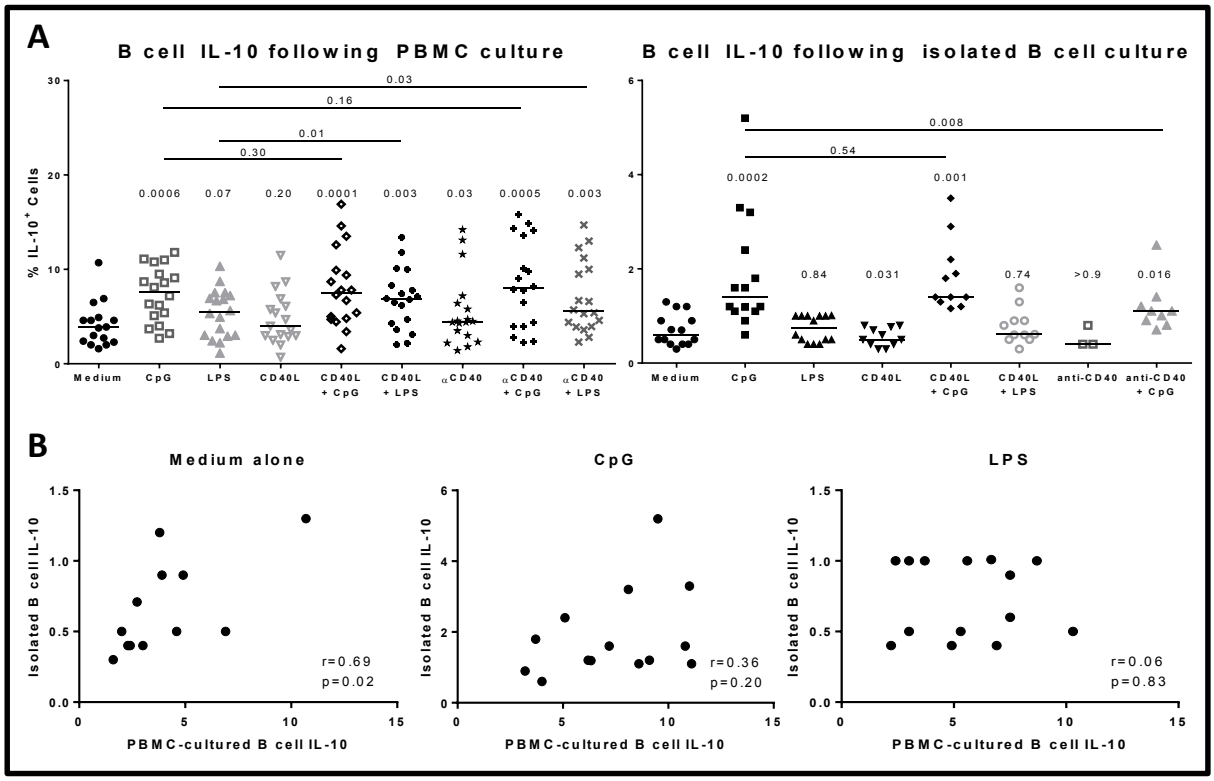


Figure 6 – Comparison of IL-10-producing B cells following stimulation in PBMC or isolated culture.

PBMCs or isolated B cells were stimulated for 48 hours in the conditions shown (**A**). Proportions of IL-10-producing B cells are subsequently detected by flow cytometry. Donor numbers differed for PBMC culture (n=17, NA=6, AR=9, AIT=2) compared to isolated B cell cultures (for medium, CpG and LPS n=13, NA=6, AR=7 – for CD40L+CpG and CD40L+LPS n=10, NA=6, AR=4 – for anti-CD40 n=4 all AR – for anti-CD40+CpG n=9, NA=6, AR=3). Wilcoxon matched-pairs signed-rank test was used to compare for differences, following significant Friedman test ($p<0.0001$ for both). Proportions of $IL\text{-}10^+$ B cells following culture in (from left to right) medium alone, CpG or LPS are shown for PBMC against isolated B cell cultures (**B**) (for medium n=11, NA=5, AR=6 – for CpG and LPS n=13, NA=6, AR=7).

3.1.3. Time course of IL-10 induction by CpG amongst B cells

Representative flow cytometry staining for IL-10 is shown following 1 μg/mL CpG at 0, 24, 48, 72 and 96 hours (Figure 7 A) and at 48 hours with 0, 1 and 3 μg/mL CpG (Figure 7 B). Flow cytometry shows an increase in IL-10 producing B cells at 48 hours following 1 μg/mL and at 72 hours following 3 μg/mL, compared to 0 μg/mL controls. qPCR showed significantly increased mRNA for IL-10 at 24 hours for 1 μg/mL and both 1 and 3 μg/mL at 48 hours relative to 0 μg/mL CpG controls (Figure 7 D). IL-10 concentrations were significantly greater in both 1 and 3 μg/mL CpG cultures relative to unstimulated cells at 48 hours, maintained through 72 and 96 hour time points.

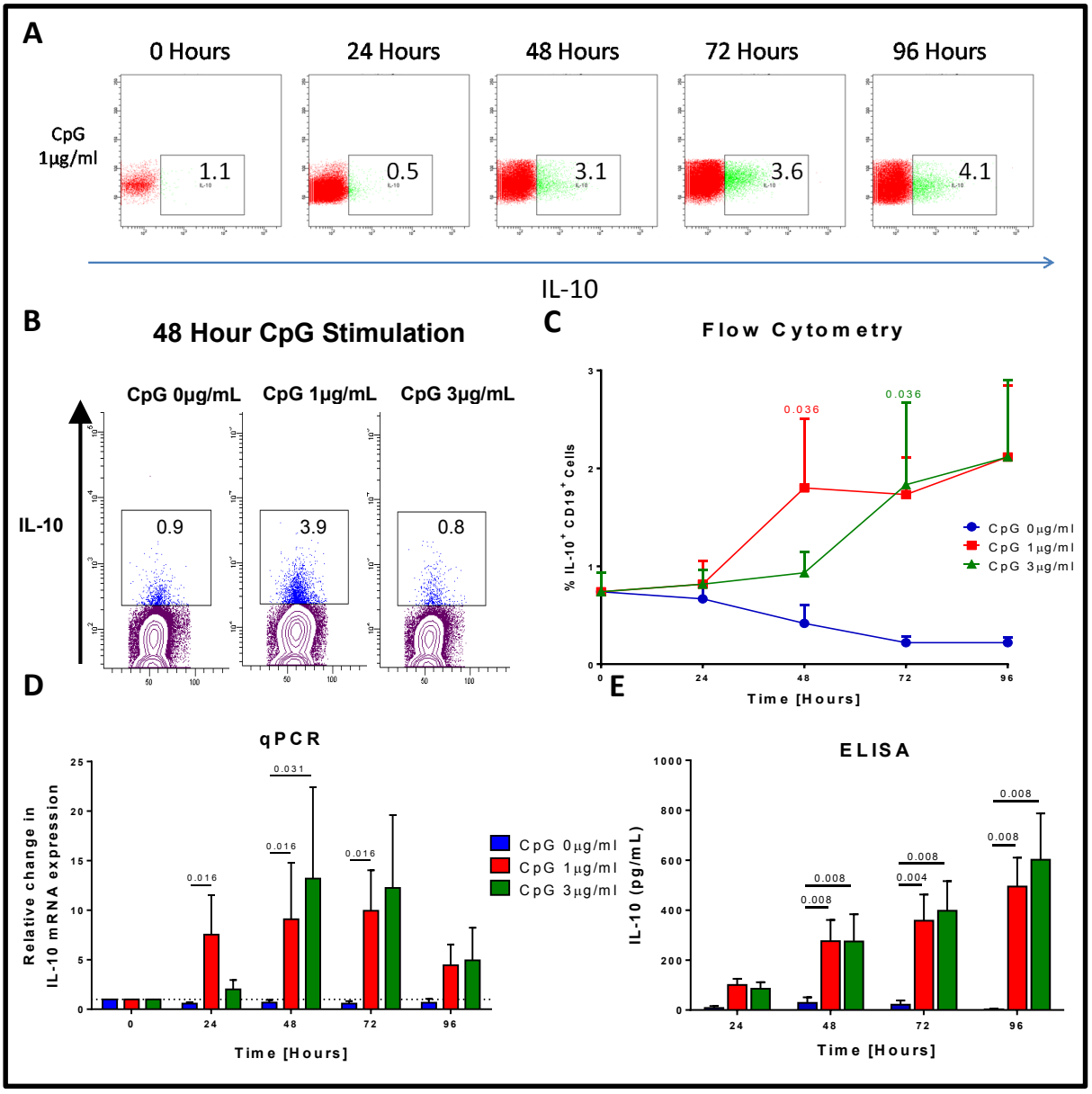


Figure 7 – Time course of IL-10 production amongst isolated B cells using CpG.

B cells were isolated from non-allergic (NA=6) donors. Cells were stimulated for 24, 48, 72 or 96 hours with 1 (red) or 3 (green) µg/mL CpG. Cells were stained for flow cytometry to detect IL-10⁺ B cells (as shown) at each time point with 1µg/mL CpG (**A**) and at 48 hours with 0, 1 and 3 µg/mL of CpG (**B**). Stimulation with 0 (blue), 1 or 3 µg/mL of CpG is shown for the percentages of IL-10⁺ B cells measured by flow cytometry (**C**), relative IL-10 mRNA expression detected by qPCR (**D**) and concentration of IL-10 supernatant protein produced quantified by ELISA (**E**). For all data sets, mean and standard error are shown. Wilcoxon matched-pairs signed rank test results are shown following significant Friedman test (all <0.01).

Isolated B cells were examined for changes in mRNA expression following 1 µg/mL CpG stimulation at 0, 3, 6, 9, 18, 24, 48 and 72 hours (Figure 8). Unfortunately, due to 4 repeats, no statistical comparison can be made of this data, however trends are clear. IL-10 mRNA increased 23 fold by 6 hours and remained 8.2 fold higher than baseline at 72 hours of stimulation (Figure 8 A). IL-10 receptor was also increased at 72 hours by 2.2 fold, but did not show induction until 18 hours of stimulation (Figure 8 B). IL-6 mRNA increased sharply by 105 fold compared to baseline expression at 6 hours, but also decreased sharply by 72 hours to only 3.5 fold that of baseline (Figure 8 C). No clear changes in mRNA were seen for TGF-β (Figure 8 D), IL-4 (Figure 8 E), IFNγ (Figure 8 F) and FoxP3 (Figure 8 G).

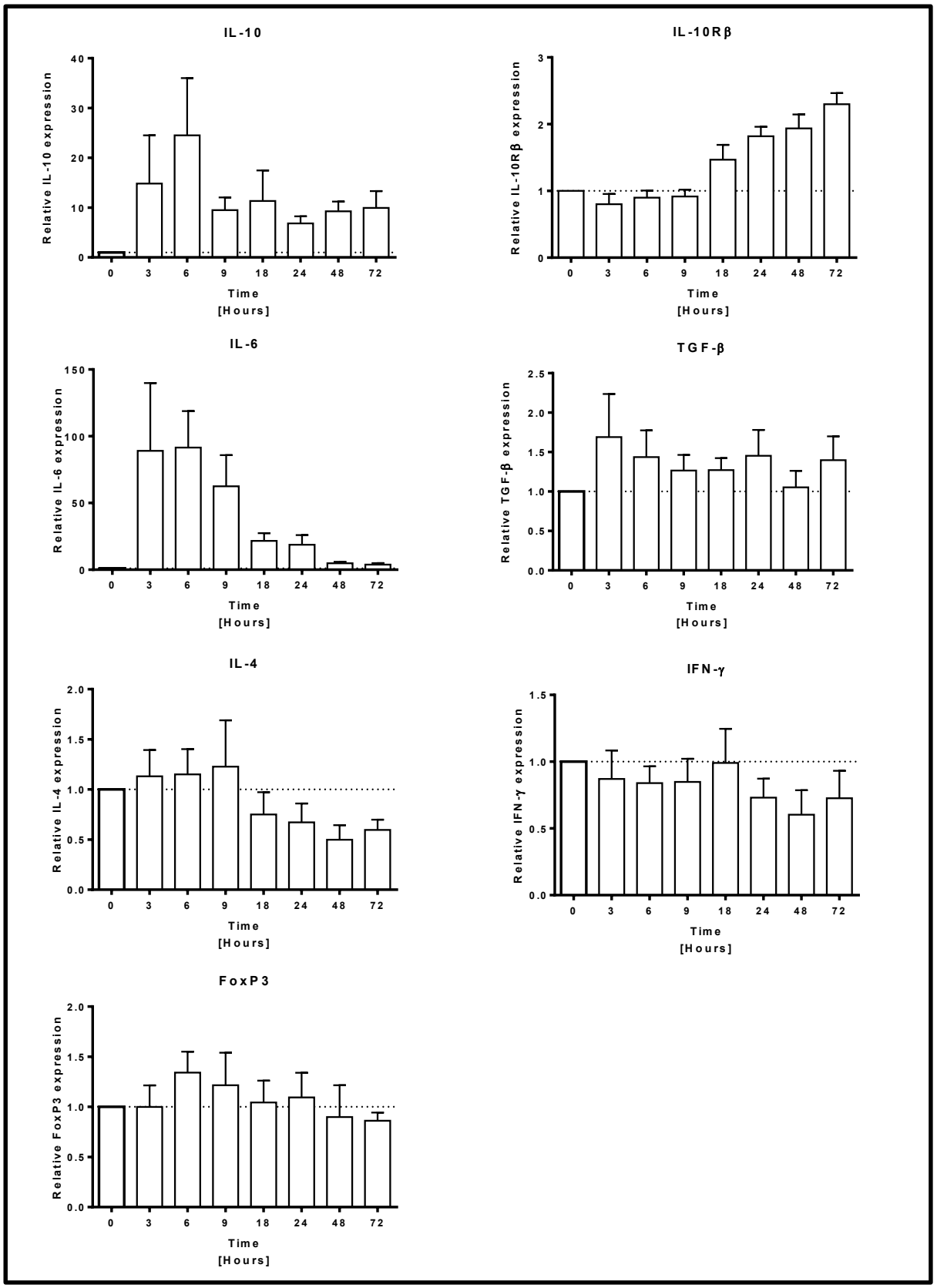


Figure 8 – Time course of mRNA induction following CpG stimulation.

B cells were isolated from non-allergic donors (n=4) and stimulated over 72 hours with 1 µg/mL CpG. Cell lysates were taken at time points shown. Lysates were assessed for relative changes against time 0 for mRNA towards IL-10 (**A**), IL-10 receptor β (**B**), IL-6 (**C**), TGF-β (**D**), IL-4 (**E**), FoxP3 (**F**) and IFNγ (**G**).

3.1.4. CpG-enhanced B cell IL-10 relates to innate capacity

Given the level of induction of IL-10 amongst B cells observed by CpG stimulation compared to culture in medium alone it was pertinent to assess whether CpG-induced responses are enhancing innate IL-10 capacity or showing a disproportionate level of IL-10 induction compared to innate capacity. *De novo* or innate IL-10 capacity was taken as the IL-10$^+$ B cells following culture in medium alone for 48 hours. Proportions of IL-10$^+$ B cells following culture in medium and those following CpG stimulation significantly correlate, both when B cells are cultured amongst PBMCs (Figure 9 A) and as isolated B cells (Figure 9 B).

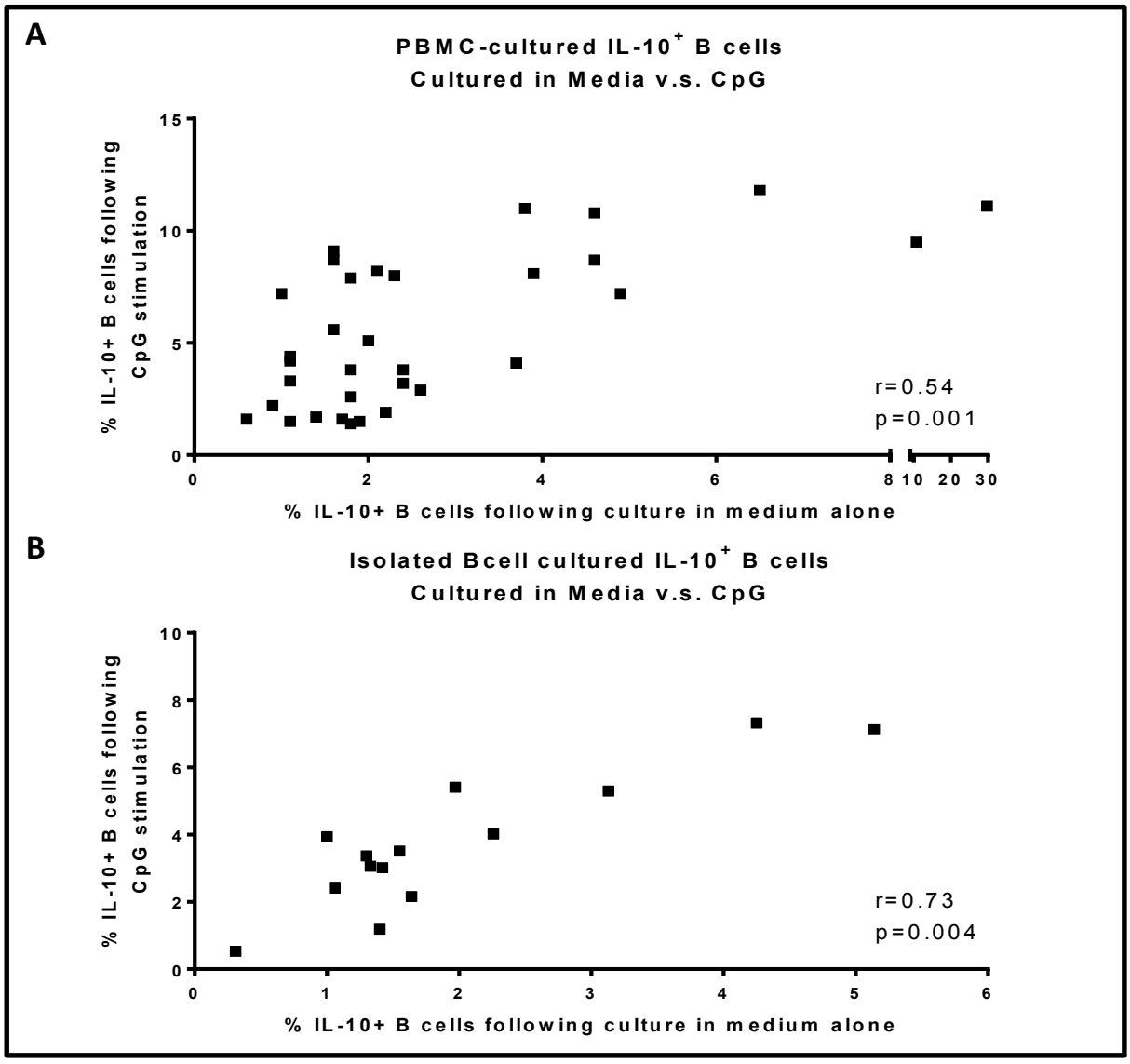


Figure 9 – Comparison of medium or CpG stimulated B cells for IL-10 capacity.

PBMCs (n=33, NA=14 AR=12 AIT=7) or isolated B cells (n=14, NA=7 AR=7) were cultured for 48 hours in medium alone or 1µg/mL CpG and stained for flow cytometry to detect CD19$^+$ B cells and IL-10. A comparison of the percentages of B cells producing IL-10 following culture in medium or CpG is shown for B cells cultured amongst PBMCs (**A**) or as cells (**B**).

3.1.5. Phenotyping of IL-10-producing B cells

Isolated B cells were examined for their expression of IL-10 in concert with B cell subsets previously identified as containing increased concentrations of IL-10-producing B cells ($CD24^{hi}CD38^{hi}$, $CD27^{+}$, $CD10^{+}$, $CD27^{+}CD24^{hi}$, PD-$L1^{+}$ and $CD5^{hi}$) (Figure 10 and Figure 11). IL-10^{+} B cells were significantly enriched amongst $CD24^{hi}CD38^{hi}$ B cells relative to $CD24^{int}CD38^{int}$ and $CD24^{hi}CD38^{lo}$ B cells following 1 or 3 µg/mL CpG (Figure 10 B and C). In the absence of CpG at 48 hours there was no enrichment within the $CD24^{hi}CD38^{hi}$ subset (Figure 10 A). All 3 subsets have significantly increased proportions of IL-10^{+} B cells following either 1 or 3 µg/mL CpG (Figure 10 E, F and G). Gating of IL-10^{+}, $CD27^{+}$, $CD10^{+}$, $CD27^{+}CD24^{hi}$, PD-$L1^{+}$ and $CD5^{hi}$ B cell subsets (Figure 11 A) has allowed the comparison of IL-10 capacity amongst individual subsets with respect to the total B cell population. Significantly greater proportions of IL-10^{+} B cells were found within $CD10^{+}$, $CD27^{+}CD24^{hi}$, PD-$L1^{+}$, $CD5^{hi}$ and $CD24^{hi}CD38^{hi}$ subsets relative to the whole $CD19^{+}$ population (Figure 11 B). $CD27^{+}$ B cells showed no change in the proportion of IL-10^{+} B cells in the absence of CpG but had significantly fewer IL-10-producing B cells post CpG stimulation. Amongst both medium cultured and CpG stimulated B cells the subset with the greatest proportion of IL-10^{+} B cells was within the $CD24^{hi}CD38^{hi}$ B cells. IL-10^{+} B cells increased from medians of 1.49% in medium to 17.9% within the $CD24^{hi}CD38^{hi}$ subset in the absence of CpG and from 3.44% to 13.6% with CpG. The phenotype within both the IL-10^{+} and IL-10^{-} B cell populations shows all B cell markers were expressed to a significantly greater extent within the IL-10^{+} subset compared to non-IL-10-producing B cells with the exception of CD27 expression on CpG-stimulated cells (Figure 11 C). Single molecule surface expressions are shown with respect to IL-10^{+} (red lines) and IL-10- (blue lines) (Figure 11 D), showing CD27 expression within IL-10^{+} B cells decreased, however CD10, CD5 and CD274 (PD-L1) clearly increased in signal intensity amongst IL-10^{+} B cells compared to IL-10^{-} B cells.

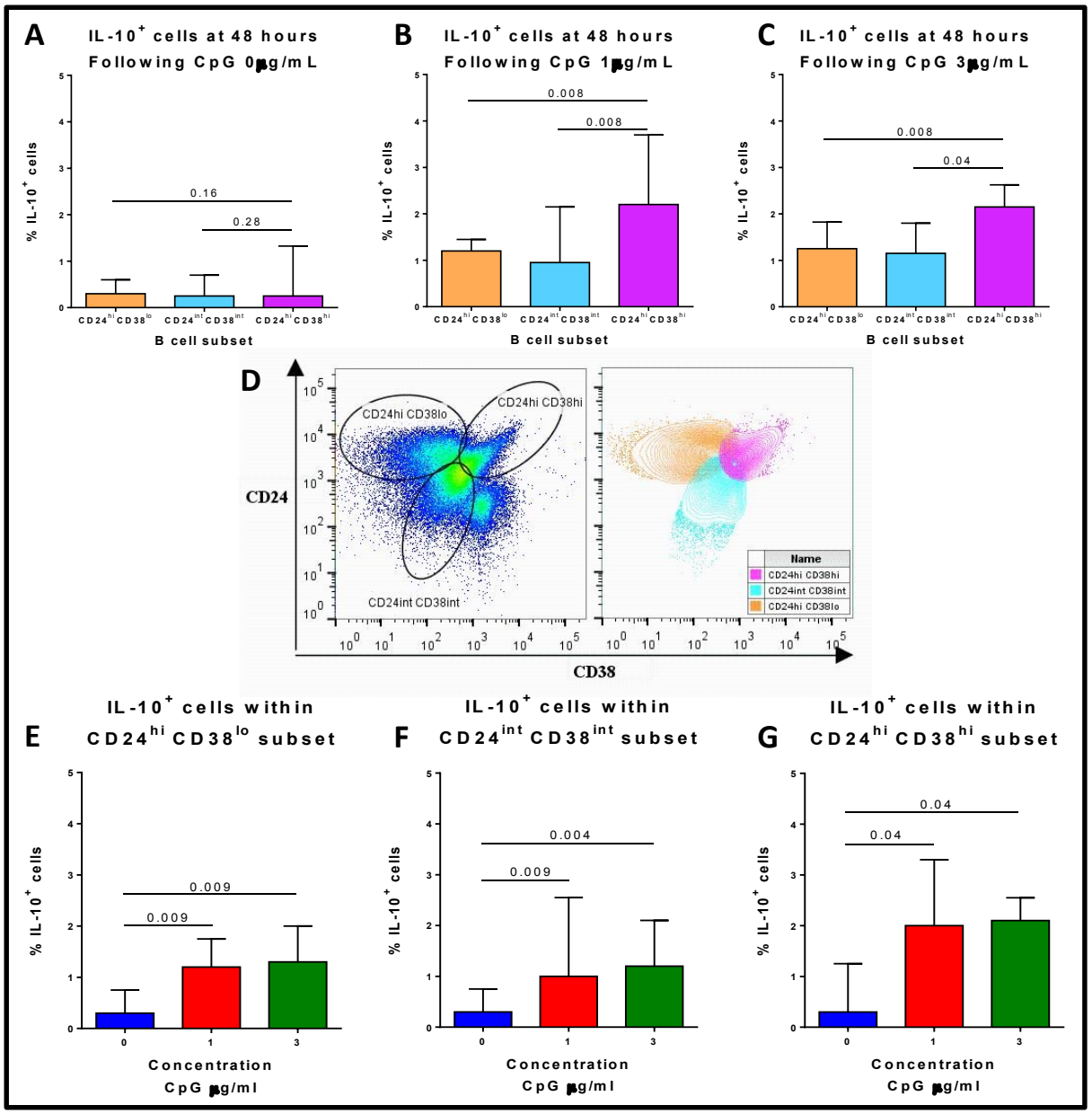


Figure 10 – Relative expressions of IL-10 amongst CD24 and CD38 B cell subsets.

B cells were isolated from non-allergic donors (n=6) and cultured for 48 hours in the presence of CpG. Cells were stained for flow cytometry and gated for IL-10 and CD24 and CD38 subsets as shown (**D**). Percentages of IL-10^{+} B cells within CD24^{hi}CD38^{lo} (**E** and orange), CD24^{int}CD38^{int} (**F** and blue) and CD24^{hi}CD38^{hi} (**G** and purple) are displayed amongst unstimulated (**A** and blue), 1µg/mL CpG (**B** and red) and 3µg/mL CpG (**C** and green). Median and IQR are shown for all.

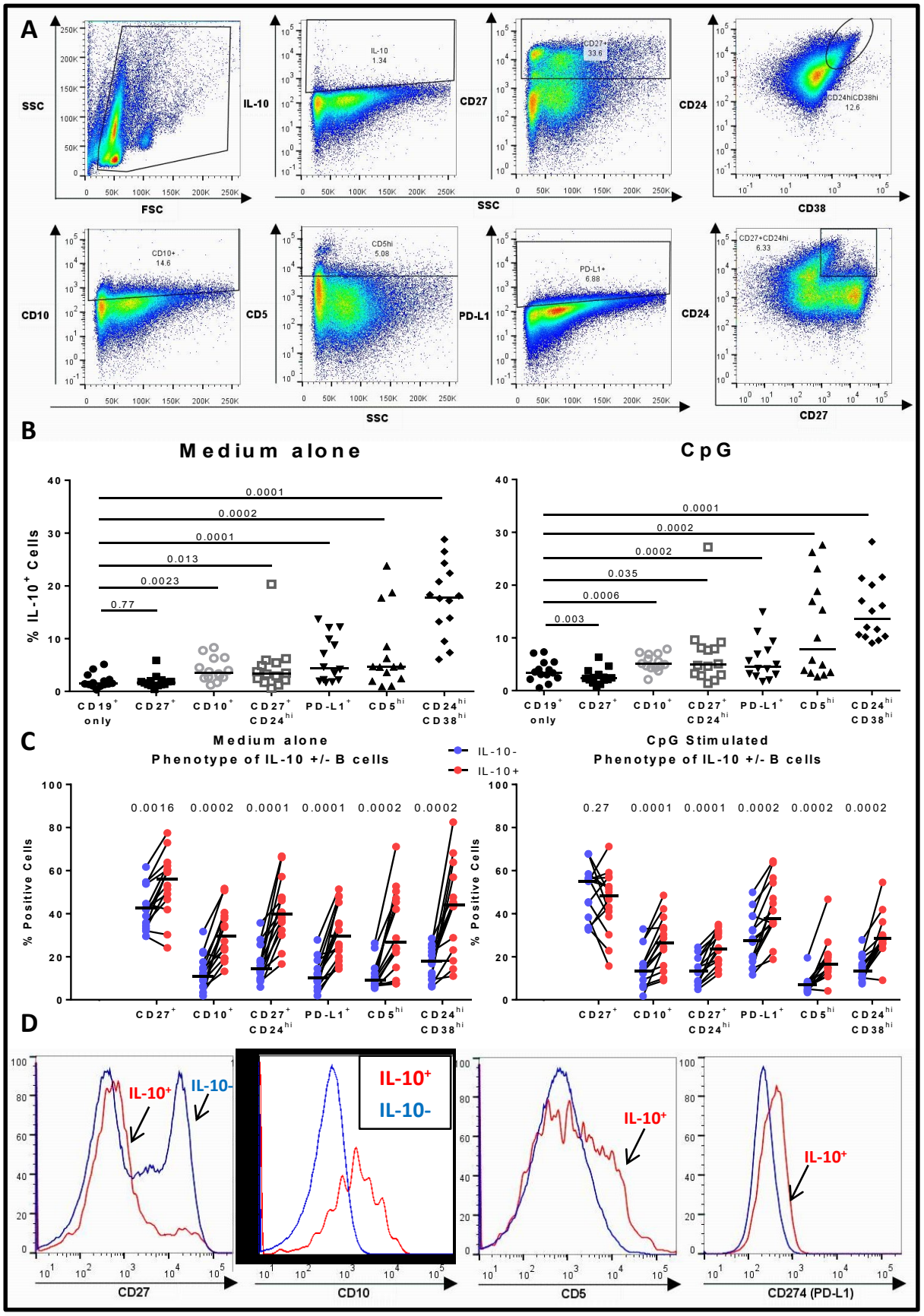
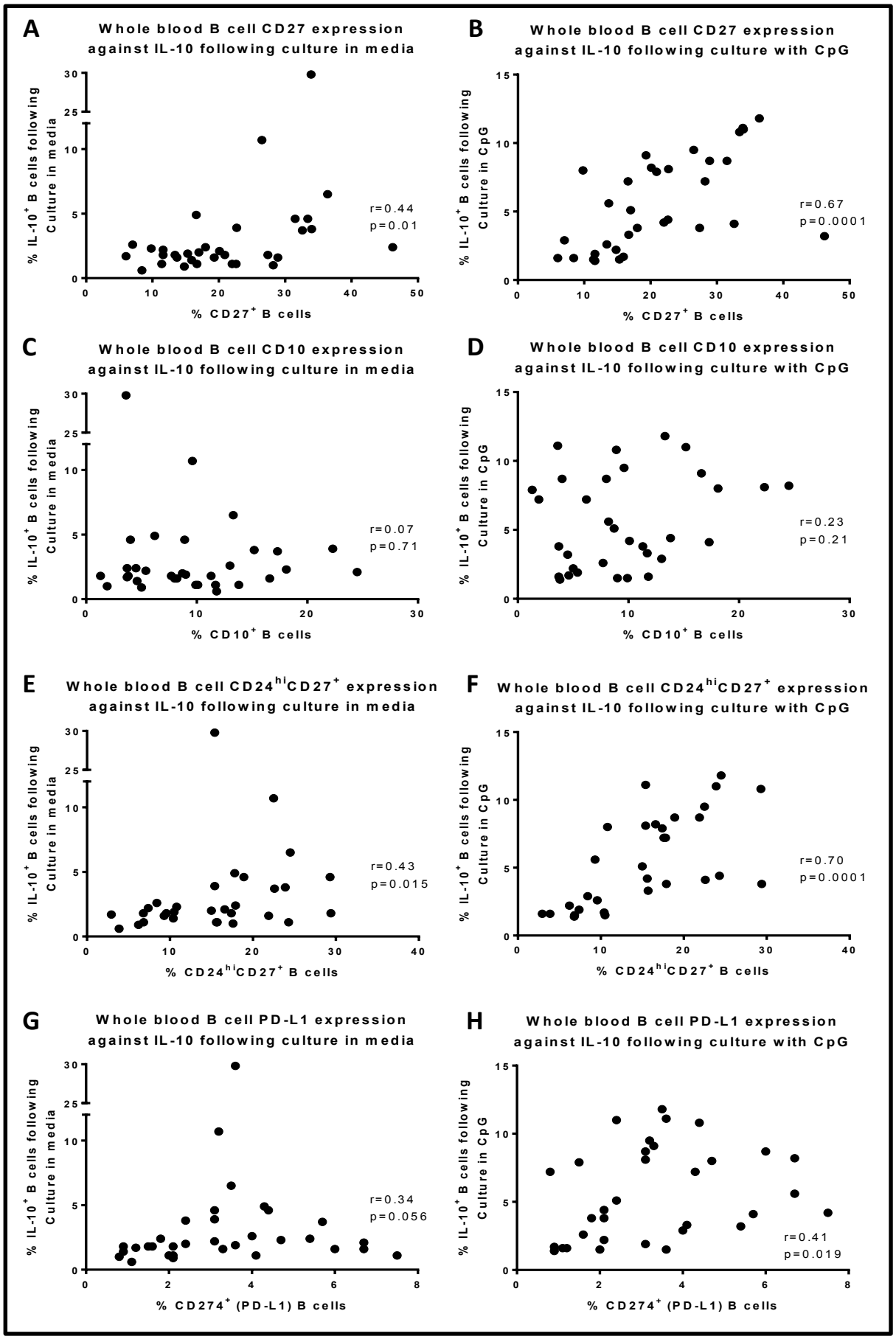


Figure 11 – Phenotypic markers of IL-10-producing B cells.

B cells were isolated (n=14, NA=7 AR=7) and cultured for 48 hours in medium alone or 1µg/mL CpG before staining for for flow cytometry. Representative gating for B cell subsets, including IL-10, is shown (**A**). Percentages of IL-10-expressing B cells which express cell subsets as shown for B cells cultured in medium alone and CpG (**B**). Within each B cell subset percentages of IL-10$^-$ (blue) and IL-10$^+$ (red) cells are shown (**C**). Overlays demonstrate representative examples of IL-10$^-$ (blue) and IL-10$^+$ (red) B cells (**D**). Wilcoxon matched-pair signed-rank results are shown (following significant Friedman test p<0.0001 for **B**).

Whole blood *ex vivo* staining was carried out within 2 hours of phlebotomy and proportions of B cell subsets were compared with matched 48 hour CpG stimulated (Figure 12 – right hand panels) or un-stimulated (Figure 12 – left hand panels) proportions of IL-10-producing B cells within PBMC culture. All comparisons showed greater correlation between phenotype *ex vivo* and proportions of IL-10-producing B cells following CpG stimulation compared to medium alone. These show greater proportions of IL-10-producing B cells after culture correlated with greater proportions of CD27 expressing B cells prior to culture both in medium and CpG (Figure 12 A and B). CD10 expression showed no correlation with subsequent proportions of IL-10-producing B cells following culture (Figure 12 C and D). $CD24^{hi}CD27^{+}$ B cells showed a significant positive correlation with respect to proportions of IL-10-producing B cells (Figure 12 E and F). Expression of PD-L1 showed a strong trend towards correlation with medium cultured $IL\text{-}10^{+}$ B cells and significant correlation with CpG culture ($p=0.056$ and $p=0.019$ respectively) (Figure 12 G and H). $CD5^{+}$ B cells prior to culture correlated significantly with subsequent proportions of $IL\text{-}10^{+}$ B cells in both medium and CpG culture (Figure 12 I and J). There was an inverse correlation observed between the proportions of $CD24^{hi}CD38^{hi}$ B cells *ex vivo* and the proportions of IL-10-producing B cells *in vitro* ($p=0.0007$ and $p=0.0004$ for cells cultured in medium and CpG respectively) (Figure 12 K and L).


A
Whole blood B cell CD27 expression against IL-10 following culture in media
% IL-10+ B cells following Culture in media
% CD27+ B cells
r=0.44
p=0.01
B
Whole blood B cell CD27 expression against IL-10 following culture with CpG
% IL-10+ B cells following Culture in CpG
% CD27+ B cells
r=0.67
p=0.0001
C
Whole blood B cell CD10 expression against IL-10 following culture in media
% IL-10+ B cells following Culture in media
% CD10+ B cells
r=0.07
p=0.71
D
Whole blood B cell CD10 expression against IL-10 following culture with CpG
% IL-10+ B cells following Culture in CpG
% CD10+ B cells
r=0.23
p=0.21
E
Whole blood B cell CD24hiCD27+ expression against IL-10 following culture in media
% IL-10+ B cells following Culture in media
% CD24hiCD27+ B cells
r=0.43
p=0.015
F
Whole blood B cell CD24hiCD27+ expression against IL-10 following culture with CpG
% IL-10+ B cells following Culture in CpG
% CD24hiCD27+ B cells
r=0.70
p=0.0001
G
Whole blood B cell PD-L1 expression against IL-10 following culture in media
% IL-10+ B cells following Culture in media
% CD274+ (PD-L1) B cells
r=0.34
p=0.056
H
Whole blood B cell PD-L1 expression against IL-10 following culture with CpG
% IL-10+ B cells following Culture in CpG
% CD274+ (PD-L1) B cells
r=0.41
p=0.019

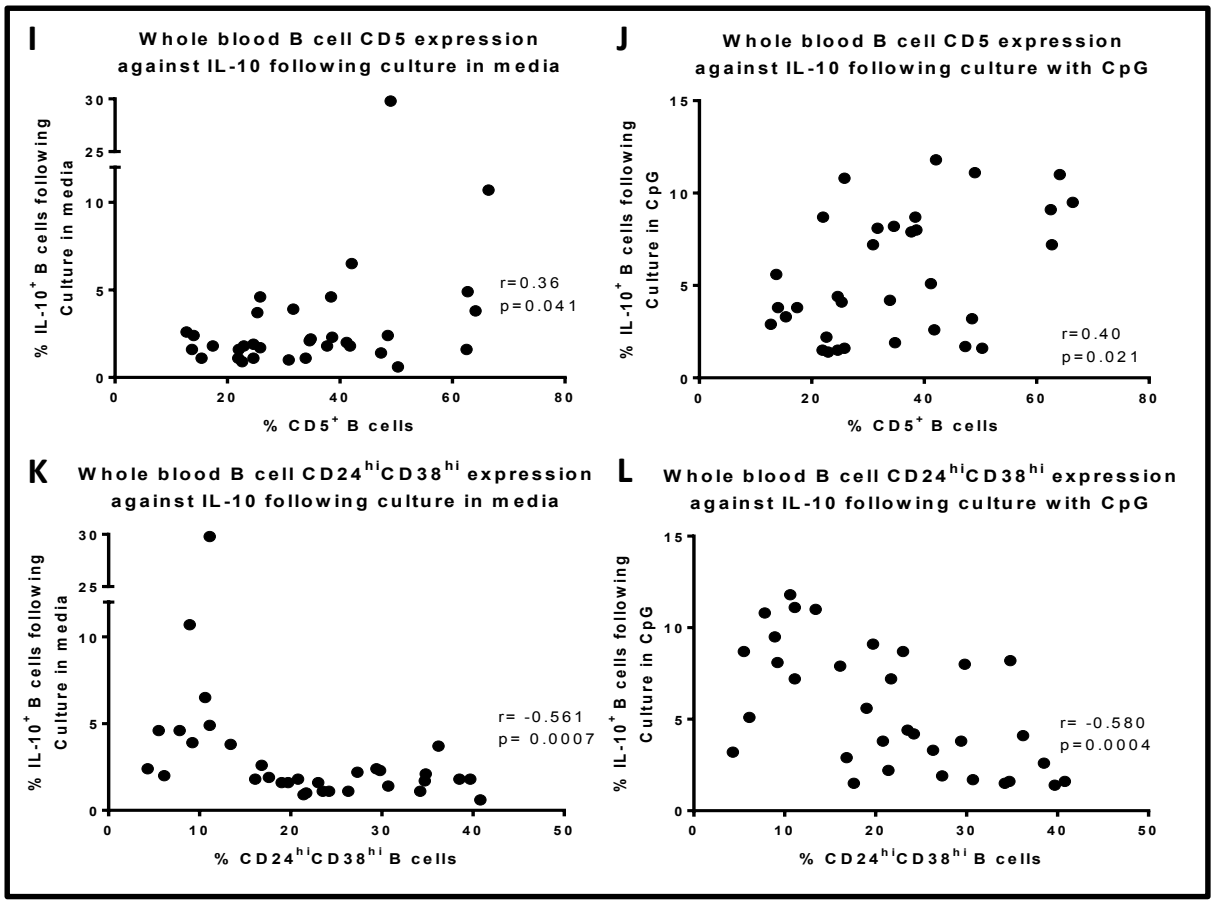


Figure 12 – Comparisons of IL-10-producing B cells following culture and B cell subsets detected ex-vivo in whole blood.

Prior to culture, whole blood flow cytometry was carried out. PBMCs were isolated from n=46 (NA=18 AR=18 AIT=10) donors and stimulated for 48 hours in medium alone (left hand panels) or 1µg/mL CpG (right hand panels). Cultured cells were stained for flow cytometry to detect CD19 and IL-10. Whole blood (pre-cultured, *ex vivo)* percentages of $CD27^+$ (**A** and **B**), $CD10^+$ (**C** and **D**), $CD24^{hi}CD27^+$ (**E** and **F**), $CD274^+$ (PD-L1) (**G** and **H**), $CD5^+$ (**I** and **J**) and $CD24^{hi}CD38^{hi}$ (**K** and **L**) B cells are compared with $IL\text{-}10^+$ B cells detected following culture in medium alone (**A**, **C**, **E**, **G**, **I** and **K**) and CpG (**B**, **D**, **E**, **H**, **J** and **L**).

Proportions of cells positive for chemokine receptors amongst both $IL\text{-}10^+$ (red) and $IL\text{-}10^-$ (blue) populations are compared (Figure 13 A), showing significantly greater chemokine receptor expression on $IL\text{-}10^+$ B cells for CCR1, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR10, CXCR1, CXCR2 and CXCR2). Significantly higher expression was accompanied by a distinct change with CCR3, CCR4, CCR7, CCR8, CCR10 and CXCR5 expression. Representative gating for single cells and IL-10 are shown with representative overlays showing signal intensity for CCR3, CCR4, CCR7, CCR8, CCR10 and CXCR5 on $IL\text{-}10^+$ (red lines), $IL\text{-}10^-$ (blue lines) and unstained cells (grey filled shape) (Figure 13 B).

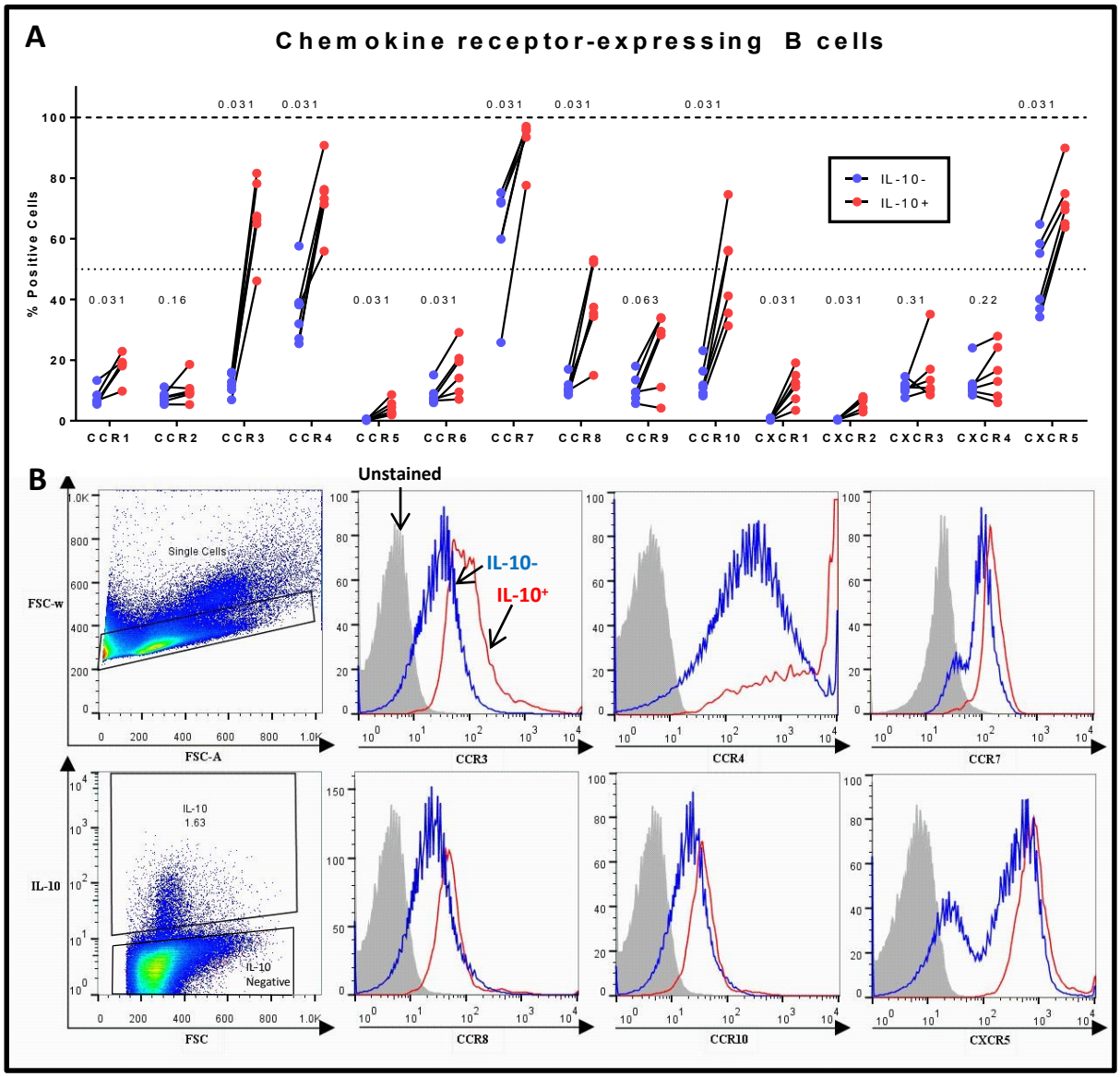


Figure 13 – Chemokine receptor expression on IL-10-producing B cells.

B cells were isolated (n=6, NA=1 AR=2AIT=3) and cultured for 48 hours in the presence of CpG. Cells were stained for flow cytometry to detect cell surface chemokine receptor expression as well as intracellular IL-10. Events were gated to exclude doublets, representative gating is shown (**B**). Individual chemokine receptors as described were gated against unstained controls cells. Percentage expression of each receptor is shown for IL-10^{-} (blue) and IL-10^{+} (red) B cells (**A**). Wilcoxon matched-pairs signed-rank test was used, p values are shown.

3.2. Regulation of T cell responses

3.2.1. Suppression of polyclonal T cell responses

The proportions of T cells proliferating in co-culture with 48 hour CpG-primed B cells (red) are shown relative to T cells proliferating with un-primed B cells (blue). Representative gating for proliferation at day 9 of co-culture illustrates suppression of T cell proliferation with CpG-primed B cells compared to un-primed B cells (Figure 14 A). Suppression is not observed at days 3 and 6 of co-culture and shows significant suppression only at day 9, maintained (but not significant) through to day 12 (Figure 14 B). B cells used in decreasing proportions with respect to T cells, beginning at in equal numbers at 1:1 of T:B cells decreasing to 32:1, shows significant suppression at 1:1, slightly decreased at 2:1, with and no suppression observed at 4:1 onwards (Figure 14 C).

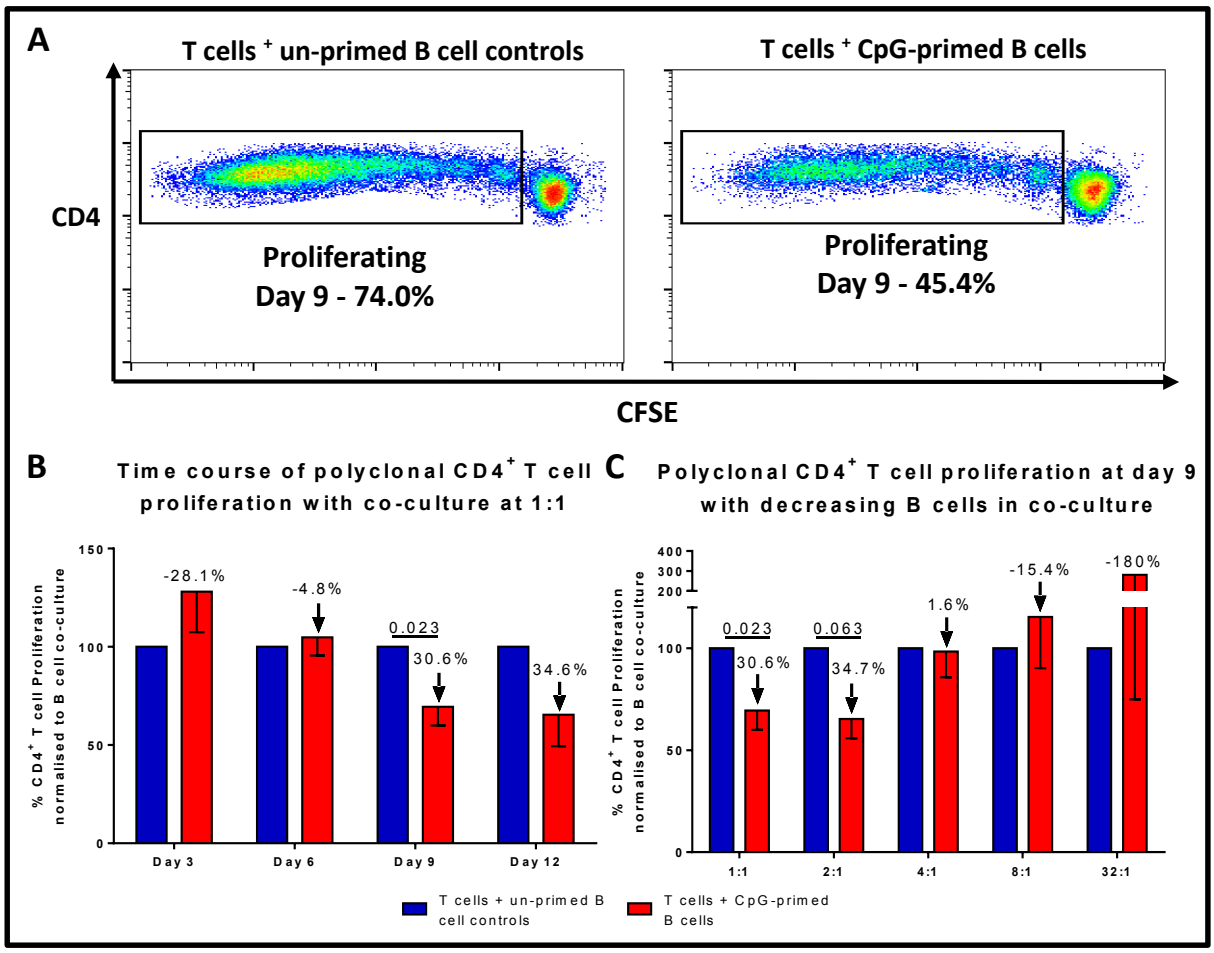


Figure 14 – Co-culture of CpG-primed B cells with polyclonally stimulated T cells

Percentages of proliferating T cells following polyclonal stimulus in co-culture with un-primed (top left/blue) and CpG-primed (right/red) B cells were gated as shown (**A**). T cell proliferation with un-primed B cells is shown as 100%, relative proliferation of T cells with CpG-primed B cells is shown as a percentage thereof (where <100% indicates reduced proliferation and vice versa). Proliferation with 1:1 of T:B cells is shown at days 3, 6, 9 and 12 (**B**). For which Days 3 and 6 n=11 (NA=5 AR=5 AIT=1), at day 9 n=8 (NA=4 AR=3 AIT=1) and at day 12 n=4 (NA=2 AR=1 AIT=1). 9 day co-cultures had decreasing ratios of B cells used with respect to T cells (ratios show T:B), for both un-primed and CpG-primed B cells (**C**). For B cell titration numbers were; 1:1 n=8, 2:1 n=6, 4:1 n=7, 8:1 n=5, 32:1 n=4 and 64:1 n=4. Mean and standard error are represented with percentage suppression. Wilcoxon matched-pairs signed-rank test was used with paired raw data.

Proportions of regulatory T cells (gated as $CD25^{hi}$ $CD127^{lo}$ $FoxP3^{+}$) and Tr1 regulatory T cells ($IL\text{-}10^{+}$ T cells) were examined by flow cytometry on days 3, 6 and 9, representative gating is shown for day 9 in Figure 15 A. Neither $FoxP3^{+}$ regulatory T cells (Figure 15 B) nor $IL\text{-}10^{+}$ T cells changed in abundance over time between the two cultures (Figure 15 D). The relative proportions of T cell populations are similar despite titration of B cells relative to T cells at day 9 (Figure 15 C and E).

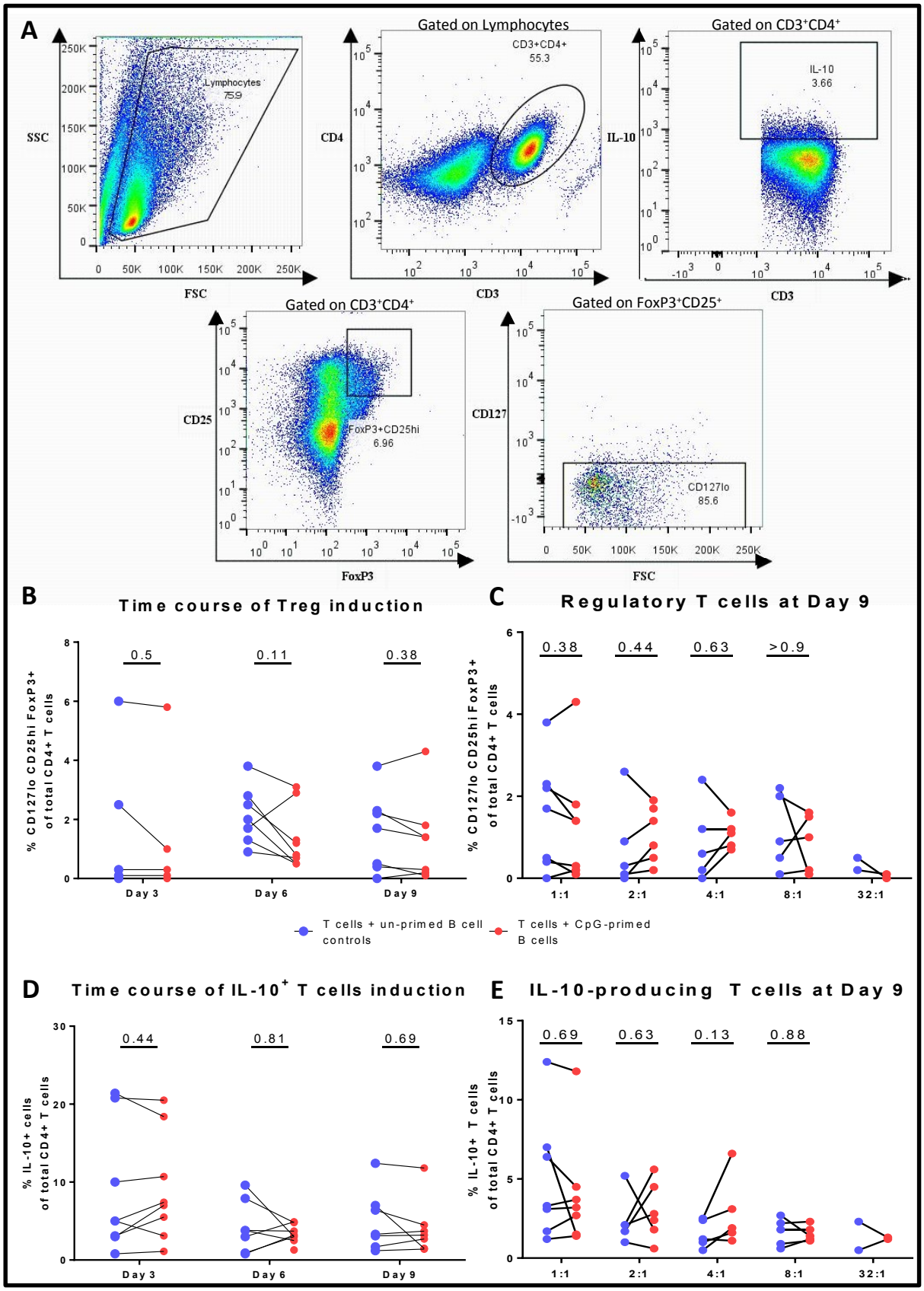


Figure 15 – Regulatory T cells following polyclonal stimulation of T cells in B cell co-culture.

Percentages of $CD4^+CD3^+FoxP3^+CD25^{hi}CD127^{lo}$ and $CD4^+CD3^+IL\text{-}10^+$ cells were determined by the representative gating shown (**A**). Changes in the proportions of $CD4^+CD3^+FoxP3^+CD25^{hi}CD127^{lo}$ T cells when cultured with un-primed (blue) or CpG-primed (red) B cells (**B** and **C**) and $CD4^+CD3^+IL\text{-}10^+$ (**D** and **E**) T cells are shown at 3-daily time points (**B** and **D**). Day 9 B cells were titrated relative to T cells (ratio shows T:B cells) (**C** and **E**). Days 3 and 6 had n=8 (NA=4 AR=3 AIT=1), day 9 1:1 n=7 (NA=4 AR=3), 2:1 n=6, 4:1 n=6, 8:1 n=5, 32:1 n=2. Wilcoxon matched-pairs signed ranked testing was used.

At day 9 of T and B cell co-cultures (with T and B cells in equal proportion) relative expression of IL-10 mRNA is significantly greater in co-cultures with CpG-primed compared with un-primed B cells (Figure 16 A). There is a notable trend towards reduced relative expression of GATA-3 mRNA with CpG-primed B cells. Supernatant cytokine concentrations were analysed by 42plex ELISA, the full data set is shown in the appendix (

Appendix Table 2). These protein concentrations showed a non-significant trend towards greater IL-10 concentrations (p=0.08) and significantly lower concentrations of IL-2, IFNγ, IL-6, IL-4, IL-5, IL-9 and IL-13 (Figure 16 B).

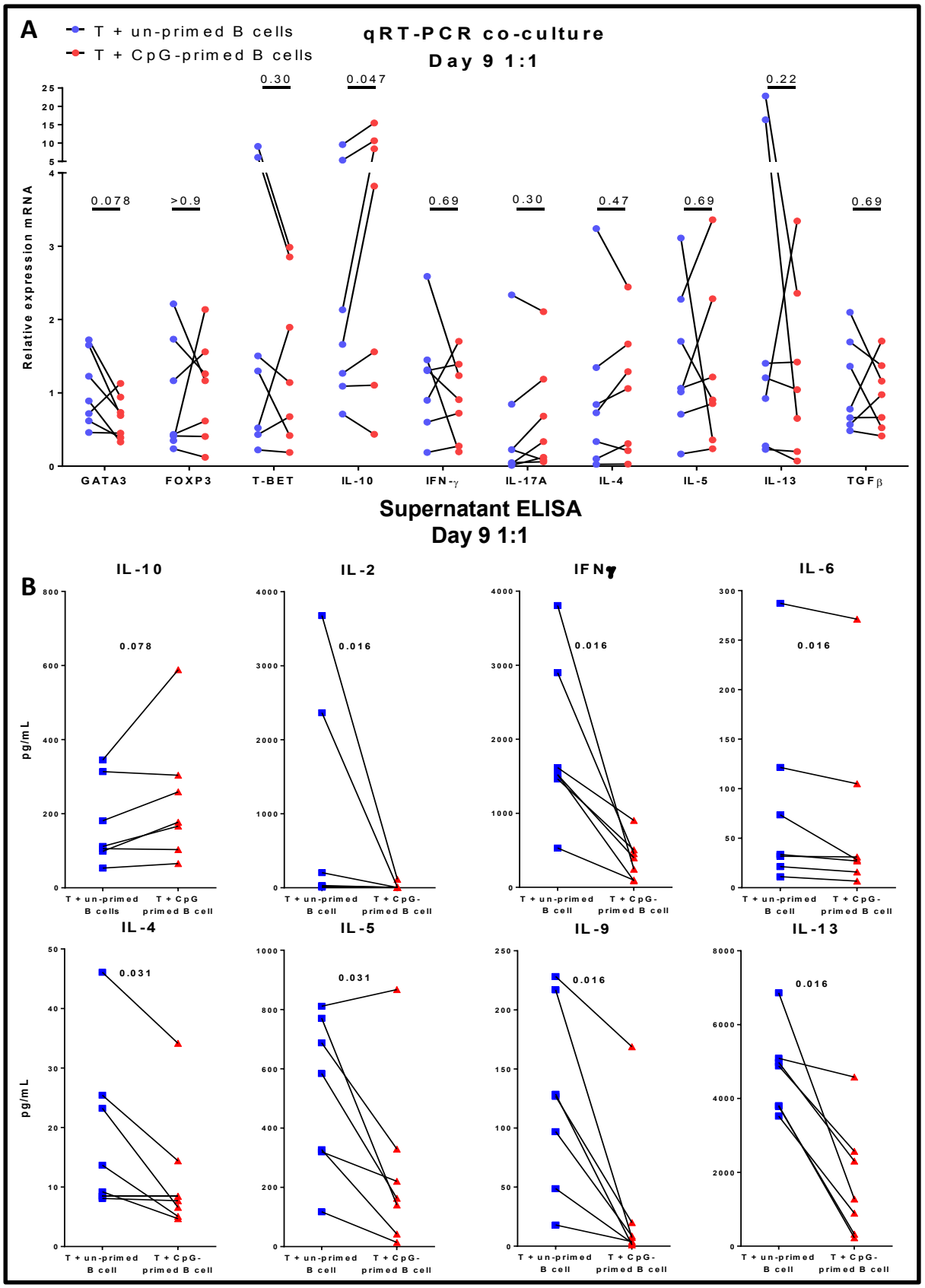


Figure 16 – Changes in supernatant cytokines following polyclonally stimulated T cells in co-culture with B cells.

Culture lysates and supernatants of T and B cell co-cultures at 1:1 are compared, where B cells are 48 hour CpG primed (red) or un-primed (blue). Total culture relative expression mRNA is shown (relative to T cells stimulated alone) between co-cultures (**A**). Supernatant protein concentrations are shown in pg/mL (**B**) for IL-10, IL-2, IFNγ, IL-6, IL-4, IL-5, IL-9 and IL-13 as measured by multiplex ELISA. For all n=7 (NA=4 AR=3). Wilcoxon matched-pairs signed-rank test results are shown.

3.2.2. Suppression of allergen-stimulated T cell responses

CpG-primed B cells were used to suppress an antigen-specific T cell stimulus, Phl p. T cells were cultured with an equal number of irradiated non-T cell PBMCs to present antigen and varying numbers of CpG-primed (red) or un-primed (blue) B cells. Significant relative suppression of T cell proliferation with CpG-primed B cells as compared with un-primed B cells is observed at days 6 and 9 (Figure 17 B). Titration of B cells with respect to T cells shows distinct suppression from 1:1 through to 32:1 (T cells : B cells) at day 9 (Figure 17 C). Representative gating of proliferating T cells in co-culture at 1:1 on day 9 shows this suppression (Figure 17 A). Although suppression of proliferation was observed at day 6, this was not sustained when B cells were titrated with respect to T cells as is the case at day 9 (Appendix Figure 4). Suppression was also not sustained when B cells were the only cells in culture along with T cells and therefore the only cells capable of presentation (Appendix Figure 6, n=2), although suppression was achieved at 1:1.

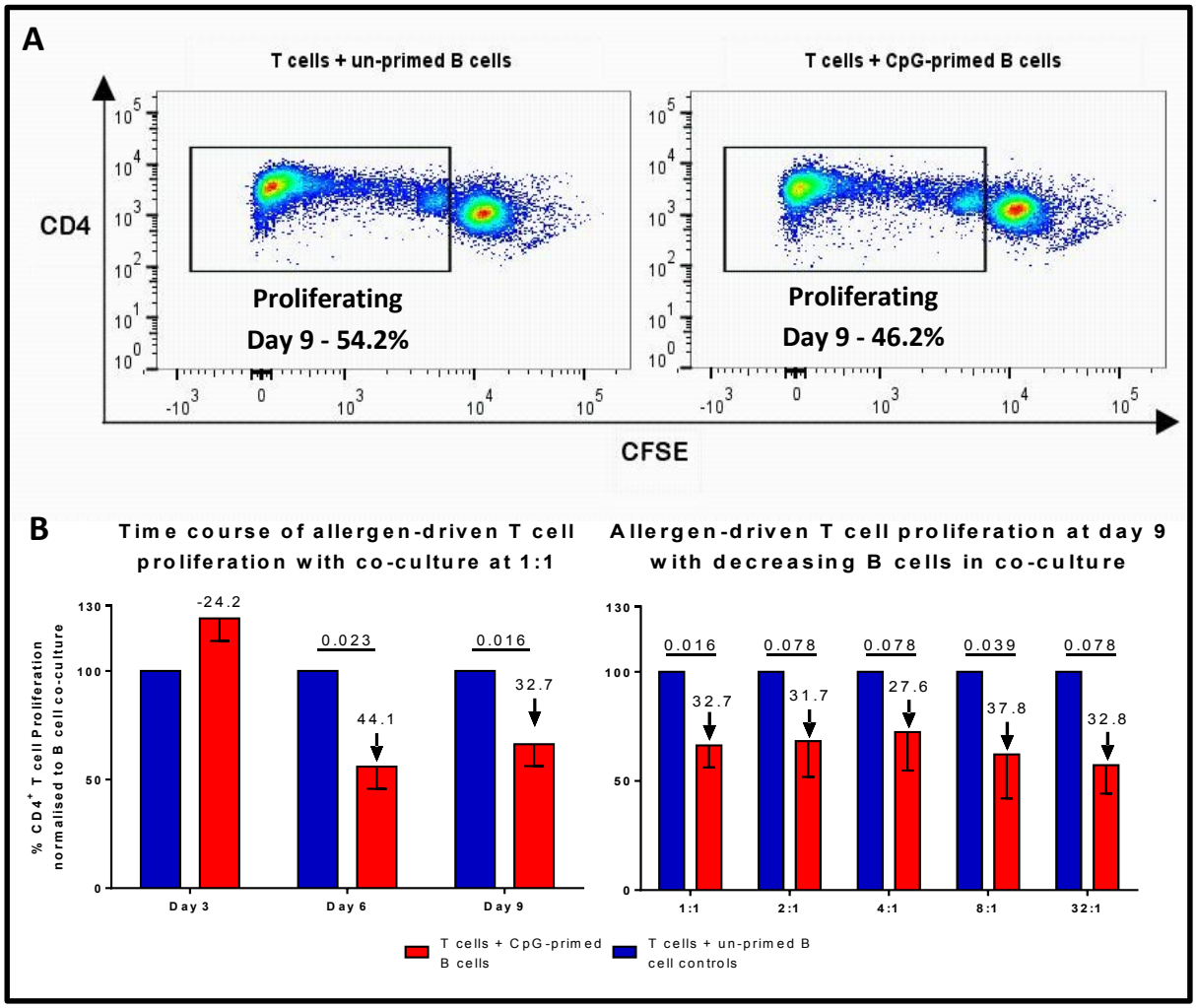


Figure 17 – T cell Proliferation following allergen-stimulated and B cell co-culture.

Percentages of Phl p stimulated T cells proliferating (with allergen presented by irradiated non-CD4+ PBMCs) following co-culture with un-primed (top left/blue) or CpG-primed (right/red) B cells were gated as shown (**A**). T cell proliferation with un-primed B cells is shown as 100%, with relative proliferation of T cells with CpG-primed B cells shown as a percentage thereof. Proliferation with 1:1 of T:B cells is shown at days 3, 6 and 9 (n=8, all AR) (**B** left hand). 9 day co-cultures had decreasing ratios of B cells with respect to T cells (ratios show T:B), for both un-primed or CpG-primed B cells (n=8 for all, AR) (**B** right hand). Mean and standard error are shown. Wilcoxon matched-pairs signed-rank test was used with paired raw data.

Regulatory T cells ($CD4^{+}CD25^{hi}FoxP3^{+}$) were stained and gated as shown in Figure 18 A. Proportions of regulatory T cells were significantly fewer in the presence CpG-primed B cells compared to un-primed B cells in equal proportions (1:1) at days 6 and 9 (Figure 18 B). No change in the proportions of regulatory T cells was observed between co-cultures when B cells were titrated relative to T cells (Figure 18 C). T cell CD25 MFI was significantly lower amongst CpG-primed B cell co-cultures at day 6 and trended towards significance at day 9 (Figure 18 D), whilst trending towards decrease throughout titration of B cells relative to T cells (Figure 18 E).

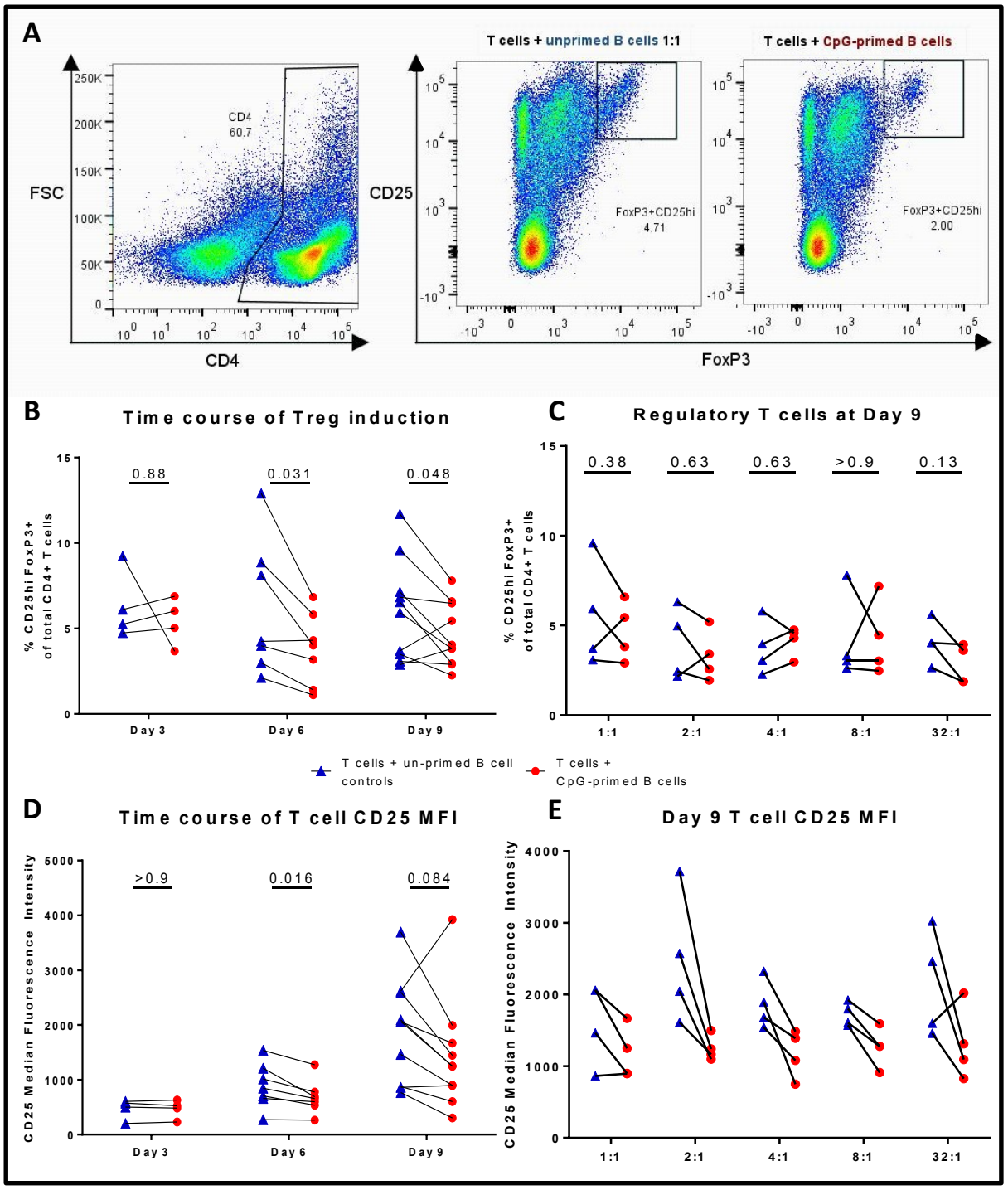


Figure 18 - Tregs following allergen-stimulated and B cell co-culture.

T cells were co-cultured with equal numbers of irradiated non-CD4⁺ PBMCs and Phl P along with B cells which had been either un-primed (blue) or CpG-primed (red). At days 3 (n=4), 6 (n=7) and 9 (n=10) CD4⁺ CD25^{hi} FoxP3⁺ were gated as shown (**A**) and percentages represented in (**B**). At day 9 B cells were titrated relative to T cells (T:B ratios are shown) (**C**). CD25 MFI is assessed amongst CD4⁺ T cells across co-cultures during days 3, 6 and 9 (**D**) and with B cell titration (**E**).

Total co-culture mRNA assessed for IL-10 and IL-17A shows a significantly greater relative increase in CpG-primed compared to un-primed B cell co-cultures (Figure 19 A). None of GATA3, FOXP3, T-BET, IFNγ, IL-4, IL-6 IL-13 and TGFβ mRNA had significantly different relative expression in co-cultures with CpG-primed or un-primed B cells. Supernatant were analysed by 25plex ELISA, the complete data set is shown in the appendix (Appendix Table 3). Significantly greater IL-10 (not IL-17A) protein concentration was found in co-cultures with CpG-primed B cells as compared with un-primed B cells (Figure 19 B). Supernatant concentrations of IL-2, IFNγ, IL-12p70, IL-4, IL-5, IL-9 and IL-13 all significantly decreased in CpG-primed compared to un-primed B cell co-cultures (Figure 19 B).

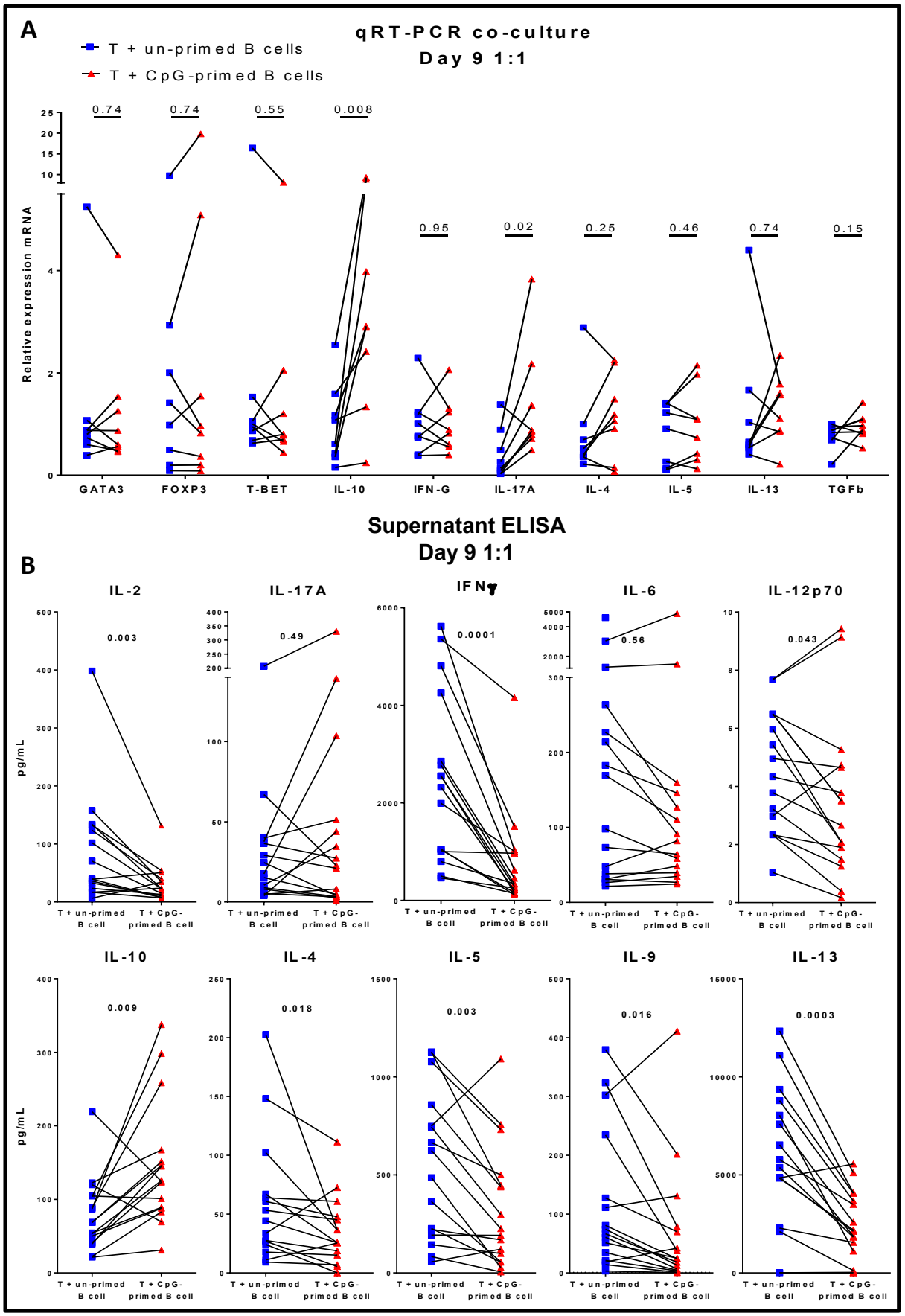


Figure 19 – Changes in supernatant cytokines following allergen-stimulated T cells in co-culture with B cells.

T cells were stimulated with Phl P in the presence of un-primed (blue) or CpG-primed (red) B cells. At 9 days supernatants were removed for ELISA and total co-culture cells lysed for mRNA analysis. Relative expression of mRNA is shown against that found in T cells stimulated in the absence of B cells (**A**). ELISA established protein concentrations (**B**). Wilcoxon matched-pairs signed-rank test results are shown.

Cell death was assessed with Annexin V and 7AAD. Double negative events are live cells (bottom left of dot plot), $7AAD^-$ and Annexin V^+ identifies pre-apoptotic dying cells (bottom right of dot plot) and dual positive events are apoptotic cells (top right of dot plot) in the data shown in Figure 20 A (top centre plot). Gating of these populations is shown for all events and separately for $CD4^+$ events alone (Figure 20 A - top centre and bottom set, respectively). At day 6 no difference in the proportions of live, dying or dead cells was observed between any of the culture conditions for either all events or $CD4^+$ events (Appendix Figure 3), especially comparing un-primed (blue) and CpG-primed (red) B cell co-cultures. This is also the case at day 9 with respect to B cell co-cultures (Figure 20 D and E), however there are greater proportions of dead cells amongst T cell conditions in the absence of B cells; T cells unstimulated (grey) and T cells stimulated (green).

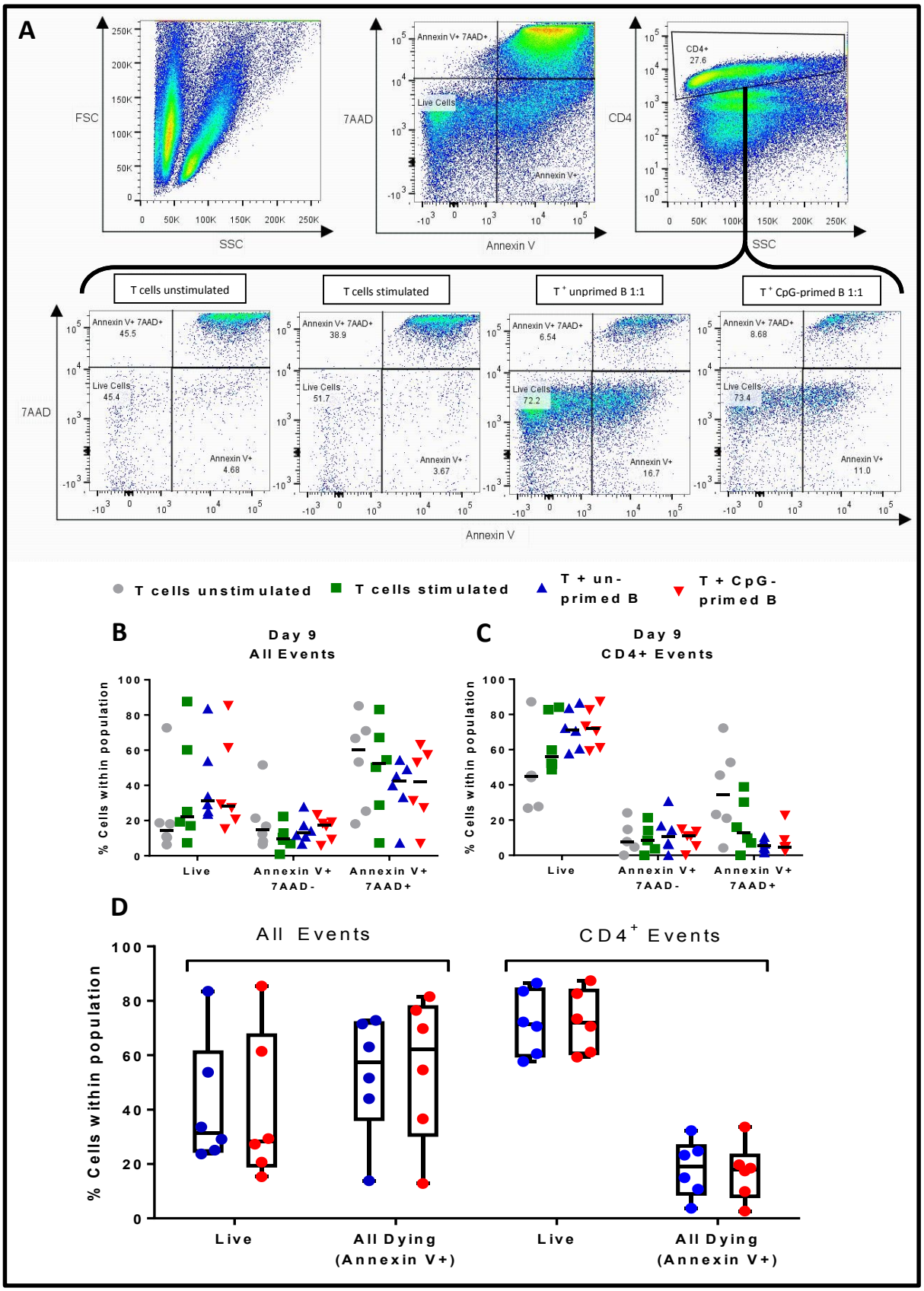


Figure 20 – Live/dead staining of allergen-stimulated T cells in co-culture with primed B cells.

T cells remained unstimulated (grey), stimulated with Phl P (green) or stimulated in the presence of un-primed B cells (blue) or CpG-primed B cells (red). Following 9 days of co-culture staining with 7AAD and Annexin V identified live, dead and dying cells as illustrated (**A**). This is quantified for all events (**B**) and CD4$^+$ events only (**C**). This is further dissected for Live or Annexin V$^+$ dying cells separately in co-cultures alone for all events and CD4+ events only (**D**).

In order to determine the mechanism of suppression by B cells in these cultures, high concentrations of blocking antibodies towards IL-10, IL-10 receptor, TGF-β, PD-L1, CD80 and CD86 were added at the start of 9 day co-cultures. No blocking antibody was able to show significant reversal of the suppression observed in the absence of blocking antibodies (Figure 21 A). Blocking antibodies were then added at days 0, 3 and 6 of co-culture (Figure 21 B), this showed a trend toward reversal of suppression in some patient samples. Half of co-cultures with anti-IL-10 showed a reversal of suppression and anti-IL-10 receptor showed 4 of 7 with decreased suppression; however 2 samples had increased proliferation beyond control. Both anti-TGFβ and anti-PD-L1 showed loss of suppression in 6 out of 7 repeats ($p=0.039$ and 0.055, respectively).

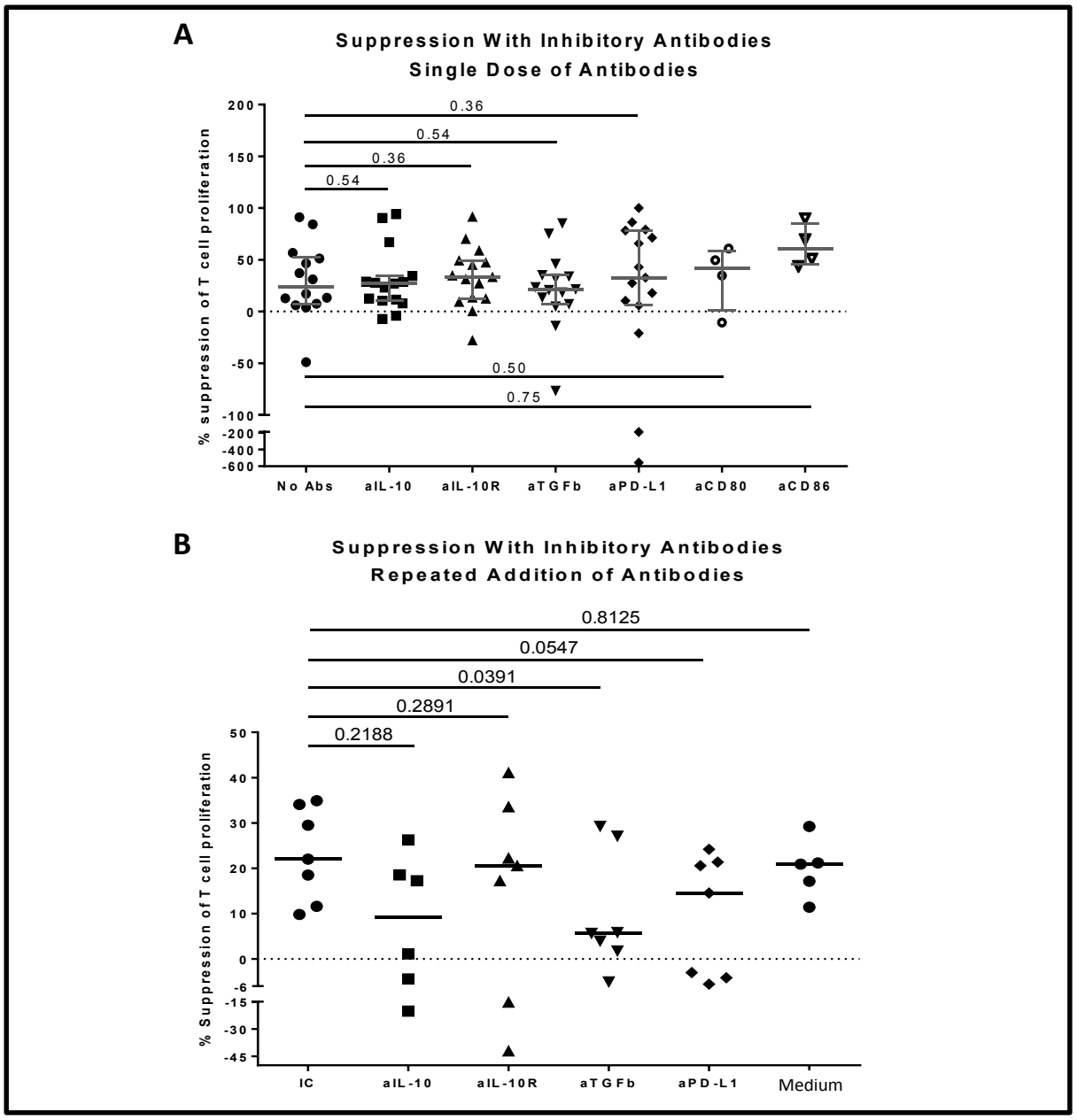


Figure 21 – Allergen stimulated T and B cell co-cultures with the addition of inhibitory antibodies.

Suppression of T cell proliferation is shown; with and without the addition of 5µg/mL of antibodies blocking the cytokine or receptor shown. Antibodies are added at the start of 9 day co-culture (**A**) or at days 0, 3 and 6 (**B**). Percentage suppression is defined by the percentage less proliferation of Phl P stimulated T cells in the company of CpG-primed B cells compared with un-primed B cells. Wilcoxon matched-pairs signed-rank test results are shown.

3.3. Discussion

3.3.1. CpG-induced IL-10

In this chapter it was demonstrated that B cells can produce IL-10 which can be further induced by CpG. Concentrations of 1 and 3 μg/mL of CpG were optimum to increase the proportions of IL-10-producing B cells, with IL-10 enhanced at 48 hours and beyond. These concentrations are consistent with published accounts of IL-10-producing B cells, increased following CpG stimulation, following 48 (Bouaziz et al., 2010, Iwata et al., 2011) or 72 hours (Blair et al., 2010, Lemoine et al., 2011, van de Veen et al., 2013).

3.3.2. LPS and CD40 responses

A dose-dependent induction of IL-10 was not observed following either LPS or CD40 stimulation. This finding is not in agreement with published data (Iwata et al., 2011, DiLillo et al., 2013, Gantner et al., 2003, Blair et al., 2010, Lemoine et al., 2011). Both LPS and CD40 stimulation were explored in further detail at published concentrations, as discussed below.

3.3.2.1. LPS

Isolated B cells do not produce more IL-10 following LPS stimulation, compared to culture in medium alone, detected by flow cytometry or supernatant ELISA. However, there was a trend of increased IL-10 amongst PBMC-cultured B cells. For this reason PBMC and isolated B cell cultures were compared. The proportions of IL-10-producing B cells in both cultures correlated significantly in medium re-stimulated cells and trended towards significance with CpG (likely due to few repeats) suggesting that under these conditions other PBMCs do not sufficiently interfere with native B cell IL-10 capacity. Lack of any correlation in B cell IL-10 capacity following LPS stimulation, however, suggests TLR4 stimulation of non-B cells may induce IL-10 amongst B cells in the mixed PBMC cultures, which was not otherwise induced or detected in the isolated B cell cultures. Increased B cell IL-10 potential may be due to other cells within these cultures bolstering the IL-10-producing capacity of B cells by cell to cell contact or secreted factors, although this was not tested further here. Indeed human B cells from healthy donors have been reported to express very low if any levels of TLR4 (Hornung et al., 2002, Ganley-Leal et al., 2010, Bourke et al., 2003), although TLR4 expression is reported to be increased *in vitro* following inflammatory stimuli (such as IL-4, IL-2 and anti-CD40) and amongst Crohn's disease patients (Ganley-Leal et al., 2010). Whilst it is clear that mouse B cells certainly express TLR4, it is unclear why LPS is reported capable of enhancing IL-10 production amongst human B cells (Iwata et al., 2011, DiLillo et al., 2013). The first of these studies showed PBMCs stimulated with LPS, which may suggest this was

a bystander enrichment of IL-10 capacity, whilst the second used isolated B cells. Both identify LPS as a weaker inducer of IL-10 than CpG.

3.3.2.2. CD40 stimulation

Studies using CpG or LPS as a method of enhancing the proportions of IL-10-competent B cells have shown further increased proportions of IL-10-producing B cells with the addition of CD40 stimuli (Gantner et al., 2003, Iwata et al., 2011, Todd et al., 2014). However, in agreement with this thesis, others have shown the addition of CD40 ligand to show no increase in the proportions of IL-10-producing B cells (Daien et al., 2014, Bouaziz et al., 2010). These studies have used isolated B cells predominantly, so this is unlikely an effect on other PBMCs as described with LPS. Some of the studies successful in inducing IL-10 may be encouraging CD40 cross-linking, which is essential for CD40-mediated activation (Pound et al., 1999), by the use of CHO-expressing CD40L (Gantner et al., 2003, Blair et al., 2010) or fibroblast-expressing CD40L cells (Lemoine et al., 2011), given that cellular-expressed CD40L forms a trimer this may be more biologically relevant. CD40 stimulation may be more biologically relevant, given the necessity of CD40 co-stimulation for competent humoral immunity (Quezada et al., 2004). In addition, CD40 has been shown to be relevant to IL-10-producing B cells since the interesting observation that transgenic over-expression of CD40L in mice (accompanied by loss of CD22, which negatively regulates CD40 signalling) produces increased proportions of IL-10-producing B cells (Poe et al., 2011). However, one study that did employ CD40L-transfected L cells to promote cross linking of CD40 (Bouaziz et al., 2010) was unable to show increased IL-10.

A clear understanding the role CD40 stimulation is able to play in promoting pan-immunological tolerance is pertinent due to the development of agonistic monoclonal CD40 therapies currently seeking approval for the treatment of both solid and circulating cancers (Khong et al., 2012). Murine data using agonistic anti-CD40 treatment of predisposed autoimmune mice shows expansion of regulatory B cells, which increased murine survival and abated disease in this model (Blair et al., 2009). Peculiarly, a clinically trialled anti-CD40 agonist was suggested to have greater T cell priming capacity *in vitro* when used with CpG (Carpenter et al., 2009). This study demonstrated significant IL-10 and IL-6 induction following anti-CD40 and CpG B cell priming. However, T cell co-cultures were carried out with primed but lethally irradiated B cells, likely incapacitating any B cell regulatory capacity. Whilst CD40 agonists likely promote anti-tumour B cell activity in an inflammatory microenvironment by presentation and cytotoxic T cell activation, there may be a risk of Breg induction in microenvironments with a paucity of inflammatory mediators. Whilst CD40 mAbs may additionally be able to target oncological B cells for destruction, the use of agonistic CD40 mAbs risks promoting

'regulatory B cell tumours'. Use of CD40 mAbs in the treatment of B cell cancers, such as chronic lymphocytic leukaemia (CLL) which has been shown to have an increased regulatory B cell potential (DiLillo et al., 2013), will require further work to fully understand the context within which CD40 stimulators induce either B cell regulators or pro-inflammatory presenters.

3.3.3. Detection of B cell mRNA following CpG stimulation

Interestingly, although both IL-10 and IL-6 mRNA peak at 6 hours following stimulation, IL-10 remains high through to 72 hours, suggesting consistently enhanced IL-10 gene induction. In comparison, IL-6 mRNA is induced to a greater extent at 6 hours, but declines rapidly and greatly by 72 hours. However, it is clear from examination of the supernatant cytokine concentrations in the next chapter that IL-6 is strongly expressed, whilst this is discussed in the next chapter (section 4.4.2.3). Induction of IL-6 to a greater degree than IL-10 may be relevant to the lack of regulatory T cells observed in co-cultures with primed B cells, as discussed in detail later (section 3.3.7). Despite the presence of IL-6, these primed B cells were able to mediate T cell suppression.

IL-10Rβ mRNA induction shown here increases from 18 hours through to 72 hours following CpG stimulation, suggesting autologous IL-10 feedback in CpG-activated B cells. Autocrine action of IL-10 may promote B cell survival (Gary-Gouy et al., 2002) and has been shown to drive plasmablast generation and later IgM or IgG secretion (Heine et al., 2014), which may feed into the production of IL-10-producing plasma cells, which have been recently detected (Shen et al., 2014). Interestingly, a case study of 5 patients with non-EBV large B cell lymphomas shows IL-10 receptor loss of function mutations as the trigger of this uncontrolled proliferation (Neven et al., 2013), suggesting the integral role of the IL-10 receptor to human B cells *in vivo*.

3.3.4. The relevance of CpG-induced IL-10

The purpose of this chapter is to identify B cells capable of IL-10 production by phenotype, but also to develop a means by which all IL-10 competent B cells can be identified. However, it may be suggested that CpG is an artificial stimulus, irrelevant *in vivo*, for this reason it was pertinent to demonstrate that proportions of IL-10-producing B cells detected following CpG stimulation closely related to those detected following culture in medium alone (before PMA and Ionomycin re-stimulation). Medium-cultured B cells, receiving only 5 hours of PMA and Ionomycin stimulation may be compared to the 'B10' population described by Tedder et al (Iwata et al., 2011), whilst the 48 hour CpG-induced IL-10 B cells detected here would have been described as 'B10 and B10-pro' B cells, detected after a period of expansion. Much like their study, these populations closely correlated in this work. Whist PBMC-cultured B cell IL-10 correlated significantly, but less closely, than B cells in isolation (r=0.54 compared

with r=0.73). However, as mentioned above (section 3.3.2.1), this may be due to other cells acting on B cells in PBMC culture.

Gray and colleagues have demonstrated that both murine and human B cells cultured with apoptotic cells, which had with exposed DNA, produced IL-10 driven by TLR9 signalling (Gray et al., 2007, Miles et al., 2012). These studies show a clear *in vivo* relevance for TLR9-mediated IL-10 B cell responses driven by apoptotic cellular DNA in the microenvironment, suggesting anti-inflammatory B cell responses contribute to prevent inappropriate inflammation in response to controlled cell death.

3.3.5. Phenotyping IL-10-producing B cells

Phenotypic characterisation showed increased proportions of IL-10-producing B cells amongst $CD24^{hi}CD38^{hi}$ B cells, compared to $CD24^{int}CD38^{int}$ or $CD24^{hi}CD38^{lo}$ populations, consistent with that found following CD40 stimulation (Blair et al., 2010). IL-10 restriction to $CD24^{hi}CD38^{hi}$ B cells in the previously cited study was induced following CD40 stimulation by CD40L-expressing CHO cells. It is possible that CD40 stimulation in the absence of BCR or cytokine signals polarises IL-10 production to this naïve subset. Despite this, it is clear that IL-10-producing B cells exist amongst the non-$CD24^{hi}CD38^{hi}$ populations and are also expanded following culture in CpG. The $CD24^{hi}CD38^{hi}$ B cells were not the sole IL-10 competent B cell population in this study or others (Das et al., 2012, Silva et al., 2012).

This thesis shows comparable proportions of IL-10-producing cells amongst all three subsets with PMA and Ionomycin stimulation in the absence of CpG. $CD10^{+}$, $CD27^{+}CD24^{hi}$, $CD274^{+}$ (PD-$L1^{+}$), $CD5^{hi}$ and $CD24^{hi}CD38^{hi}$ B cells separately all showed significantly increased proportions of IL-10-producing cells compared to the whole $CD19^{+}$ B cell population, as well as significantly greater expression amongst IL-10^{+} B cells than IL-10^{-}. Of the markers examined, CD27 was the only one not to show increased proportions of IL-10-producing B cells, or to be increased in the IL-10 population (following CpG stimulation). This is consistent with the mixed data within the literature. Tedder and colleagues, who identified the $CD27^{+}CD24^{hi}$ subset as a regulator of monocytes, showed increased CD27 expression amongst IL-10-positive B cells following CpG (Iwata et al., 2011). A number of other studies have found IL-10^{+} human B cells do not reside entirely within the $CD27^{+}$ population (Bouaziz et al., 2010, Lemoine et al., 2011, Heine et al., 2014). A clear phenotype with which to define the IL-10-competent B cell has yet to emerge, however this study highlights that CD5-expressing or $CD24^{hi}CD38^{hi}$ B cells are beneficial phenotypes to identify in order to determine B cell IL-10 capacity.

3.3.6. Exploring the predictive value of *ex vivo* phenotyping on *in vitro* IL-10 capacity

Given the identification of markers which identified B cell populations with greater IL-10 competency, it stands that the proportions of B cells expressing these markers in whole blood, established directly *ex vivo*, should correlate with subsequent proportions of IL-10-producing B cells *in vitro*.

In most cases, expression of phenotypic markers *ex vivo* correlated with proportions of IL-10-producing B cells at 48 hours, in the presence or absence of CpG. Where correlations did occur, CpG stimulation only served to increase the confidence and significance of the relationship present with medium re-stimulated B cells alone. This is likely due to the greater spread in the proportions of IL-10-producing B cells detected between patients following CpG stimulation, allowing for a greater sensitivity in detecting variation. Proportions of $CD10^+$ B cells were the only subset not to correlate with later IL-10 capacity, surprisingly as CD10 is a marker of naïve/transitional B cells identified as strongly IL-10 competent (Blair et al., 2010).

Expression of CD27 and $CD24^{hi}CD27^+$ amongst B cells *ex vivo* showed a greater correlation coefficient and higher significance with CpG-induced IL-10 than medium alone. This may be due to $CD27^+$ B cells preferentially responding to TLR9 stimulus (Bernasconi et al., 2002), however, CD27- B cells still express and respond to TLR9 stimulation. Although, as this relationship was significant in the absence of CpG, this may be due to CpG producing greater IL-10 responses, as mentioned above.

Weakly significant correlations were observed between the *ex vivo* proportions of PD-$L1^+$ ($CD274^+$) or $CD5^+$ B cells and subsequent *in vitro* IL-10. CD5 expression has been established to itself induce IL-10 amongst B cells (Garaud et al., 2009, Garaud et al., 2011, Gary-Gouy et al., 2002, Mageed et al., 2012). IL-10-producing B cells have been shown to express PD-L1 (Kubo et al., 2012, van de Veen et al., 2013), potentially as a result of CpG stimulation, which may act as a mechanism of B cell mediated suppression of Th2 cells (Kubo et al., 2012). The fact that this study showed weak correlation with PD-L1 expression prior to culture reinforces this marker as indicative of IL-10 capacity in spite of CpG stimulation.

Surprisingly, the most significant correlation of *in vitro* proportions of IL-10-producing B cells observed was an inverse correlation with respect to the proportions of $CD24^{hi}CD38^{hi}$ B cells *ex vivo*. This suggests that greater proportions of $CD24^{hi}CD38^{hi}$ B cells in whole blood indicates fewer proportions of $IL\text{-}10^+$ B cells likely to be detected. The reverse was shown in a recent study (Todd et al., 2014), in which a highly significant positive correlation was drawn between proportions of $CD24^{hi}CD38^{hi}$ B cells and proportions of CpG^+CD40L-induced IL-10-producing B cells. In addition, many studies have used

proportions of $CD24^{hi}CD38^{hi}$ B cells as a measure of the proportions of regulatory B cells in a given patient population (Li et al., 2012, Blair et al., 2010, Das et al., 2012, Silva et al., 2012, Flores-Borja et al., 2013, van der Vlugt et al., 2014, Saussine et al., 2012, Duggal et al., 2013, Todd et al., 2014, Lepse et al., 2014, Cherukuri et al., 2014). In all but three of these studies cited, the patient group with greater proportions of $CD24^{hi}CD38^{hi}$ B cells had a correspondingly greater proportion of IL-10-producing B cells. Two studies have shown significantly fewer proportions of $CD24^{hi}CD38^{hi}$ B cells amongst either active vasculitis or renal graft rejection patients compared to controls (Lepse et al., 2014, Cherukuri et al., 2014); despite no change in the proportions of IL-10-producing B cells following CpG stimulation. The only one of these studies to show a reversal in this relationship assessed a group of allergic asthmatics who had greater proportions of $CD24^{hi}CD38^{hi}$ B cells but fewer LPS-induced (from isolated B cell cultures) IL-10-producing B cells compared to healthy controls (van der Vlugt et al., 2013).

It is unclear why fewer $CD24^{hi}CD38^{hi}$ B cells in whole blood result in greater proportions of IL-10-producing B cells in culture, despite this subset being the dominant source of IL-10-producing B cells detected in this study, as discussed above.

The chemokine receptors CCR3, CCR4, CCR7, CCR8, CCR10 and CXCR5 showed both a significant and clearly defined increase in expression amongst $IL\text{-}10^{+}$ B cells compared with $IL\text{-}10^{-}$ B cells. Whilst this data is limited to the staining of surface receptor expression, and has not shown a functional migratory response, this is suggestive of a potential homing (or identifiable) phenotype. Significant with respect to allergic inflammation is the expression of CCR3 and CCR4, expressed by Th2 cells (Sallusto et al., 1998, Bonecchi et al., 1998). CCR4 in particular identifies Th2 cells able to home to the lung (Mikhak et al., 2013) and is critical for allergic inflammation (Banfield et al., 2010, Kaminuma et al., 2012), which may suggest the co-localisation of Th2 and IL-10-producing B cells *in vivo*. CCR3 expression on B cells has been shown to be induced by IL-4, inducing no chemotactic capacity but driving the expression of CD95 and CD95L, making these cells more susceptible to apoptosis (Jinquan et al., 2003), although no assessment of CD95 or CD95L took place in this thesis. Additionally, the expression of CCR10 has been associated with IgE-secreting B cells predisposed to home to the asthmatic lung (Scanlon et al., 2011). CCR10 expression is also associated with recruitment to the mucosa, especially for IgA-expressing plasmablasts (Liang et al., 2011).

Increased expression of both CXCR5 and CCR7 may further, or separately, identify these IL-10-producing cells as predominantly naïve B cells, able to enter and interact with both DCs and T cells within the germinal centre (Okada and Cyster, 2006). Finally, CCR8 expression has been suggested to synergise with TLR4-mediated stimulation of murine monocytes to induce expression of IL-10, IL-6 and

TNFα (Oshio et al., 2014), which bears some similarity to the phenotype observed here. However, very little is known of CCR8 expression amongst B cells.

3.3.7. B cell mediated suppression of stimulated T cells

The use of CpG-primed (and therefore IL-10-enriched) B cells in co-culture with $CD4^+$ T cells showed no suppression of proliferation until day 9 of co-culture, suggesting a much delayed response beyond that which would be expected with IL-10 alone. A similar time course of CpG-primed suppression has been previously reported, in which suppression was not apparent at day 4 and only began from day 5 of co-culture (Lemoine et al., 2011). Suppression was maintained when a T:B ratio of 1:1 or 2:1 was used but not when B cells were diluted beyond this, in the context of a polyclonal T cell stimulus. This suggests a T:B ratio of 2:1 is required for B cells to achieve potency for suppression. Conversely ratios of 8:1 and 32:1 showed increased $CD4^+$ T cell proliferation in CpG-primed compared to un-primed B cell co-cultures, suggesting that CpG priming enhanced T cell activation or proliferation. This may be due to IL-6 and TNFα drowning out IL-10 or cell-cell mediated suppression at these ratios, although this observation was not pursued further in this thesis.

Given polyclonal T cell activation is scarcely representative of activation *in vivo*, a system of Th2 T cell stimulation, using purified grass pollen allergen in T cell cultures, presented by irradiated PBMCs, was used to demonstrate suppression by CpG-primed B cells. Suppression of allergen-driven T cell proliferation, conversely, was maintained at T:B ratios of up to 32:1. This is likely due to the infrequency of grass pollen allergen-specific $CD4^+$ T cells. A study using T cell peptide antigen tetramers identifies the most common antigens recognised by as few as 1 in 10,000 T cells (Wambre et al., 2014). Experiments using EliSpot showed roughly 1 in 10,000 PBMCs secreting IL-4 in response to allergen stimulation (Gabrielsson et al., 1997, Horiguchi et al., 2008). Given IL-4 is only produced by $CD4^+$ T cells which make up approximately 30% of PBMCs, this would suggest around 1 in 3,000 $CD4^+$ T cells is capable of an allergen-specific IL-4 response. Studies of IL-4-producing T cells following re-stimulation with PMA and Ionomycin were not included to account solely for allergen-induced responses. The paucity of allergen-specific T cells, even amongst allergics as used for allergen-driven co-cultures here, suggests few T cells capable of responding to allergen stimulation and may explain the increased potency of suppression at 32:1. This protocol activates the so-called 'B10 and B10-pro' B cells, as described by Tedder et al., which have IL-10 capacity following TLR9 (or, in their hands, TLR4) stimulation (Iwata et al., 2011). However, the potency shown here of CpG-primed B cells may be considered surprising given CpG priming merely increased the proportions of IL-10-producing B cells from 0.6% to 1.4% (medians following culture in medium or CpG). However, this may be explained by their active role in IL-10 production, with IL-10 protein detected by ELISA in supernatants, at up to 4

days following CpG stimulation, with no detectable IL-10 from medium-cultured B cell conditions. Both primed and un-primed B cell co-cultures will also contain the identical regulatory subsets, although the suggestion is that these may be either expanded or 'activated' by CpG-priming and thus have regulatory activity subsequently. A previous study of isolated (un-primed) IL-10-competent B cell subsets achieved a much reduced potency of suppression with antigen-specific stimulation (van de Veen et al., 2013). $CD73^{-}CD25^{+}CD71^{+}$ B cells used to suppress PPD-stimulated PBMCs showed loss of $CD4^{+}$ T cell suppression at a ratio of 25 PBMCs to 1 B cell (roughly, this is a T:B ratio of 4:1).

With allergen stimulation suppression was also observed earlier at day 6, suggesting that suppression of proliferation is also achieved earlier with fewer responding T cells, although this was not maintained with dilution of B cells at this time point. B cells are potent APCs, and B cell co-cultures resulted in greater T cell proliferation than T cells achieved with irradiated PBMCs alone. It is therefore likely that B cells in allergen-driven cultures maintained T cell survival or drove maturation to a greater degree than in polyclonal T cell stimulation co-cultures; as such there was further potential interaction between B and T cells through which active, or passive, suppression may have taken place.

Suppression of proliferation at day 9 with a T:B cell ratio of 1:1 was accompanied by significant suppression of Th1 and Th2 cytokine concentrations in supernatants where CpG-primed B cells were used, with the exception of IL-10 which was greater. It is no surprise that greater IL-10 was detected in CpG-primed B cell co-cultures, likely from the IL-10-induced B cells. The reduced proliferation of T cells (and therefore fewer cells in the respective culture) may explain the reduced concentrations of inflammatory cytokines. However, whilst some cytokines had a modest reduction in concentration, others reduced by twice the percentage than the decrease in proliferation, such as IL-13 which reduced by 73% and 61% in polyclonal and allergen-driven cultures, respectively compared to a suppression of proliferation of 31% and 33%, respectively. Cytokine suppression may in part be attributed to fewer cells able to produce cytokine, but B cell inhibition of cytokine production directly should not be discounted.

Suppression of proliferation in both cases was not due to the induction of $FoxP3^{+}$ regulatory T cells, nor IL-10-producing T cells (examined in the polyclonal condition only). In fact, it was a surprise to find that in the allergen-driven co-cultures, CpG-primed B cells were accompanied by significantly fewer proportions of $CD25^{hi}FoxP3^{+}$ Tregs than un-primed B cell co-culture. This may be due to the reduced IL-2 detected in these supernatants, which is required for Treg survival (Setoguchi et al., 2005), or due to decreased overall T cell activation. Suppression of total T cell activation (including that of Treg, which may impact survival) is supported by the persistent suppression of the activation marker CD25 amongst T cells at days 6 and 9, in spite of B cell titration relative to T cells. This suggests a regulatory

mechanism which arrests T cell activation, resulting in impaired T cell responsiveness globally (proliferation *and* cytokine production).

Alternatively, or additionally, the significant reduction in Tregs may be due to the previously observed production of significant amounts of IL-6 by CpG-stimulated B cells. IL-6 drives regulatory T cells into a Th17 (IL-17-producing) phenotype (Komatsu et al., 2014). This is supported by the significant induction of IL-17A mRNA from allergen-driven co-cultures and lack of suppression observed in IL-17A protein concentration (unlike all other cytokines shown, with the exception of IL-6 (also no significant change) and IL-10 which significantly increases). Although one may expect IL-6 to increase significantly in CpG-primed B cell cultures if this were a significant factor, much like IL-10 concentration.

Reduced T cell CD25 expression or suppression of proliferation may have been due to T cell exhaustion and subsequent early death or B cell-induced killing of activated T cells. This was investigated using cell death assays. No change in total or T cell death was observed between B cell co-cultures either at day 6 or 9, although death was increased amongst cultures lacking in B cells and, unsurprisingly, unstimulated T cells showed the greatest death. Both suggesting that stimulation, and furthermore B cells in co-culture, sustain T cell survival. Comparable proportions of live, dying and dead cells between CpG-primed and un-primed B cells, however, indicate cell killing or activation induced cell death is not a mechanism of suppression in this system.

3.3.7.1. Use of inhibitory antibodies

It is clear that the exploration of the effects of inhibitory antibodies on Breg-induced suppression of T cells in these co-culture experiments are in need of further optimisation. The single addition of blocking antibodies was unable to show any impact on the suppressive capacity of these cells at day 9. Due to the long term nature of these cultures, inhibitory antibodies were added repeatedly at the same concentrations, which achieved partial reversal of suppression in some conditions, likely limited by few repeats. Reversal of suppression was achieved in about half of cases, which may suggest differing suppressive actions between individuals. The reversal of suppression by anti-TGFβ antibodies which achieved statistical significance, in contrast to IL-10, suggests IL-10 may not be the only suppressive cytokine produced by Bregs, although unfortunately TGFβ levels in supernatants from co-cultures were not checked by ELISA. Furthermore, ELISA may have been useful to confirm successful blockade of supernatant cytokines within these culture. The quantity of anti-IL-10 added was at least 5x the OD_{50} required to neutralise a lower concentration of recombinant protein.

3.4. Future work

I was unable to confirm work by others that may suggest cross linking of CD40, but not CD40 stimulation alone (as carried out here), induced or increased IL-10 competence. It would be of interest to compare the use of anti-CD40 antibodies with the addition of cross-linking antibody to form complexes. In addition, the effect of other cells producing IL-10 or responding to LPS appeared to act on B cells in these experiments. Future experiments should explore B cell dynamics with other cells, which are likely to play a greater role *in vivo*. The autocrine and exogenous actions of IL-10 on B cells may further act on B cells within this microenvironment and may be relevant *in vivo,* given the infrequency of IL-10-producing B cells, requires further exploration.

Little previous work has explored the triad of IL-10, IL-6 and TNFα responses observed following B cell stimulation with CpG. Further work would be required to dissect the cytokine responses towards IL-10-inducing stimuli, the ratio of pro and anti-inflammatory cytokines as well as characterising the differently cytokine competent B cells. Experiments performed by other groups have shown the suppressive capacity of isolated B cell subsets demonstrated here to have greater IL-10 capacity. However, although suppression of Th2 responses were shown here using CpG-primed B cells as a whole, this may differ if IL-10^{+} or subsets of IL-10 capable B cells were sorted. Further work is required to determine whether specific subsets of IL-10 capable B cells are unique or dissimilar from one another with respect to phenotype or mechanism of suppression. Whilst every other reported and confirmed IL-10 competent phenotype shown by proportion of expression in whole blood correlated with later IL-10 release, this was the inverse for percentages of CD24^{hi}CD38^{hi} B cells. There is a clear need to confirm and identify why or how increased proportions of CD24^{hi}CD38^{hi} B cells limit the later proportions of IL-10-producing B cells.

The potential for reversal of Breg mediated suppression of Th2 T cell activation by inhibitory antibodies requires further work. Further work should investigate the potential for TGFβ or IL-35-production by these B cells to mediate T cell suppression, particularly in light of recent published data that showed significant reversal of suppression by TGFβ inhibitory antibodies and demonstrating IL-35-producing B cells (Lee et al., 2014, Shen et al., 2014).

4. Influence of grass pollen immunotherapy on peripheral IL-10$^+$ Breg responses *ex vivo*

Studies which have compared healthy and allergic rhinitic allergen-induced IL-10 production, by either PBMCs or T cells, do not show a loss of IL-10 capacity amongst allergic donors, by examination of supernatant cytokine concentration (Kailaanmaki et al., 2014, Domdey et al., 2010, Akdis et al., 2004). Furthermore, allergics have been shown by some studies to have increased proportions of IL-10-producing T cells in response to allergen, compared to non-allergic controls (Han et al., 2010, Akdis et al., 2004, Yamanaka et al., 2011), likely due to IL-10 associated with Th2 responses rather than production by Tr1 or Treg cells.

In contrast, prior to this study the B cell compartment has scarcely been examined amongst allergic individuals, compared to either healthy controls or AIT-treated allergic donors. One study has shown fewer IL-10-producing B cells in response to LPS stimulation amongst allergic asthmatic subjects compared to healthy controls (van der Vlugt et al., 2014). However, proportions of IL-10-producing B cells were unaltered in the context of with CpG or anti-IgM stimulation or re-stimulation following culture in medium alone. The authors partly accounted for this by showing reduced expression of TLR4 on B cells from allergic asthmatics compared to healthy controls. A single study examined IL-10-producing B cells in relation to AIT administered for bee venom anaphylaxis (van de Veen et al., 2013). In patients undergoing bee venom AIT there were greater proportions of IL-10-producing B cells that were allergen-specific and accounted for a much greater proportion of total circulating B cells compared to those from untreated donors. Interestingly, healthy 'tolerant' bee keepers, who were naturally heavily exposed to venom through bee stings independent of AIT, had an even greater proportion of specific IL-10-producing B cells than observed for bee venom allergic individuals undergoing AIT.

This chapter will examine the *ex vivo* IL-10 capacity of B cells in grass allergic individuals compared with non-allergic control subjects and grass allergic individuals who are grass pollen tolerised following high dose allergen immunotherapy. Identification of IL-10-producing B cells following CpG stimulation, using methodology optimised in the previous chapter and described above (section 2.3), their proportions and phenotypic characteristics will be compared between clinical categories.

Hypotheses

1) Allergic individuals have fewer regulatory B cells compared to non-allergic controls, which are restored following successful allergen immunotherapy.

2) Allergic individuals have impaired regulatory B cell responses to allergen that are restored following successful allergen immunotherapy.

Aims

To use a cross-sectional study of non-allergic, grass pollen allergic and grass pollen immunotherapy-treated allergic participants to investigate:

1. Proportions of peripheral IL-10-competent B cells
2. Regulatory B cell subsets in peripheral blood
3. Proportions of regulatory B cell subsets after nasal allergen challenge

4.1. Pilot comparisons of B cells from allergic and non-allergic participants

PBMCs and isolated B cells were cultured from non-allergic and allergic volunteers (2 SCIT samples were available for PBMCs) and compared by flow cytometry (Figure 22 A and B, respectively). PBMC culture showed significantly reduced proportions of IL-10-producing B cells amongst allergic donors compared to non-allergic controls following stimulation with CpG or LPS (Figure 22 A). However no differences were observed for medium alone. In contrast, amongst isolated B cell cultures the proportions of IL-10-producing B cells were identical between groups (Figure 22 B). Since the disparity in IL-10-producing B cells between allergic and non-allergic donors cannot be reproduced in isolated B cells, FluoroSpot was used as an alternative method for detecting IL-10 production amongst isolated B cells (in a subset of the same donors). Significantly fewer IL-10$^+$ B cells were observed amongst allergic individuals compared to healthy controls following CpG stimulation (Figure 22 C). Few B cells produced IL-10 with LPS stimulation or in medium alone.

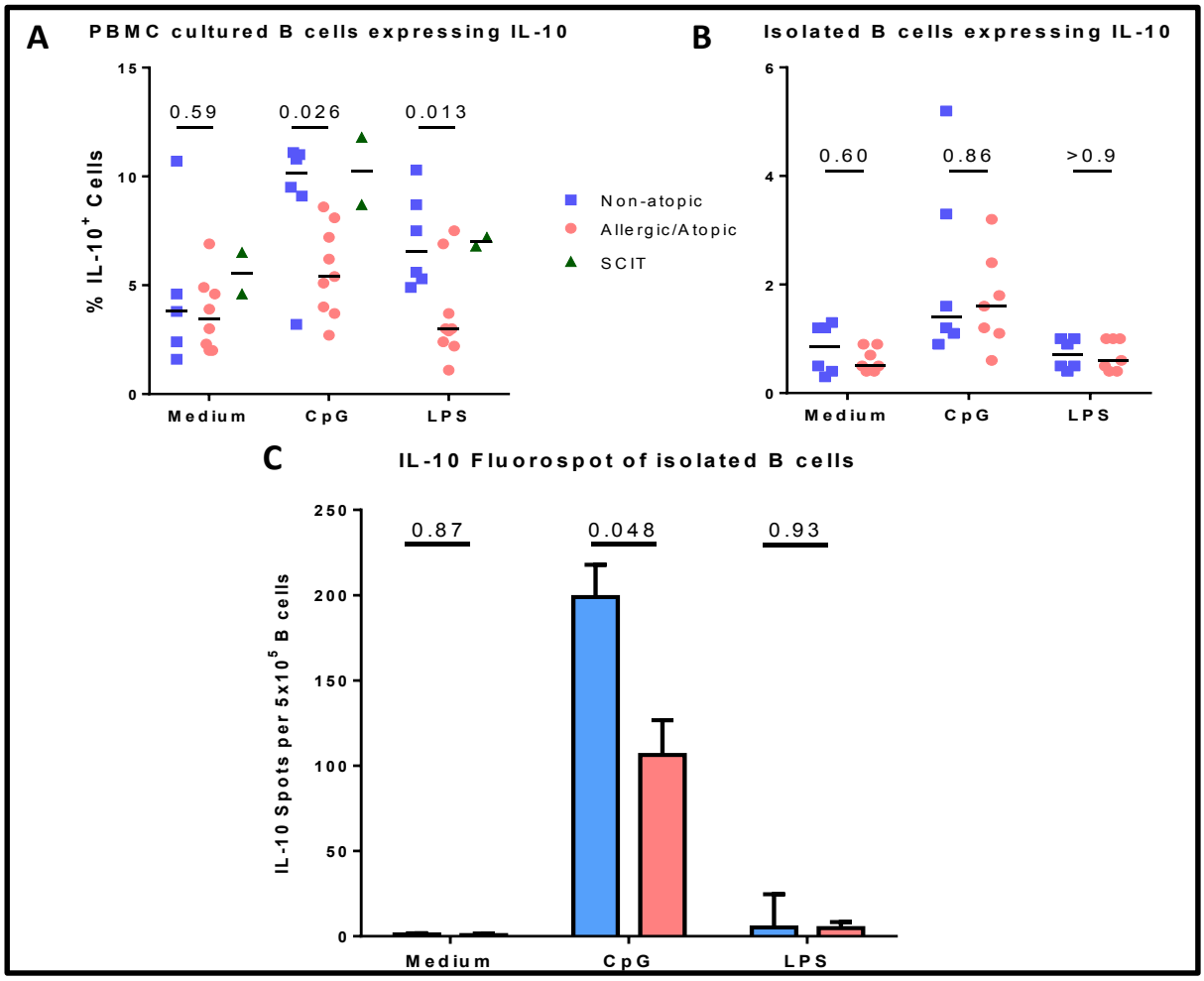


Figure 22 –Comparison of B cell IL-10 from non-allergic, allergic and immunotherapy-treated donors.

PBMCs (n=17, NA=6, AR=9, AIT=2) or isolated B cells (n=13, NA=6 AR=7) were cultured from NA (blue), AR (red) or AIT (green) donors. Cells were stimulated for 48 hours with 1µg/mL CpG, LPS or left in medium alone. Cells stained for flow cytometry were gated for their percentages of IL-10$^+$ B cells following PBMC (**A**) or isolated B cell culture (**B**). IL-10 detection by fluoroSpot (n=9, NA=6, AR=3) was used to quantify numbers of B cells producing detectable IL-10 (**C**). Data shows median with interquartile range.

4.2. Effect of allergen-specific immunotherapy on IL-10-producing Bregs

4.2.1. Cross-sectional participant's baseline demographic and clinical data

On the basis of the above preliminary data a larger cross-sectional study was initiated to confirm the preliminary findings and compare the impact of allergen immunotherapy on IL-10-producing Bregs, as described in section 2.10, on p58. Allergic donors were recruited from the Royal Brompton hospital allergy clinic in response to advertisements, along with 14 non-allergic control donors from external sources. Allergic donors were untreated (n=14) or AIT-treated (n=8 SCIT and n=6 SLIT).

Demographic details of the volunteers show a similar age across all groups (Table 6). There was a gender bias towards females amongst non-allergic and SLIT donors and towards males amongst untreated seasonal allergic, SCIT and AIT withdrawal donors. SLIT donors had a greater duration of AIT compared to SCIT donors (median of 34 compared to 6 months, respectively), whilst the withdrawal group have been treated the longest (median 62 months). Serological testing showed raised total and allergen-specific IgE amongst all allergic participants.

	Non-Allergic controls	Seasonal Allergic Rhinitic	SCIT	SLIT	Withdrawal	P value
Age [Years]	35.5 (26.75-53)	31 (25.75-39.5)	38.5 (29-49)	34.5 (22.75-55.75)	46.5 (37.25-56.5)	0.38
Gender [M/F]	5/9	9/5	6/2	0/6	4/0	
Duration of AIT [Months]	N/A	N/A	6 (5.25-13.75)	34 (17-48.5)	62 (52-73.5)	0.0002
Duration on maintenance dosing [Months]	N/A	N/A	1 (1.0-7.25)	N/A	N/A	N/A
Duration of Withdrawal [Months]	N/A	N/A	N/A	N/A	18.5 (16.0-21.75)	N/A
Total IgE [KU/L]	12.85 (3.53-50.25)	138.5 (91.05-209.3)	220.5 (115.8-361.3)	105.9 (25.73-370.3)	112.5 (79-614)	<0.0001
Phl p 5-specific IgE [KU/L]	0.01 (0.01-0.02)	8.91 (6.53-36.5)	27.1 (4.66-51.68)	24.7 (1.48-38.8)	11.5 (4.17-15.2)	<0.0001

Table 6 – Demographic data of cross-sectional participants.

For this study participants were NA), AR, receiving SCIT, SLIT or had completed a course of AIT (withdrawal) (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT). All patients having received immunotherapy are referred to as AIT. The age distribution, gender split, durations of AIT (where relevant), total and allergen-specific (Phl p 5) IgE are shown. For all, median data is shown above interquartile ranges in brackets. P values represent Friedman test results.

Patient symptom questionnaires (described in section 2.10.2, p59) were completed by participants at screening, asking retrospectively about the severity of their hay fever symptoms the previous summer. AIT patients were similarly questioned at screening, with the exception of those who had not been through a pollen season following initiation of treatment, who completed symptom questionnaires after the following grass pollen season. Global total nasal symptoms were significantly lower amongst SCIT or SLIT treated allergics compared with untreated allergics (Figure 23 A). Change in perceived pollen symptoms during the last pollen season compared to previous seasons, prior to treatment for AIT donors, showed AIT-treated donors reporting a maximum modal improvement whilst untreated donors reported no change in symptoms (median 0), both SCIT and SLIT treated donors reported equal improvement (Figure 23 B). Pollen season quality of life is significantly impacted amongst untreated allergics compared to normal controls (Appendix Figure 5), AIT-treated patients (p=0.03) however not compared with SLIT or SCIT treatment alone.

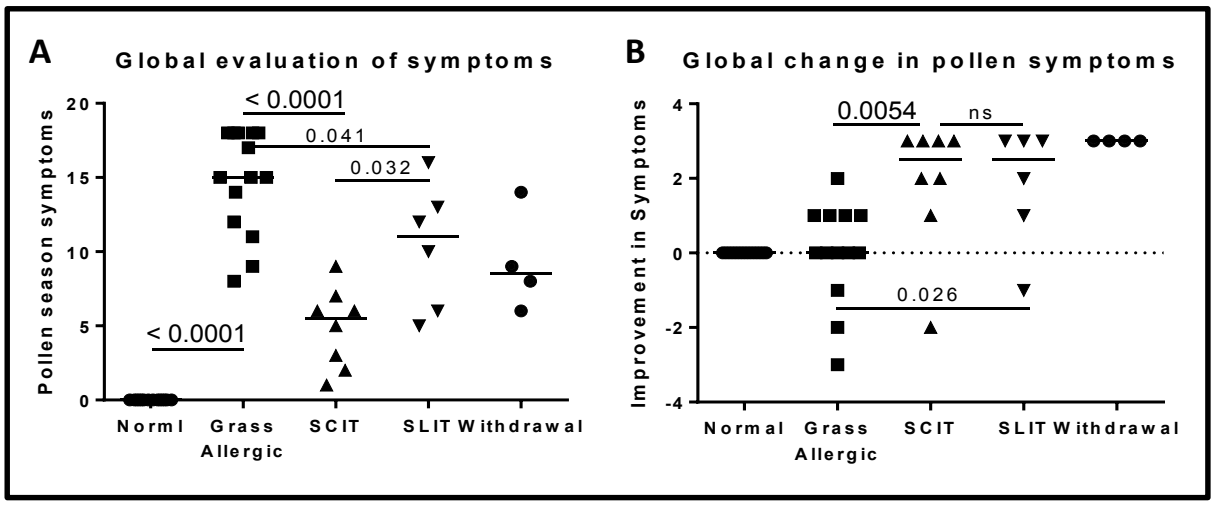


Figure 23 – Self-assessment data of cross-sectional study participants.

NA, AR and AIT donors (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT). Data shows subjective global nasal symptom score (**A**), subjective changes in pollen season rhinoconjunctivitis symptoms (compared to before treatment for AIT donors) (**B**).

Study participants received an intradermal allergen challenge (detailed in section 2.10.3, on p60). Significant suppression of allergen induced early (wheal at 1 hour) and late (wheal at 8 hours) phase skin responses were observed in AIT-treated patients compared to untreated allergics (Figure 24 A and C). Early and late phase suppression of wheal size is shown separately for the SCIT and SLIT groups relative to untreated grass pollen allergic subjects (Figure 24 B and D). A marked and significant suppression of both the early (15 minutes) and late (eight hour) responses were observed for both SCIT- and SLIT-treated subjects. This was apparent for the AIT-withdrawn patients compared with the untreated allergics for the late but not the early phase response, possibly due to the small number of AIT-withdrawal subjects studied.

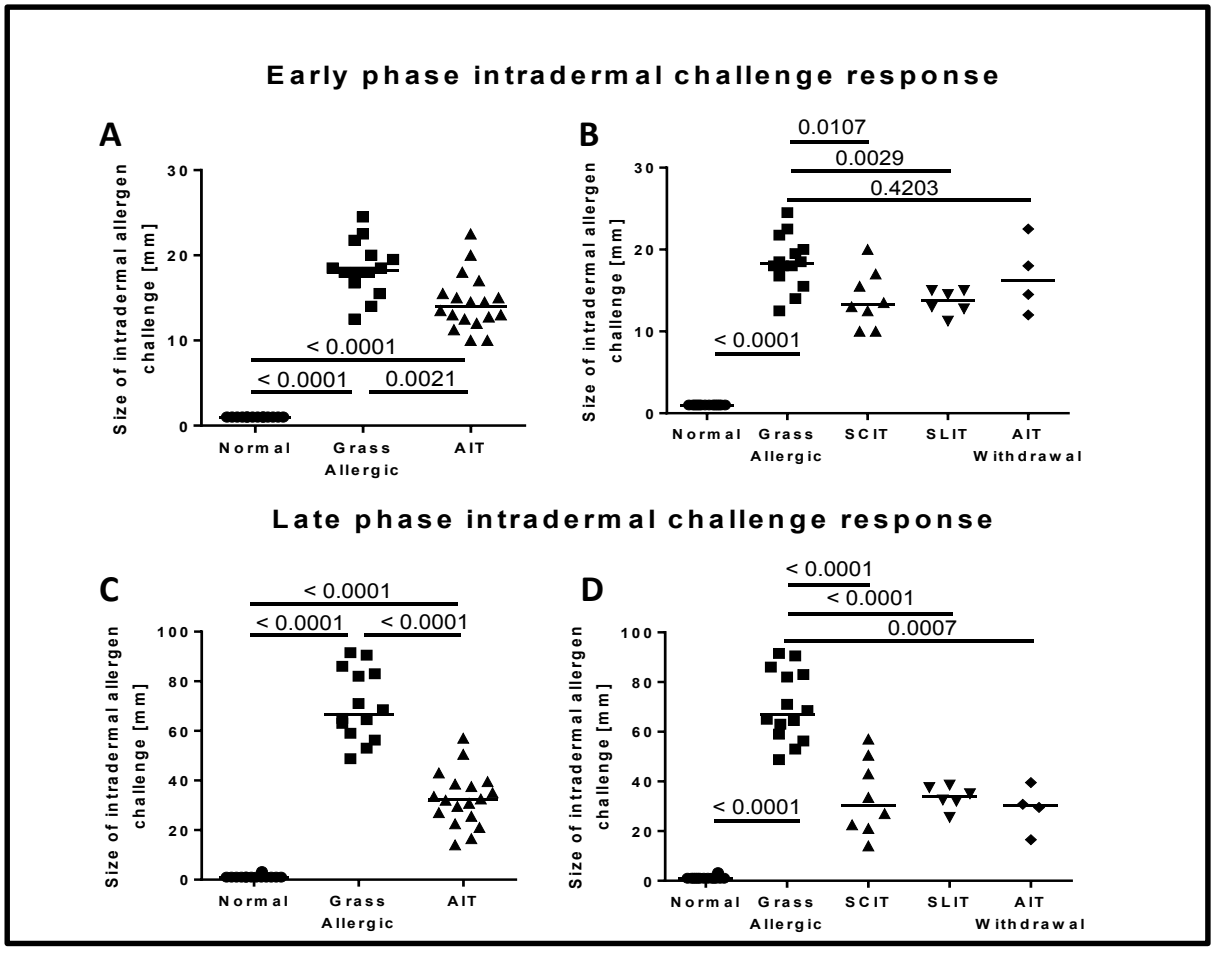


Figure 24 – Intradermal challenge results of cross-sectional study participants.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donors (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT) donors received 1 BU of allergen via intradermal injection. Size of resulting wheal was measured at 15 minutes (early phase – **A** and **B**) and 8 hours (late phase – **C** and **D**). Results of Mann-Whitney tests are shown.

4.2.2. IL-10 FluoroSpot of B cells

B cells were isolated from all patients where samples allowed and stimulated on IL-10-capture FluoroSpot plates. Both CpG alone and CpG with Phl p showed significant numbers of IL-10-producing B cell spots (medians of 392 and 441, respectively, compared to medium or Phl p alone with a median number of spots of 7 and 12, respectively) (Figure 25). Interestingly, paired analysis between CpG alone or with Phl p shows significantly greater numbers of IL-10 spots detected in the latter condition. PHA stimulation is shown as a whole PBMC stimulus.

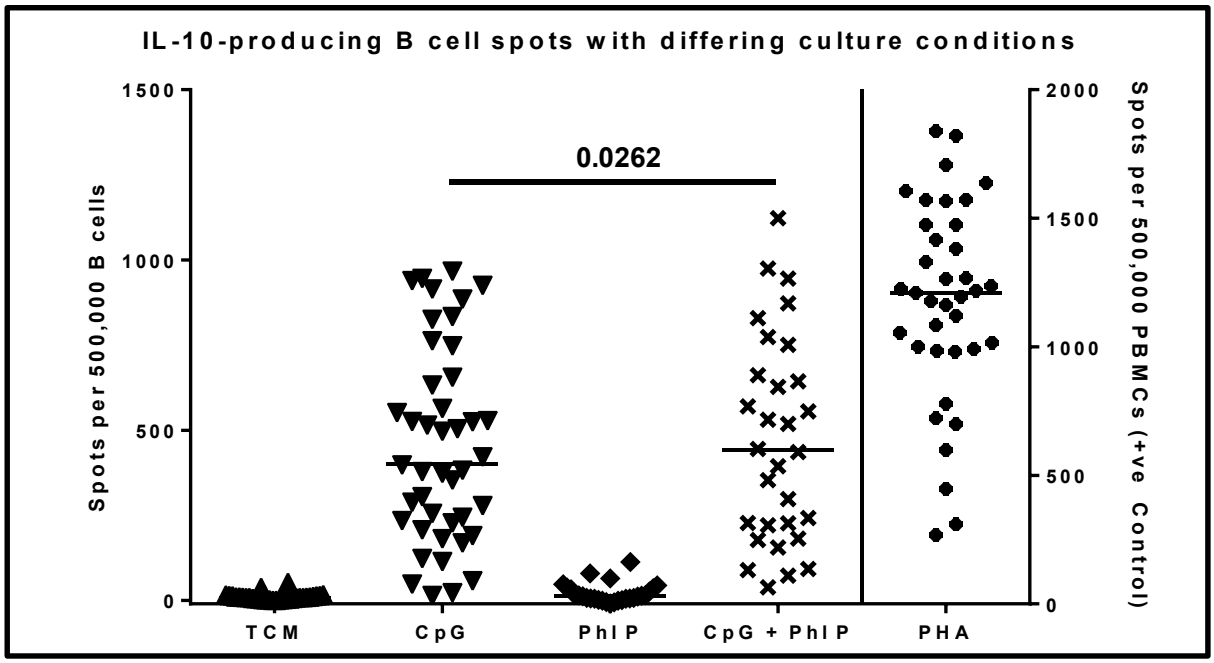


Figure 25 – IL-10 B cell FluoroSpot of cross-sectional participants.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT) were isolated and stimulated on pre-coated IL-10 FluoroSpot plates. PBMCs were stimulated with 5µg/mL PHA (right hand of graph), whilst B cells were unstimulated (medium) or cultured with 1µg/mL CpG, 5 µg/mL Phl P or Phl P + CpG. Line is shown at median. Wilcoxon matched-pairs signed-rank test result is shown.

Given significantly greater proportions of B cells produced IL-10 in response to a combination of Phl p and CpG compared to CpG alone (Figure 25). Comparisons between donor phenotypes show the AIT population is most likely to show increased IL-10-producing B cells with the addition of Phl p to CpG with a trend toward significance (p=0.068) and 10 of the 13 pairs showing increased numbers of IL-10 spots with the addition of Phl p (Figure 26). This is compared to 5 of 8 of the non-allergics and 5 of 9 of the untreated grass allergics.

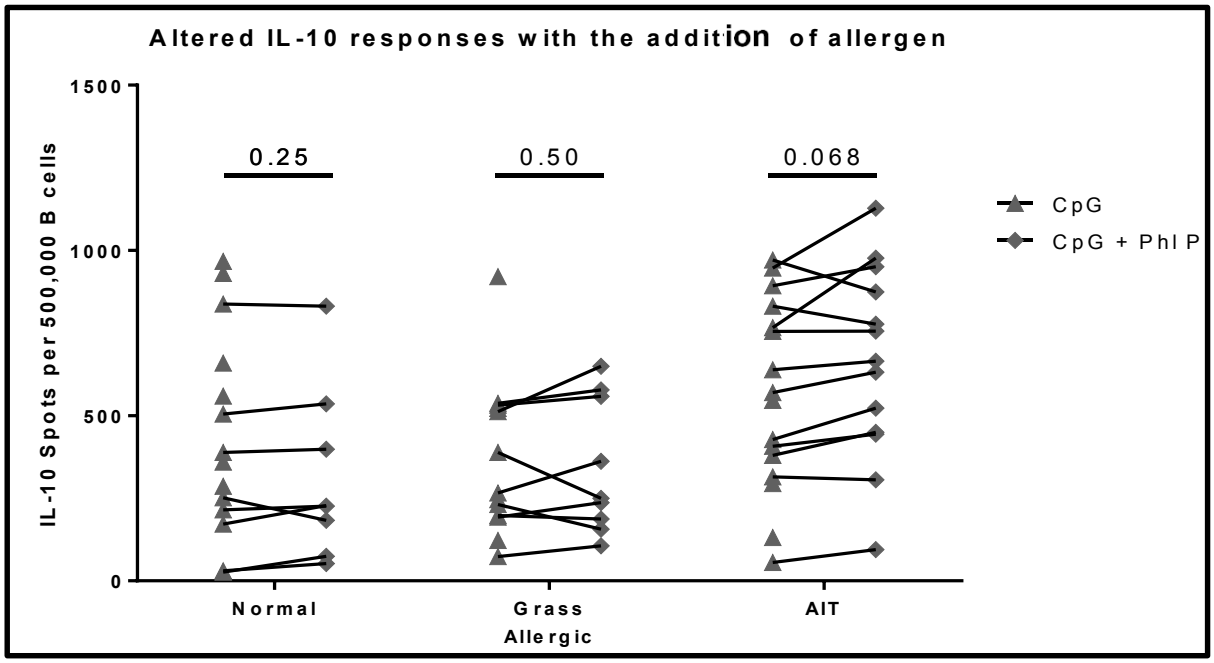


Figure 26 – Comparison of IL-10 B cell spots between CpG and CpG + Phl P stimulation.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT) were isolated and stimulated on pre-coated IL-10 FluoroSpot plates. B cells were unstimulated 1µg/mL CpG or (where numbers permitted) 5 µg/mL Phl P or Phl P in addition to CpG. The results of Wilcoxon matched-pairs signed-rank tests are shown.

No change was observed in the proportions of IL-10-producing B cells between grass allergic and normal control patients following CpG stimulation alone (p=0.54), however a strong trend toward greater proportions of IL-10 B cell spots amongst AIT-treated donors compared to grass allergic individuals (p=0.061) (Figure 27 A). Examination of AIT-treated sub-populations shows SLIT patients had significantly greater proportions of IL-10-producing B cells compared to both SCIT and untreated allergic patients (Figure 27 B). SCIT B cells did not differ in the proportions of IL-10-producing B cells compared to untreated grass allergics (p=0.94).

As previously highlighted, where cell numbers permitted, B cells were also separately stimulated with a combination of CpG and Phl p. Numbers of IL-10-producing B cells in this condition were significantly greater amongst the AIT group compared to both healthy controls and untreated grass allergic patients (Figure 28 A). Untreated grass allergics and normal controls did not differ in their proportions of IL-10 competent B cells. When AIT-treated subpopulations are separated, SLIT-treated patients are again responsible for significantly greater proportions of IL-10-producing B cells against both untreated allergic controls and SCIT-treated allergics (Figure 28 B).

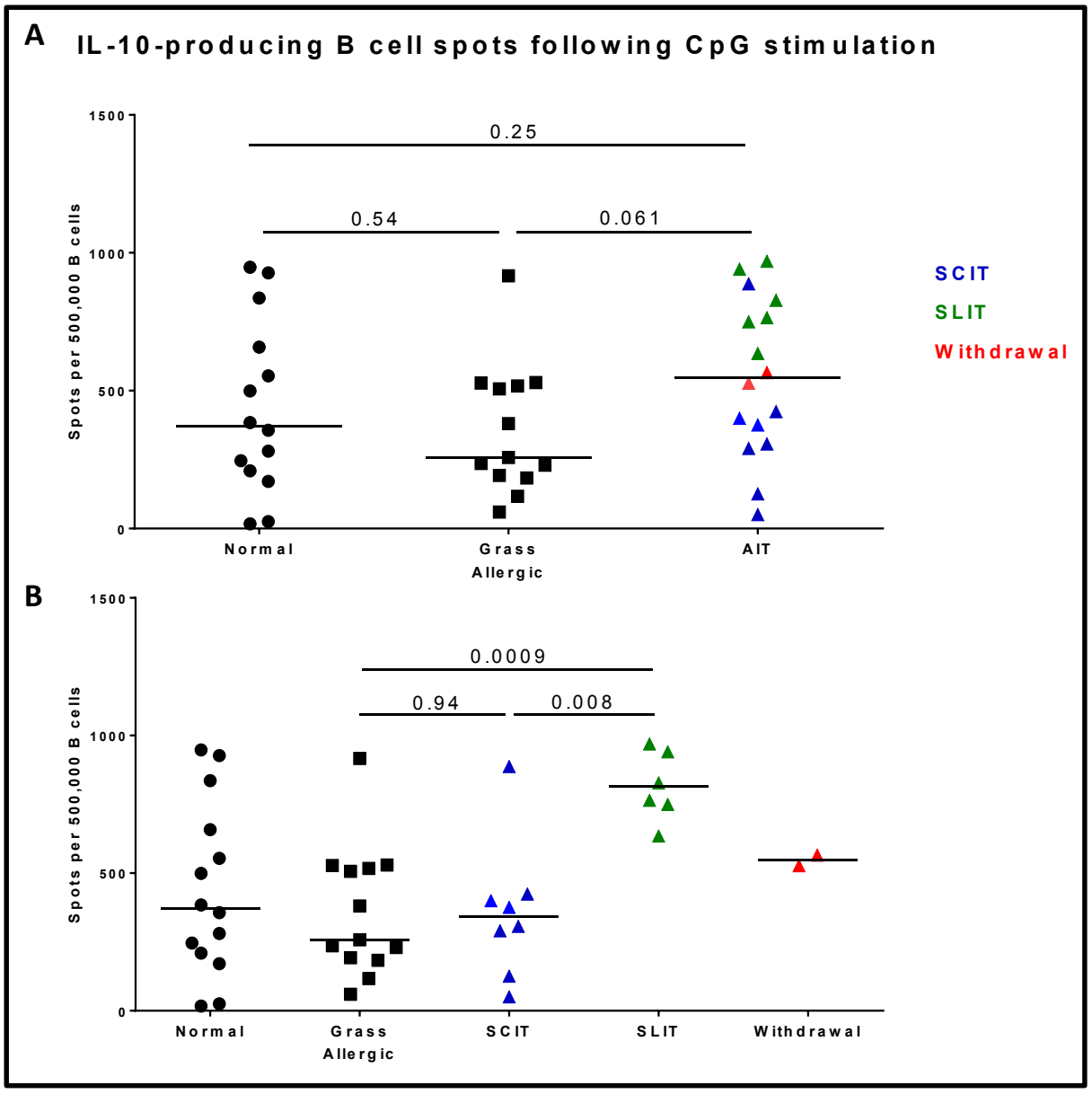


Figure 27 – IL-10 spots following CpG stimulation.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells were isolated for IL-10 capture FluoroSpot (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT). B cells were stimulated with 1µg/mL CpG and numbers of IL-10 B cell spots per 500,000 cells are shown for patient populations with AIT groups combined (**A**) and individual AIT patient populations evaluated in (**B**).

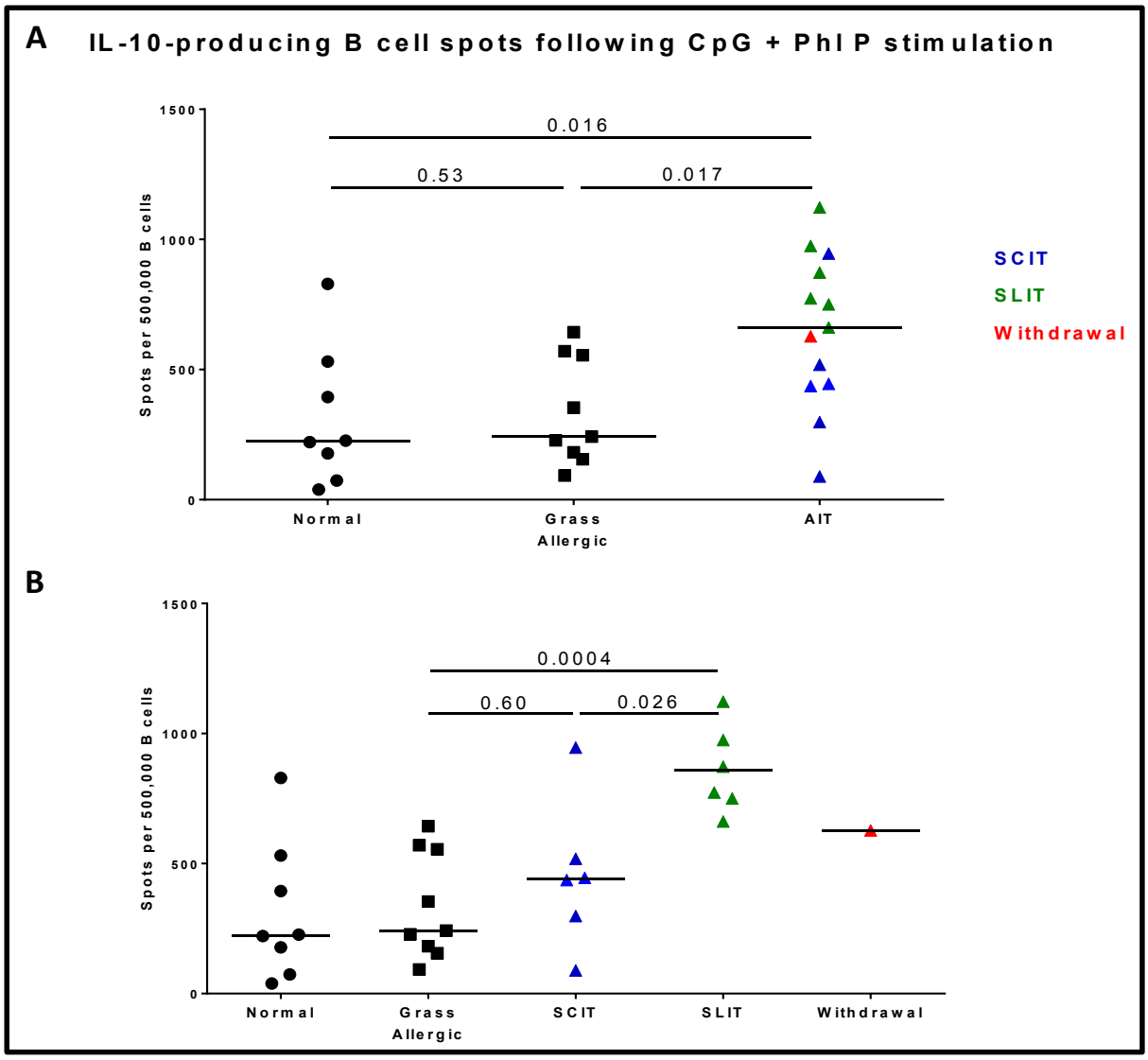


Figure 28 – IL-10 B cell spots following Phl P $^{+}$ CpG stimulation.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells were isolated for IL-10 capture FluoroSpot (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT). B cells were stimulated with 1µg/mL CpG with 5µg/mL Phl P and numbers of IL-10 B cell spots per 500,000 cells are shown for patient populations with AIT groups combined (**A**) and individual AIT patient populations evaluated in (**B**).

Phl p stimulation alone across all groups showed no significant differences in the proportions of IL-10-producing B cells (Figure 29). Non-allergic controls and AIT-treated patients show a trend towards greater proportions of IL-10-producing B cells compared to untreated grass allergic patients (p=0.12 and p=0.11 respectively). SCIT (blue) and SLIT (green) treated patients do not show disparate proportions of B cell IL-10 spots. Relatively few IL-10-producing spots are shown, however proportions of IL-10-producing B cells in the presence of allergen compared to medium alone is significantly greater (for all volunteers, p=0.0027). Negative values exist in this condition as some wells had a greater number of spots on background wells containing medium alone.

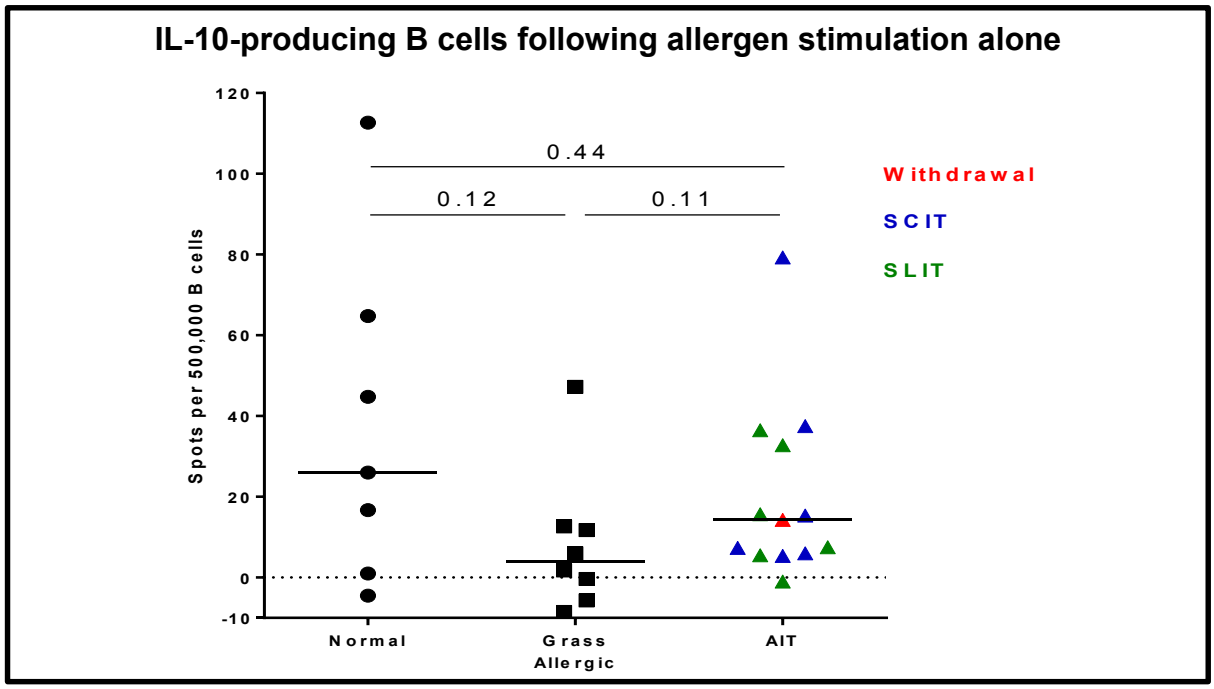


Figure 29 – B cell IL-10 spots following allergen stimulation.

NA, AR, SCIT (blue), SLIT (green) or AIT completed (withdrawal - red) donor B cells were isolated for IL-10 capture FluoroSpot (n=28, NA=7, AR=8, SCIT=6, SLIT=6, withdrawal=1 – SCIT). B cells were stimulated with 5µg/mL Phl P and numbers of IL-10 B cell spots per 500,000 cells are shown for patient populations with AIT groups combined.

4.2.3. CpG-induced changes in mRNA at 5 hours post stimulation

The mRNA from isolated B cells stimulated for 5 hours with CpG was compared with 0 hour controls (Table 7). These data show mRNA for cytokines IL-10, IL-6 and TGF-β and cell surface markers IL-10Rβ, CD39, PD-L1 and TIM-1. IL-10, IL-6 and PD-L1 were strongly induced in all samples with median relative fold changes in mRNA of 63, 156 and 10, respectively. However none showed a significant change within patient subpopulations following Kruskal-Wallis tests, however a trend towards significance was observed with IL-6 (p=0.07).

	All samples	Normal controls	Grass Allergic	SCIT	SLIT	AIT Withdrawal
Number	36	11	12	7	6	1
IL-10	63.2 (31.5-88.1)	67.2 (53.6-101.6)	33.3 (28.7-83.55)	68.7 (17.5-110.2)	55.7 (35.7-139.4)	89.4
IL-6	156.2 (105-203)	145.1 (83.0-191.7)	138.4 (103.8-157.5)	167.7 (154.9-195.5)	202.9 (152.9-243.1)	242.1
IL-10R β	0.93 (0.79-1.2)	0.96 (0.8-1.0)	0.95 (0.73-1.2)	0.91 (0.78-0.98)	0.91 (0.68-1.6)	0.76
TGF-β	1.1 (0.99-1.3)	1.1 (1.0-1.2)	1.1 (1.0-1.3)	1.1 (0.98-1.4)	1.1 (0.8-1.3)	1.2
CD39	0.78 (0.65-1.0)	0.72 (0.65-0.81)	0.93 (0.64-1.2)	0.75 (0.64-1.2)	0.84 (0.55-1.5)	1.0
PD-L1 (CD274)	10.2 (8.1-12.2)	10.4 (7.4-12.0)	9.6 (7.7-11.5)	11.5 (9.7-12.7)	9.0 (8.5-14.3)	14.2
TIM-1	0.97 (0.82-1.1)	1.0 (0.68-1.1)	0.98 (0.82-1.0)	0.93 (0.84-1.2)	0.96 (0.66-1.1)	1.1

Table 7 – qPCR or mRNA after 5 hour CpG stimulation of B cells from cross-sectional participants.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells were isolated and stimulated for 5 hours with CpG. Relative change expression of mRNA compared to 0 hours for IL-10, IL-6, IL-10R β, TGF-β, CD39, PD-L1 (CD274) and TIM-1 is shown. Median values are shown with interquartile ranges in brackets below.

This data has been examined in more detail with respect to relative changes in IL-10 and IL-6 mRNA (Figure 30). IL-10 mRNA did not show any significant changes between allergic groups (Figure 30 A), however there is a trend towards a reduced induction of IL-10 mRNA induced amongst untreated allergics (median 33) compared to non-allergic controls (median 67) (p=0.059). Collectively AIT-treated patients had a greater induction of IL-6 mRNA compared with grass allergic patients (p=0.022) (Figure 30 B). SLIT and SCIT patients independently suggested a trend towards increased 5 hour IL-6 mRNA against grass allergics (p=0.13 and p=0.068).

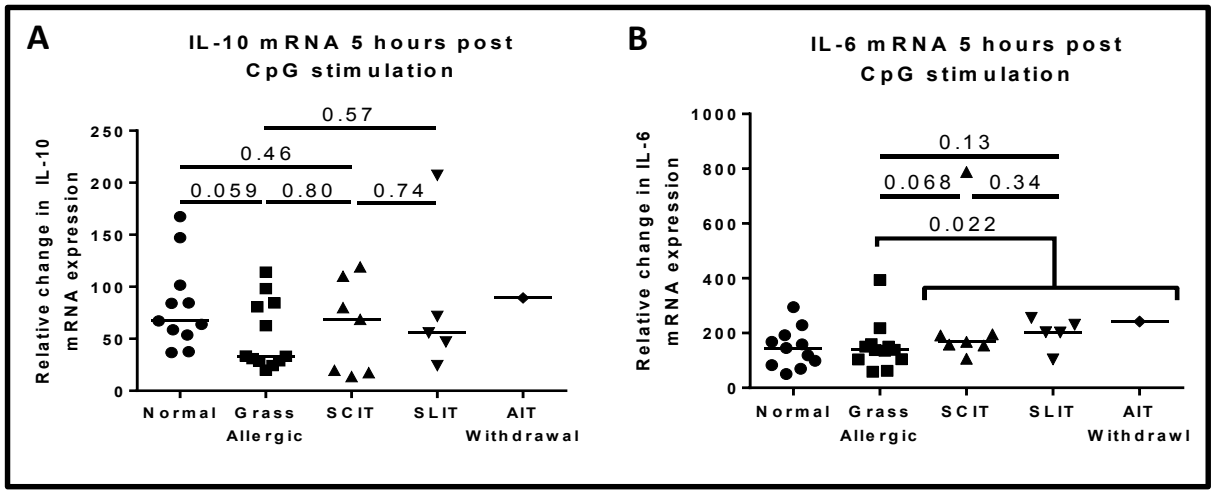


Figure 30 – Relative IL-10 and IL-6 mRNA expression in cross-sectional populations after stimulation.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells were isolated and stimulated for 5 hours with CpG. Relative change in expression of mRNA compared to 0 hours for IL-10 and IL-6 is shown for all clinical phenotypes. P values are shown as the result of Mann-Whitney tests.

4.2.4. B cell mRNA expression at baseline compared between clinical groups

Given the reliance on CpG stimulation, and thus TLR9 signalling, throughout the comparison between clinical phenotypes, it was pertinent to assess levels of TLR9 mRNA at baseline between these groups. TLR4 and immunoglobulin mRNA expression was also compared (Figure 31). No change was observed between groups for relative levels of expression of TLR9 (Figure 31 A), TLR4 (Figure 31 B), total IgG (Figure 31 C), total IgE (Figure 31 E) and total IgA (Figure 31 F). However IgG4 expression was significantly greater within the AIT population compared to normal controls ($p=0.026$) but not untreated grass allergics ($p=0.6$).

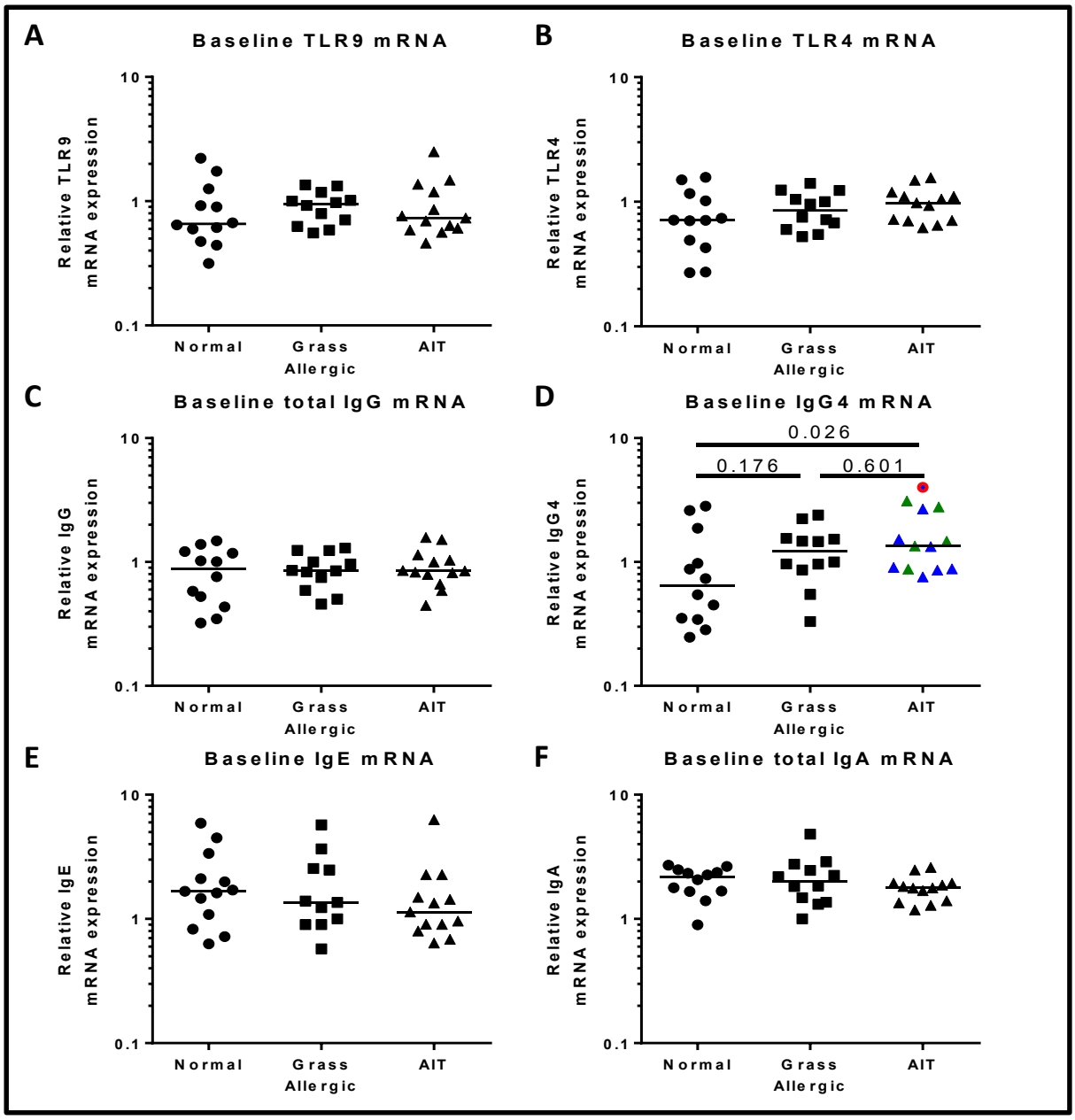


Figure 31 – Comparison of mRNA in time 0 B cell lysates from cross-sectional patients.

NA, AR), AIT donors (n=37, NA=12, AR=12, SCIT=7, SLIT=5, withdrawal=1 –SCIT) had isolated B cells lysed at time 0 on collection. Expression of mRNA for TLR9 (**A**), TLR4 (**B**), IgG (**C**), IgG4 (**D**), IgE (**E**) and IgA (**F**) is presented relative to a single random patient. Mann-Whitney test results are shown.

4.2.5. B cell supernatant proteins following 48 hour CpG stimulation

48 hour CpG stimulated B cells were assessed for concentrations of 25 analytes by multiplex ELISA (Appendix Table 4); only IL-10, IL-6 and TNFα were present in significant concentrations (Table 8). Although IL-6 and TNFα were present in the highest concentrations (medians of 635 and 248 pg/mL, respectively), IL-10 (median 162 pg/mL) showed the greatest degree of variance, with a trend towards a significant difference between groups (p=0.11).

Sub division of IL-10, IL-6 and TNFα cytokine concentrations by volunteer subsets shows significant changes amongst IL-10 populations alone (Figure 32). IL-10 concentration is significantly greater amongst SLIT-treated patients relative to SCIT or untreated grass allergics (p=0.013 and p=0.046) (Figure 32 A). This is not replicated within the IL-6 or TNFα analysis of sub groups (Figure 32 B and C).

	All samples	Normal controls	Grass Allergic	SCIT	SLIT	AIT Withdrawal	P Value
Number	45	14	13	8	6	4	N/A
IL-10	162.3 (100.7-247.7)	219.5 (117.8-285.7)	127.9 (93.4-234.5)	124.8 (43.2-182.1)	224.0 (203.5-264.6)	131.4 (119.8-223.6)	0.11
IL-6	635.3 (418.1-795.5)	716.1 (489.6-1055)	703.7 (562.8-836.4)	575.8 (401.3-789.3)	828.8 (568.8-1099)	509.9 (402.8-964.5)	0.44
TNF-α	248.1 (169.9-345.6)	205.3 (157.2-367.8)	248.1 (187.3-309.5)	228.1 (160.8-441.3)	323.8 (271.1-475.9)	261.8 (154.5-424.1)	0.66

Table 8 – ELISA results of 48 hour CpG stimulated B cell supernatants.

NA, AR, SCIT, SLIT or completed AIT (withdrawal) donor (n=45, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=4 –of which 2 received SCIT and 2 SLIT) isolated B cells were cultured for 48 hours with 1 μg/mL CpG. B cell supernatants were assessed for protein concentrations of IL-10, IL-6 and TNFα. Results are shown for all samples and samples separated by clinical group. Median is shown with interquartile range in brackets. P values represent Kruskal-Wallis tests.

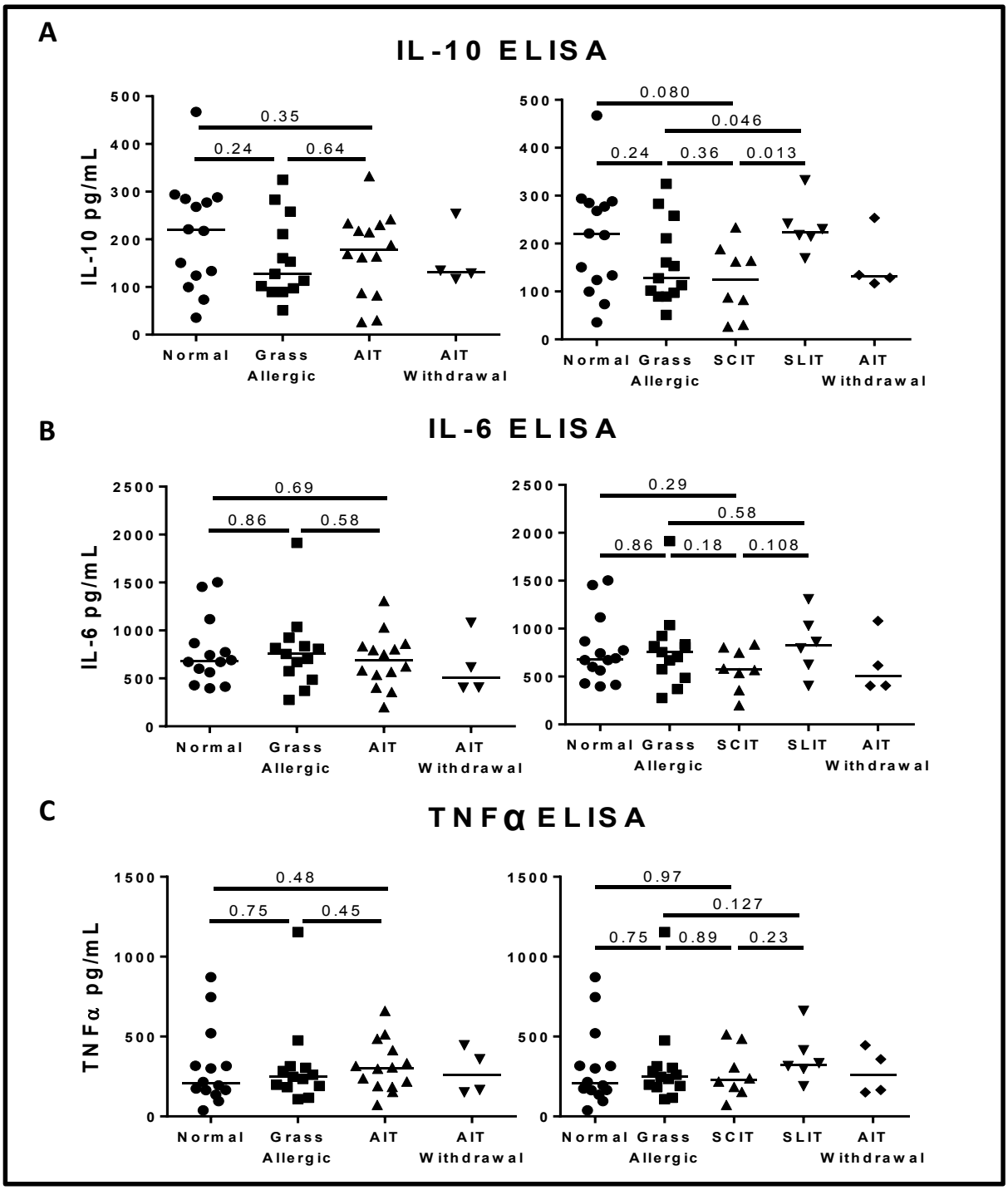


Figure 32 – ELISA of CpG-stimulated B cells from cross-sectional donors.

NA, AR, SCIT, SLIT or completed AIT (withdrawal) donor (n=45, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=4 –of which 2 received SCIT and 2 SLIT) isolated B cells were cultured for 48 hours with 1μg/mL CpG. B cell supernatants were assessed for protein concentrations of IL-10 (**A**), IL-6 (**B**) and TNFα (**C**). Results are shown for AIT groups combined (left panels) and each AIT group separately (right panels). Mann-Whitney p values are shown.

4.2.6. Comparing measures of B cell produced IL-10

Three measures of B cell IL-10 competency have been shown following CpG stimulation, proportions of IL-10-producing B cells by FluoroSpot, IL-10 concentration by ELISA and IL-10 mRNA induction by qPCR. The relative increase in IL-10 mRNA is compared with proportions of IL-10-producing B cells (Figure 33 A), showing a significant correlation (p=0.039). More significant relationships were observed between IL-10 mRNA induced and the IL-10 concentration (p<0.0001) (Figure 33 B) and proportions of IL-10-producing B cells and IL-10 concentration (p<0.0001) (Figure 33 C).

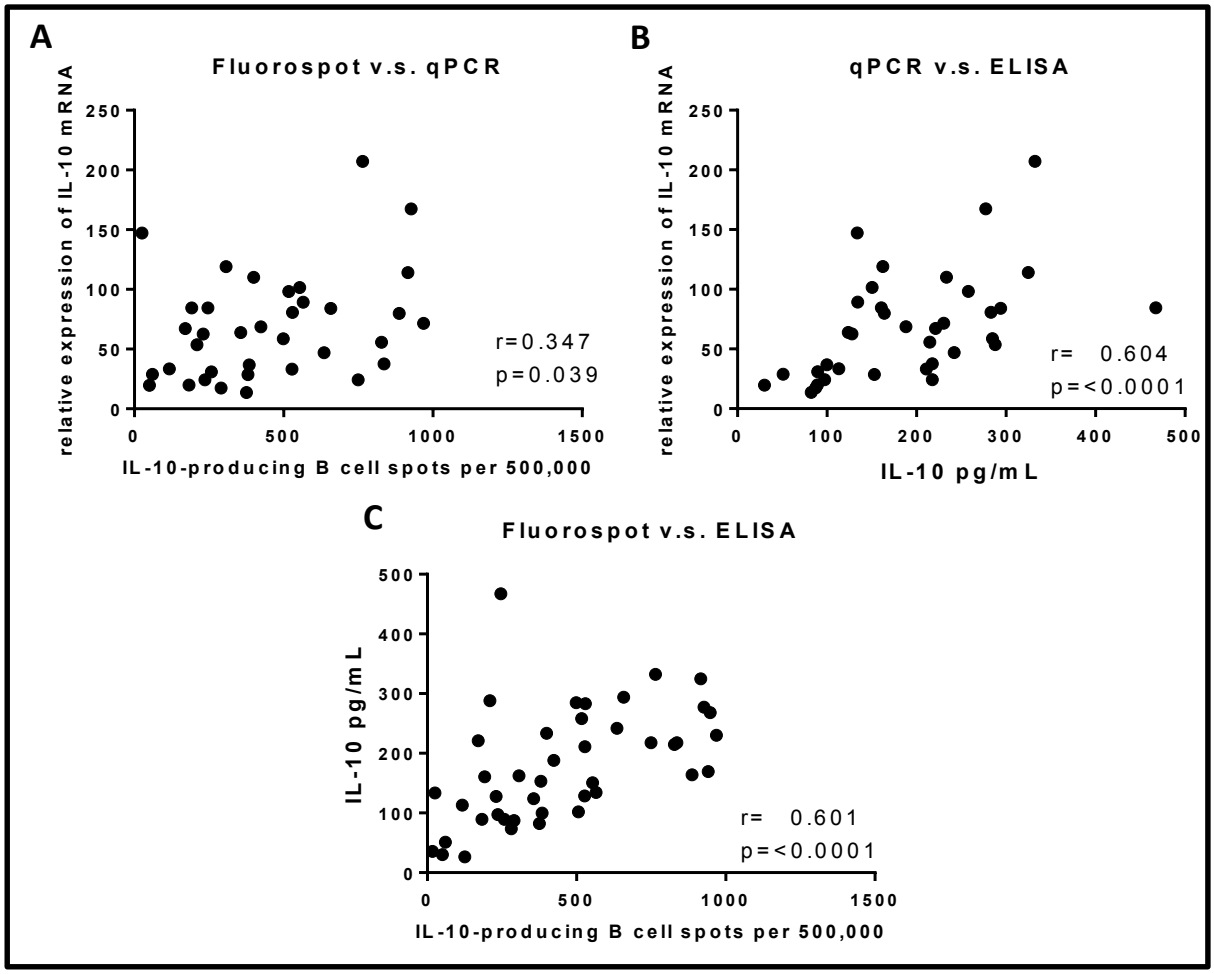


Figure 33 – Correlations between IL-10 FluoroSpot, IL-10 mRNA and IL-10 ELISA.

Cross sectional B cells were isolated and cultured with 1µg/mL CpG. Relative change in IL-10 mRNA and proportions of IL-10-producing B cells are correlated (**A**) (n=36, NA=11, AR=12, SCIT=7, SLIT=5, withdrawal=1 –SCIT) as are changes in mRNA to IL-10 protein concentration at 48 hours (**B**) (n=36 – distributed as in **A**). Finally IL-10 protein is correlated against proportions of IL-10-producing B cells (**C**) (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 –of which 1 received SCIT and 1 SLIT). Spearman correlations were carried out, r and p values are shown.

4.2.7. B cell IL-10 responses in relation to patient demographics

SLIT-treated allergic patients showed the greatest proportions of IL-10-producing B cells (Figure 25 B and Figure 26 B) and had the greatest concentrations of IL-10 within stimulated B cell supernatants amongst the allergic groups (Figure 32 A), but were also the AIT-treated group who had received the longest course of AIT treatment and are exclusively female (Table 6). These two factors are explored in further detail (Figure 34 and Figure 35). Duration of AIT treatment for AIT-treated patients only shows a trend towards a positive correlation with CpG and CpG $^{+}$ Phl p stimulated B cell IL-10 spots (p=0.095 and p=0.12) (Figure 34 A and B). No correlation was observed with respect to Phl p stimulated B cell IL-10 spots or CpG-stimulated B cell IL-10 supernatant concentration against AIT treatment duration (Figure 34 C and D). Similar correlations, with greater significance, are observed when approximate total cumulative allergen dose was correlated with these factors in place of duration of AIT (Appendix Figure 9).

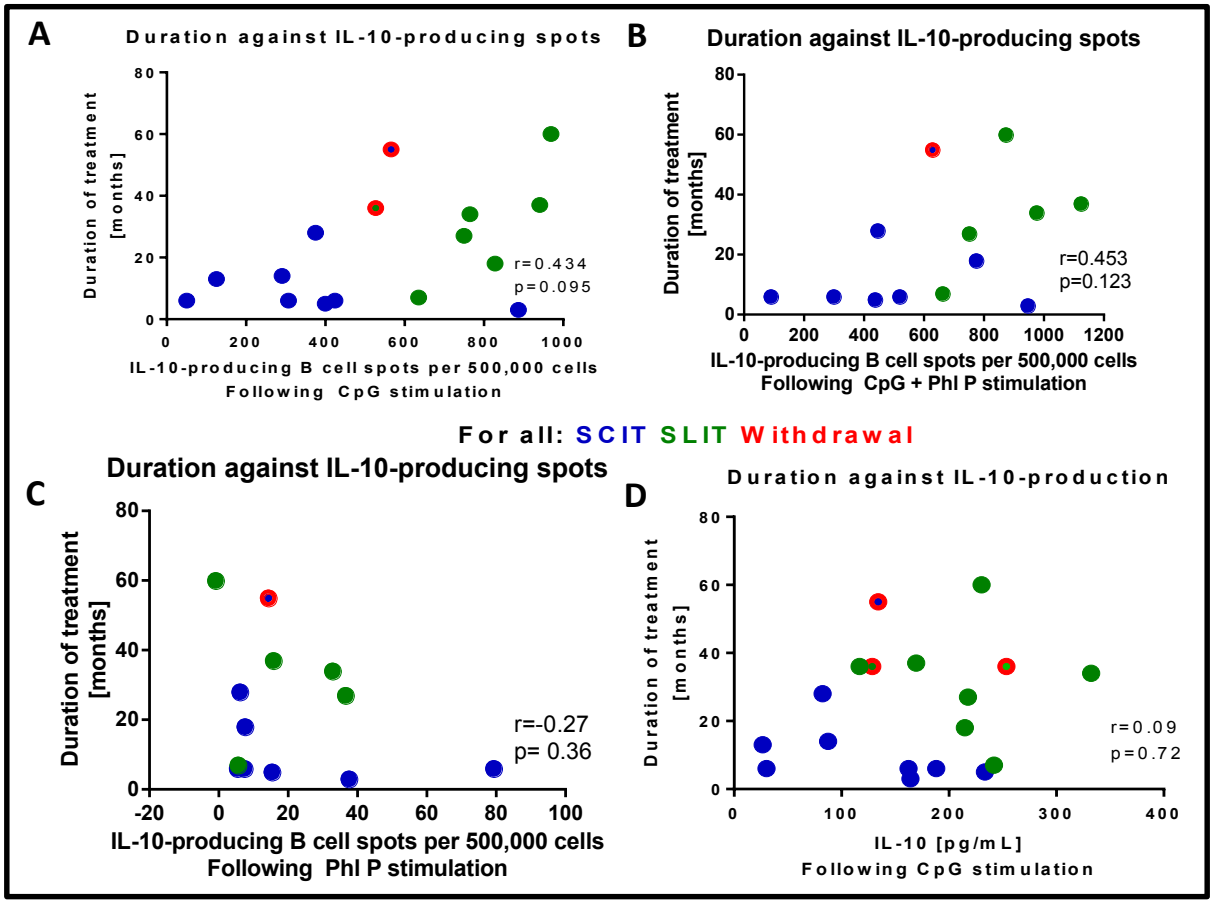


Figure 34 – Comparison between duration of treatment and IL-10 spots or IL-10 produced.

Participants received SCIT (blue), SLIT (green) or had completed a course of AIT (withdrawal - red). B cells were isolated and cultured for 42 or 48 hours with 1µg/mL CpG or for 42 hours with 5 µg/mL Phl P $^{+}$/- 1µg/mL CpG. B cells to be cultured for 42 hours were plated at 5x10^{5} cells per well on IL-10 FluoroSpot plates and IL-10 spots were counted. 48-hour cultured B cells had supernatant removed and IL-10 protein concentration was quantified. Numbers of IL-10 B cell spots were correlated with treatment duration when B cells had been stimulated with CpG alone (**A**), CpG $^{+}$ Phl P (**B**) or Phl P alone (**C**). IL-10 protein concentration following CpG stimulation is also correlated with treatment duration (**D**).

Age shows a weak negative trend towards correlation against both proportions of IL-10-producing B cells and IL-10 protein concentration following CpG stimulation ($p=0.14$ and $p=0.102$) (Figure 35 A and B). When these same measures are compared for males v.s. females there is a trend towards greater proportions of IL-10 spots amongst females ($p=0.096$) with a significantly greater concentration of IL-10 amongst females ($p=0.0007$) for all donors (Figure 35 C and D). When the AIT samples are excluded the trend is lost with IL-10 spots, but significance remains with respect to IL-10 protein produced ($p=0.0033$) (Figure 35 E and F). IL-6 and TNFα protein concentrations were also significantly greater amongst the female population as a whole, but show no significant difference when AIT donors are excluded (Appendix Figure 10).

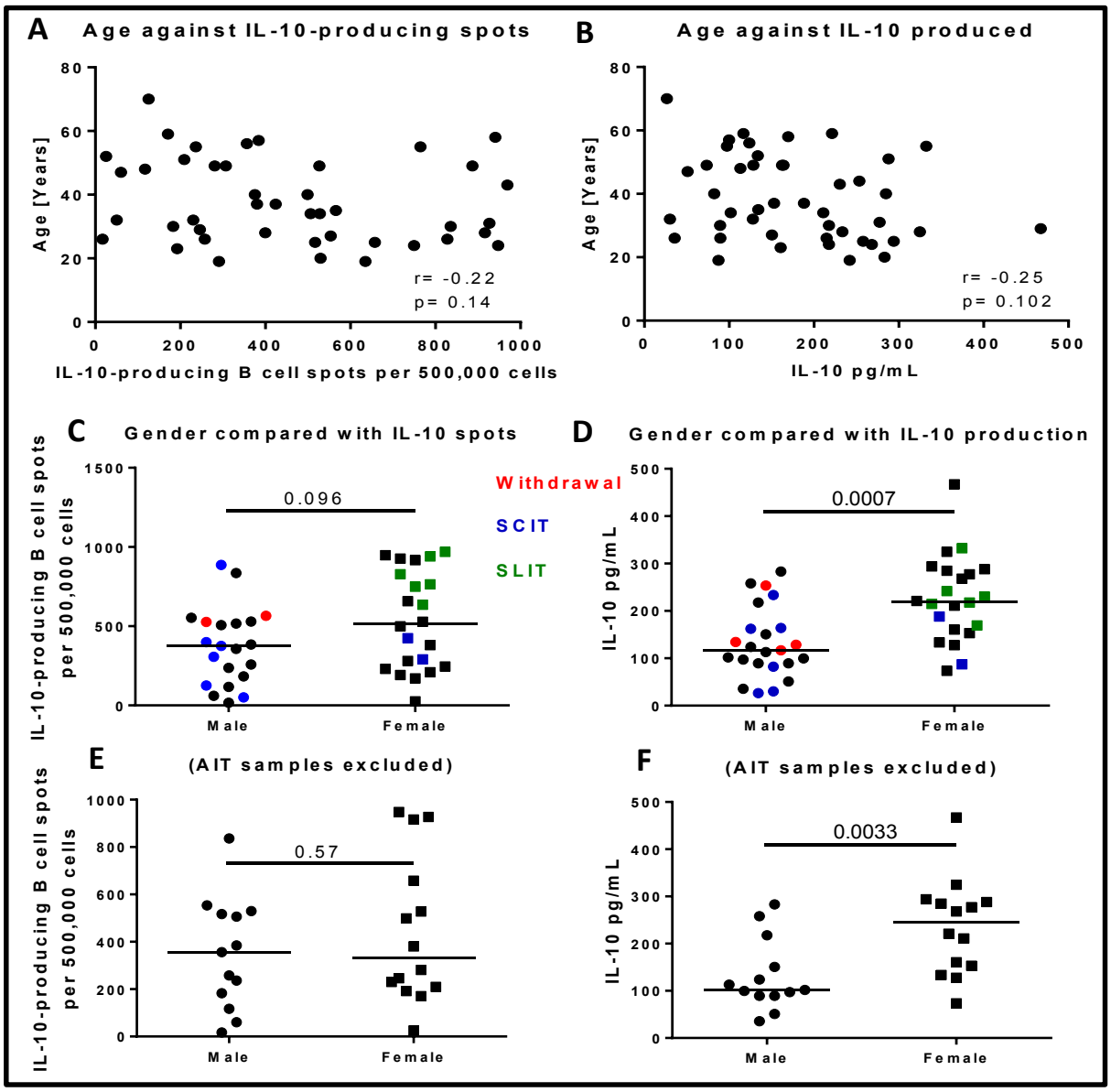


Figure 35 – Age and gender comparisons with IL-10-producing B cell spots and IL-10 production.

NA, AR, SCIT (blue), SLIT (green) or AIT completed (withdrawal - red) donor B cells were isolated and cultured with CpG. Numbers of IL-10-producing B cell spots by FluoroSpot are shown against age (**A**) and gender (**C** and **E**) (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT). IL-10 protein concentration is also shown against age (**B**) and gender (**D** and **F**) (n=45, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=4 –of which 2 received SCIT and 2 SLIT). Gender comparisons show either all data (**C** and **D**) or all participants with the exception of AIT (**E** and **F**). For age comparisons Spearman correlations were carried out, r and p values are shown. For comparisons between IL-10 and gender Mann-Whitney tests were carried out, p values are shown.

4.2.8. B cell IL-10 responses in relation to participants' intradermal challenge responses

Proportions of IL-10-producing B cells following CpG stimulation correlate significantly inversely with wheal size at 15 minutes post intradermal challenge but not at 8 hours (Figure 36 A and B). Conversely a trend towards inverse correlation was observed between CpG $^{+}$ Phl p stimulated B cell IL-10 spots and wheal at 1 hour, but achieved a significant inverse relationship at 8 hours (p=0.041) (Figure 36 C and D). IL-10 concentration following CpG stimulation showed a trend towards inverse correlation at 1 hour (p=0.079) with wheal size but not at 8 hours (Figure 36 E and F).

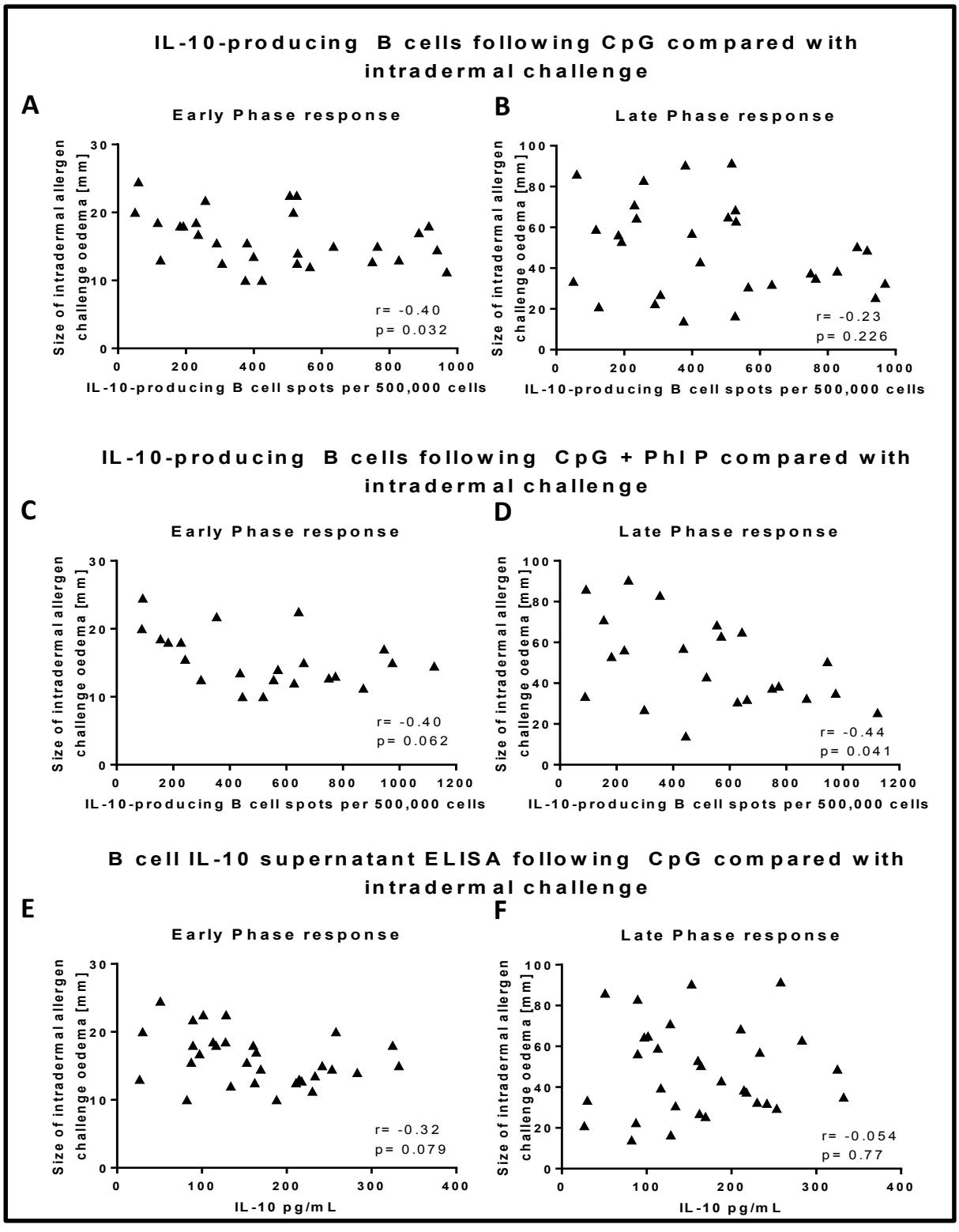


Figure 36 - Comparisons between intradermal challenge and CpG-induced B cell IL-10.

Untreated allergic rhinitis or AIT donor intradermal wheal responses are shown correlated against isolated B cell IL-10 spots following CpG alone (n=29, AR=13, SCIT=8, SLIT=6, withdrawal=2 – 1 SCIT and 1 SLIT), CpG with Phl P (n=21, AR=9, SCIT=5, SLIT=6, withdrawal=1 – SCIT) or IL-10 B cell supernatant protein concentration (n=29, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT). Wheal at 15 minutes (early phase – left hand panels) or 8 hours (late phase – right hand panels) is shown against CpG induced IL-10 spots (**A and B**), CpG with Phl P IL-10 spots (**C and D**) and CpG-induced IL-10 protein concentrations (**E and F**). Spearman correlations are shown.

4.2.9. Clinical outcomes of nasal allergen challenge

Nasal allergen challenge (NAC) was carried out on participants of this study (as described in 2.10.4, on p60). Clinical outcomes are reported to demonstrate successful allergen challenge (Figure 37 and Appendix Table 5) (data presented with kind permission of Dr. Guy Scadding, unpublished data). Physiological response to NAC is measured by change in peak nasal inspiratory flow (PNIF) from baseline. Data shows area under the curve for change in the first hour (early phase response) in addition to change over the remaining 7 hours (late phase response) combined equally. Significant reduction in nasal inspiratory flow was observed amongst untreated grass allergics compared to normal controls. Significantly reduced nasal blockage amongst AIT-treated donors combined is observed as compared with untreated grass allergic donors. Significance is achieved when SCIT-treated donors alone are compared with untreated allergics, however, SLIT-treated donors alone do not obtain significantly reduced nasal blockage compared to untreated allergics. Despite just 4 donors, AIT-withdrawal donors also had significantly reduced nasal blockage compared to untreated donors.

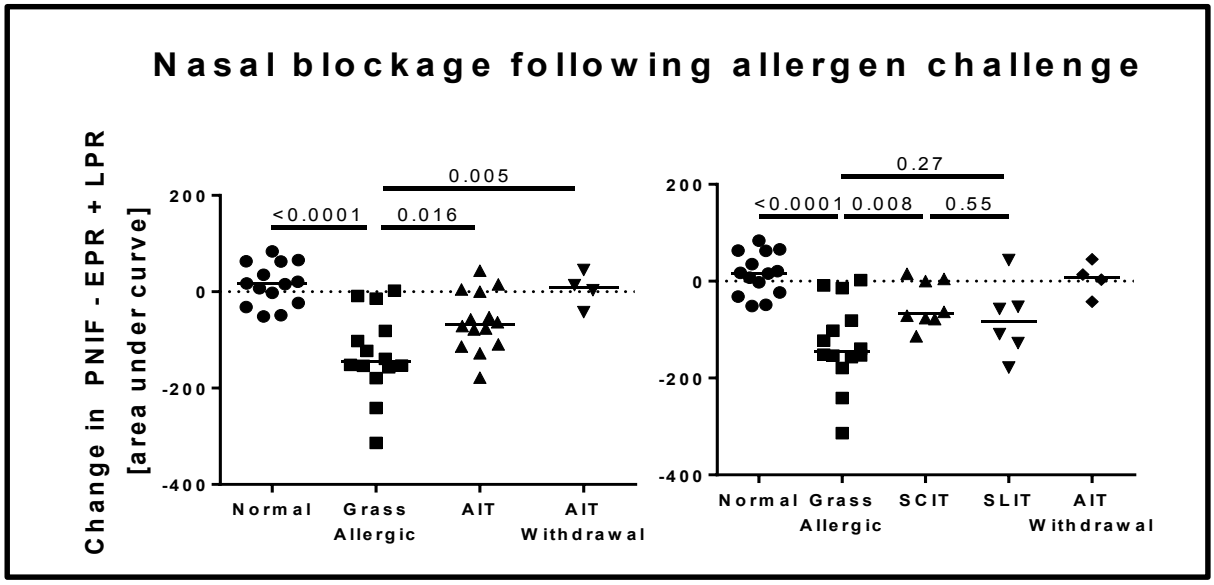


Figure 37 – Change in peak nasal flow demonstrates nasal blockage following allergen challenge.

Patients were monitored before and throughout nasal allergen challenge for nasal blockage by measurement of peak nasal inspiratory flow (PNIF). Data shows change in PNIF from baseline over the first hour (EPR) and during hours 1-8 (LPR) equally weighted (EPR+LPR) as change in PNIF, area under the curve for each. Values shown are p values resulting from Mann-Whitney tests. Line shows median values for each group. Data courtesy of Dr Guy Scadding (Scadding, G.W. et al., unpublished data).

4.2.10. B cell sub populations before and after nasal allergen challenge

Proportions of B cell subsets previously examined for IL-10 capacity (Figure 11) are explored across the clinical phenotypes within this study (Figure 38). Study participants received an NAC. Pre and post NAC blood was taken and directly stained for B cell surface markers in conjunction with counting beads, and gated as shown (Figure 38).

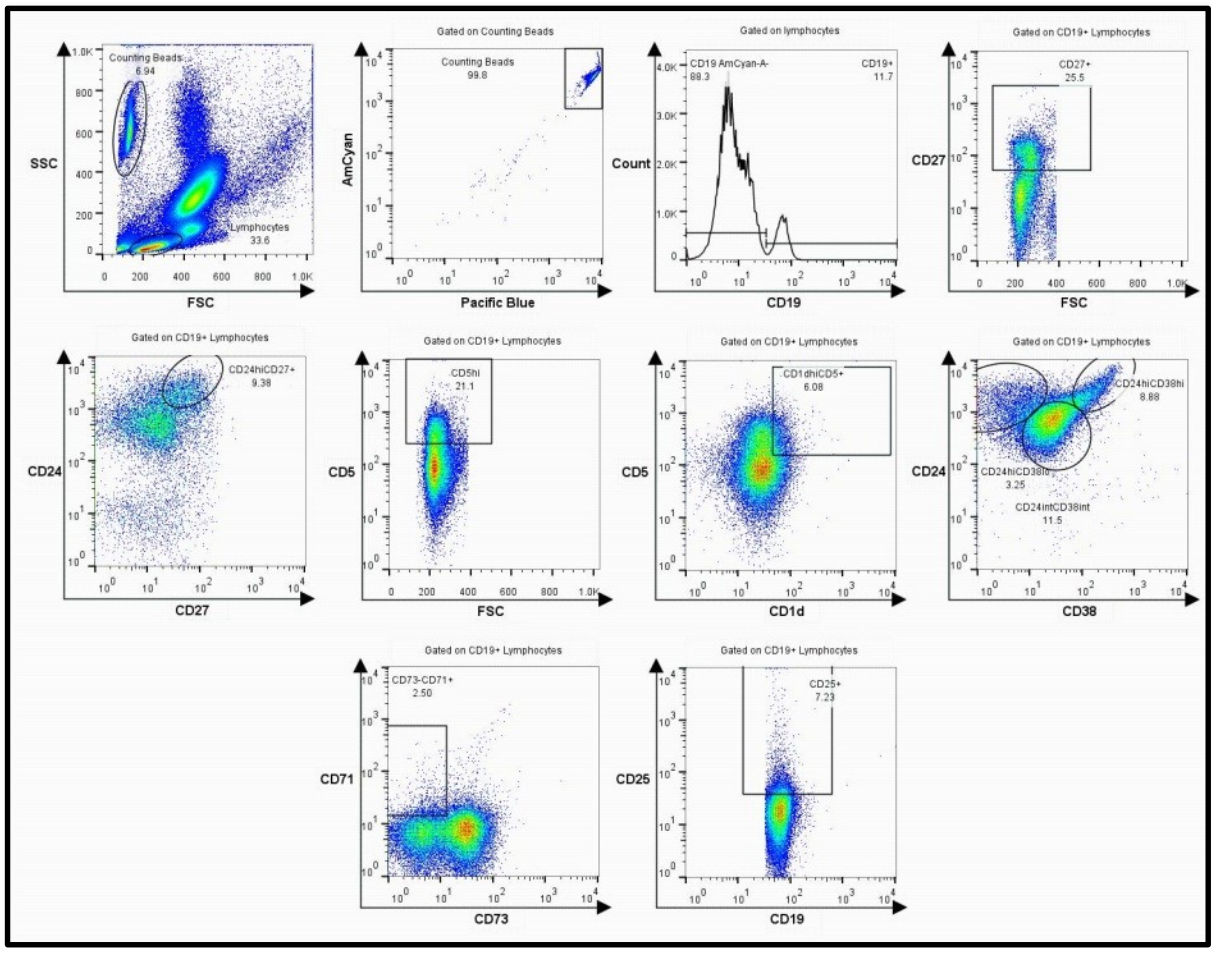


Figure 38 – B cell subset analysis of nasal allergen challenge during cross-sectional study.

NA, AR, SCIT, SLIT and AIT completed (withdrawal) (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT) volunteers underwent grass-pollen nasal allergen challenge during a single day. Blood was taken before and 8 hours following allergen challenge. Whole blood was stained for flow cytometry and run with cell counting beads in order to quantify B cell subsets. Representative staining of B cell subsets is shown, top to bottom and left to right: whole population by size (FSC/SSC), gating of counting beads, gating for CD19, and within CD19 population $CD27^{+}$, $CD24^{hi}CD27^{+}$, $CD5^{hi}$, $CD5^{+}CD1d^{hi}$, $CD24^{hi}CD38^{hi}$, $CD71^{+}CD73^{-}$ and $CD25^{+}$ are gated.

4.2.10.1. Baseline comparison of B cell subsets between study participants

Comparison of numbers of circulating total CD19^{+} B cells in the absence of challenge at baseline shows significantly greater numbers of total B cells amongst grass allergics compared to normal controls (p=0.022), and a trend towards greater numbers amongst AIT-treated allergics against normal controls (p=0.076) (Figure 39). Possibly as a result of these differences in overall numbers, all subsets show similar differences between the clinical phenotypes.

Thus percentages of B cell subsets are shown within the clinical groups (Figure 40). No differences were observed between non-allergic controls, allergic patients and immunotherapy-treated patients where percentages of CD24^{hi} CD27^{+} (Figure 40 B) or CD5^{hi} (Figure 40 D) are compared. CD27-expressing B cells amongst AIT-treated patients showed a trend towards greater proportions compared to normal controls (p=0.13) (Figure 40 A). Grass allergics had a trend toward greater proportions of CD5^{+} CD1d^{hi} B cells compared to non-allergic controls (p=0.13) and significantly greater than AIT-treated patients (p=0.012) (Figure 40 C). A non-significant trend towards increased grass allergic CD24^{hi} CD38^{hi} B cells against normal controls (p=0.13) (Figure 40 E). Proportions of CD71^{+} CD73^{-} B cells trended towards greater AIT-treated patients compared with non-allergic controls (p=0.14) (Figure 40 F). AIT-withdrawal patients had significantly greater proportions of CD71^{+} CD73^{-} B cells compared to untreated allergics (p=0.046). Proportions of CD25^{+} B cells show a trend to be fewer within the AIT-treated group compared to untreated patients (p=0.15) (Figure 40 G).

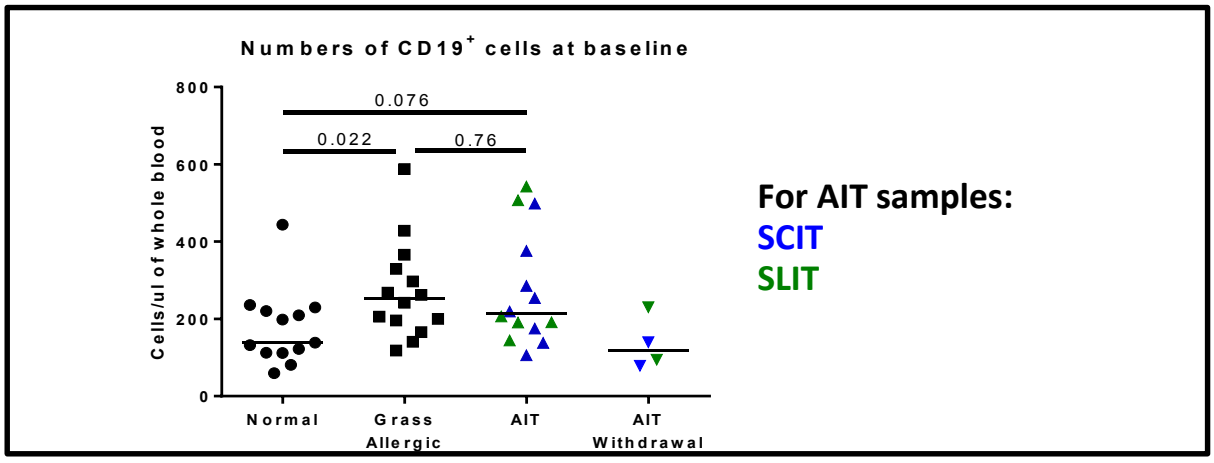


Figure 39 – Numbers of CD19^{+} B cells at baseline between clinical groups

NA, AR, SCIT, SLIT and AIT completed (withdrawal) (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT) volunteers underwent grass-pollen nasal allergen challenge during a single day. Blood was taken before and 8 hours following allergen challenge. CD19^{+} B cell numbers are shown, compared by Mann-Whitney test, p values are shown.

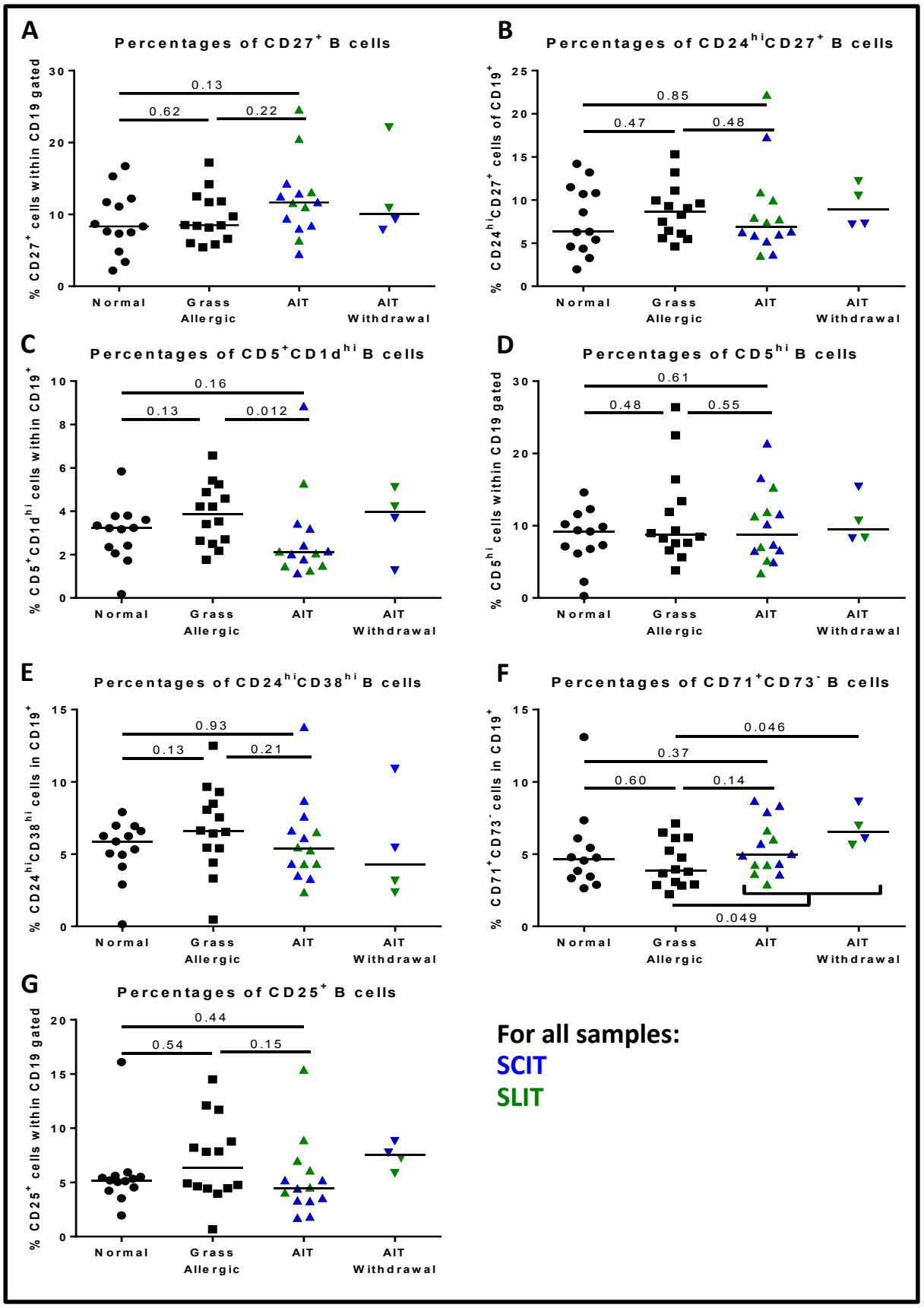
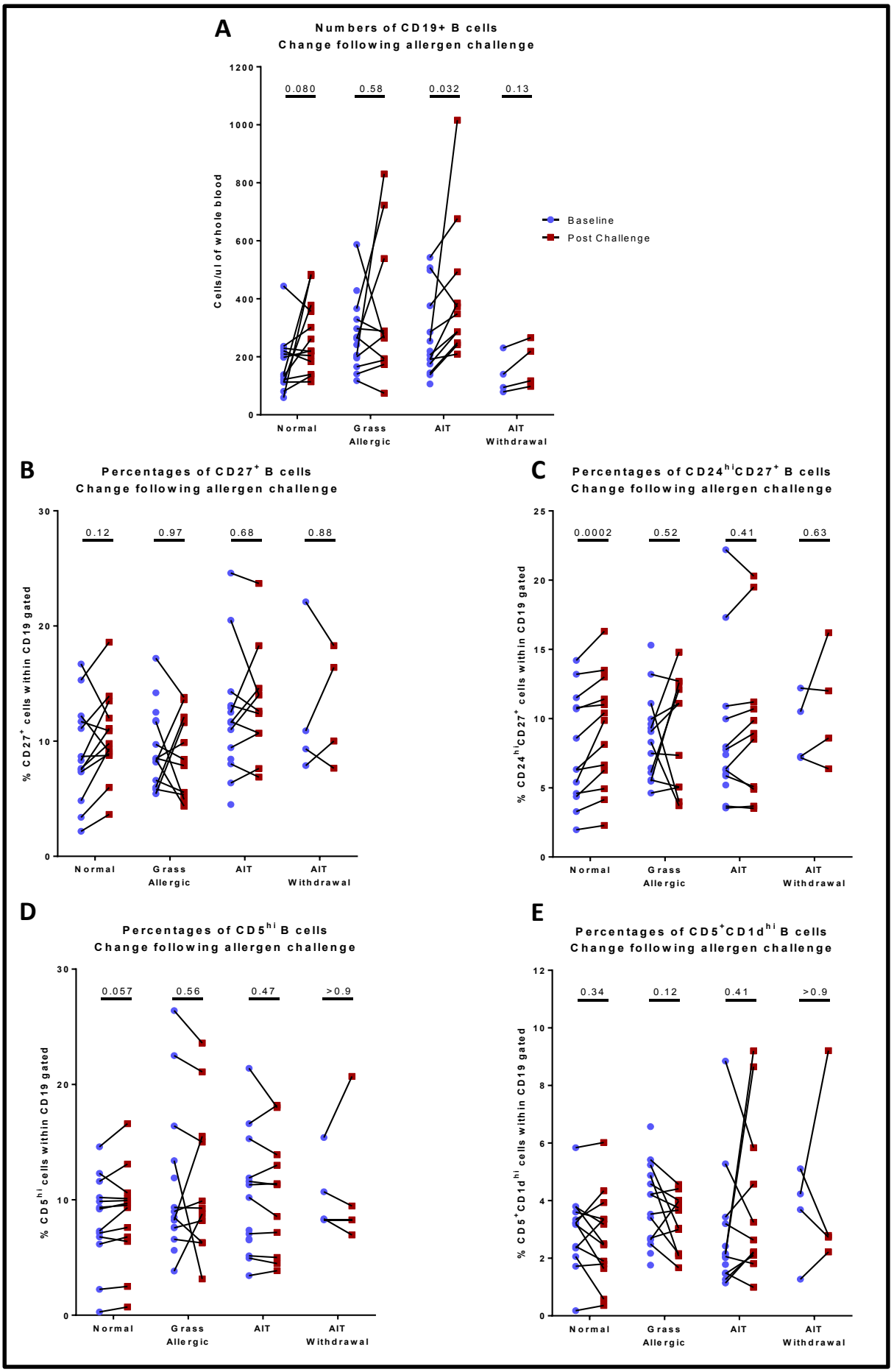


Figure 40 – Comparison between cross-sectional groups of baseline B cell subsets.

NA, AR, SCIT (blue), SLIT (green) and AIT completed (withdrawal) (n=45, NA=13, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT). Blood was taken prior to NAC, stained for flow cytometry. Percentages of $CD27^{+}$ (**A**), $CD24^{hi}CD27^{+}$ (**B**), $CD5^{hi}$ (**C**), $CD5^{+}CD1d^{hi}$ (**D**), $CD24^{hi}CD38^{hi}$ (**E**), $CD71^{+}CD73^{-}$ (**F**) and $CD25^{+}$ (**G**) B cell subsets are shown with respect to clinical phenotype. Mann-Whitney tests were carried out, p values are shown.

4.2.10.2. Changes in B cell subsets following nasal allergen challenge (NAC)

Numbers of B cells per µL of blood showed a clear trend towards an increase following allergen challenge (compared to baseline) in normal controls, AIT-treated allergic patients and completed AIT-treated allergics (p=0.08, p=0.032 and p=0.13 respectively) (Figure 41 A), however grass allergic patients did not respond in the same way, of whom just 6 of the 11 patients had increased numbers of B cells post NAC (p=0.58). This increase in total number of B cells in all groups except grass allergics is repeated in all B cell subsets showing the same trend for numbers of $CD27^{+}$, $CD24^{hi}CD27^{+}$, $CD5^{hi}$, $CD5^{+}CD1d^{hi}$, $CD24^{hi}CD38^{hi}$ and $CD25^{+}$ B cells (Appendix Figure 13) increased following NAC. Due to this trend largely being attributed to changes in the whole $CD19^{+}$ population, percentages of B cell subsets will take the focus of examination post NAC. Percentages of CD27-expressing B cells trend towards an increase following NAC in the normal control group alone (p=0.12) (Figure 41 B), furthermore the percentages of $CD24^{hi}$ $CD27^{+}$ B cells were significantly increased following NAC in normal controls alone (p=0.0002) (Figure 41 C). $CD5^{hi}$ B cells show a non-significant increase following NAC in non-allergic controls alone (p=0.057) (Figure 41 D), despite only a minor change in median percentages of cells (from 9.2% to 9.53%). No significant change was observed following NAC in the proportions of $CD5^{+}$ $CD1d^{hi}$ B cells (Figure 41 E), despite a trend towards a decrease within the grass allergic population (p=0.12). The only B cell subset in which the change in *proportions* of B cells (replicated by the change in *numbers*) following challenge is within the $CD24^{hi}CD38^{hi}$ population (Figure 41 F). The percentages within both normal control and AIT-treated groups increased significantly (p=0.027 and p=0.005) (Figure 41 K), without any significant change in the grass allergic group. Percentages of $CD71^{+}CD73^{-}$ B cells show a non-significant trend towards a decrease in the AIT-treated population alone (p=0.08) (Figure 41 M). Finally, the proportions of $CD25^{+}$ B cells significantly increased following challenge in the non-allergic group alone (Figure 41 O).


A
Numbers of CD19+ B cells
Change following allergen challenge
Cells/ul of whole blood
0.080
0.58
0.032
0.13
Baseline
Post Challenge
Normal
Grass Allergic
AIT
AIT Withdrawal
B
Percentages of CD27+ B cells
Change following allergen challenge
% CD27+ cells within CD19 gated
0.12
0.97
0.68
0.88
C
Percentages of CD24hiCD27+ B cells
Change following allergen challenge
% CD24hiCD27+ cells within CD19 gated
0.0002
0.52
0.41
0.63
D
Percentages of CD5hi B cells
Change following allergen challenge
% CD5hi cells within CD19 gated
0.057
0.56
0.47
>0.9
E
Percentages of CD5+CD1dhi B cells
Change following allergen challenge
% CD5+CD1dhi cells within CD19 gated
0.34
0.12
0.41
>0.9

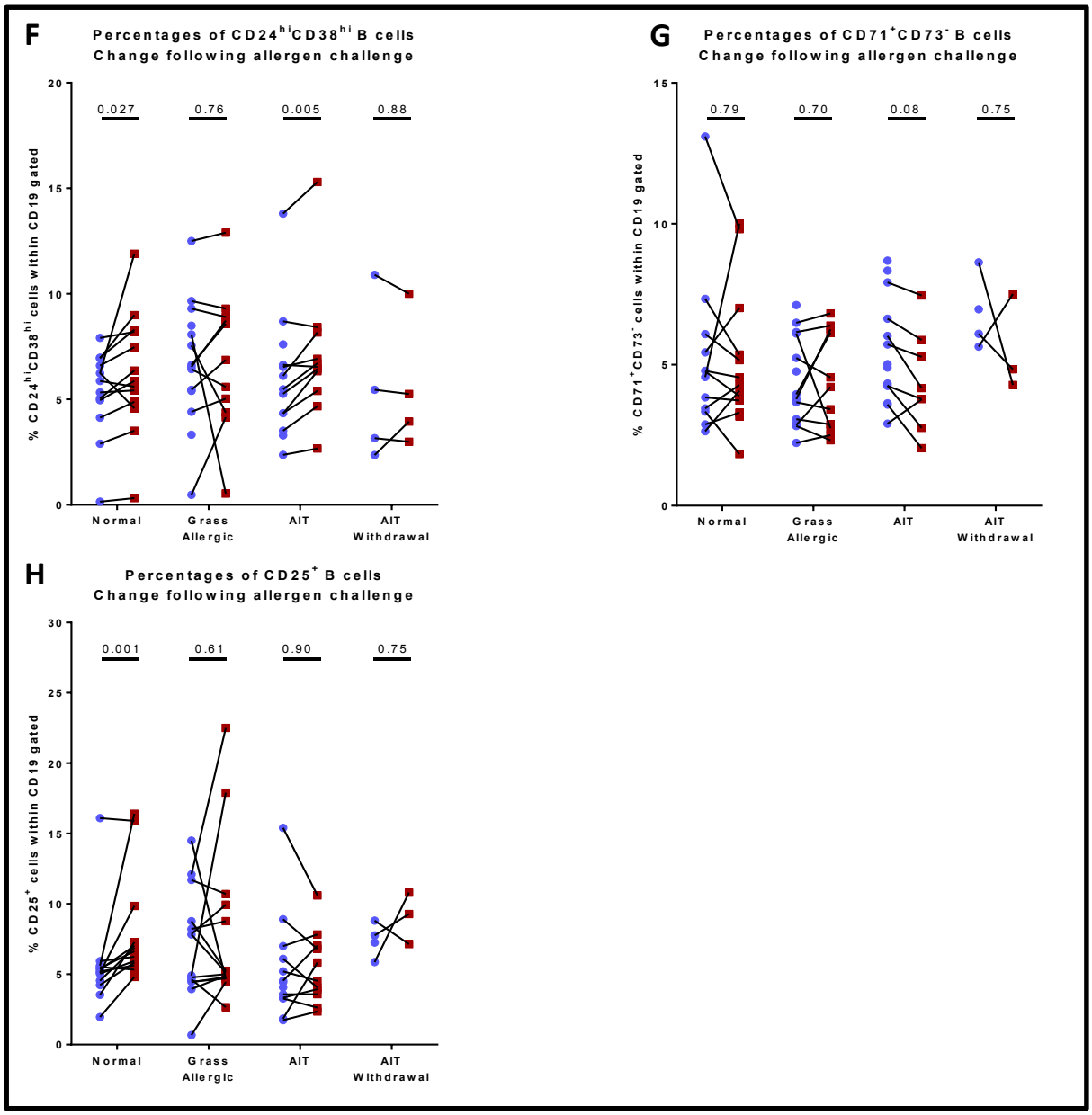


Figure 41 – Changes in B cell subsets after nasal allergen challenge.

NA, AR, SCIT, SLIT and AIT completed (withdrawal) (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT) volunteers underwent grass-pollen NAC. Blood was taken before and 8 hours following NAC. Whole blood was stained for flow cytometry and run with cell counting beads to quantify cell numbers relative to the amount of blood used. Numbers (**A**) of B cells are shown per µl of blood, whilst percentages of B cell subsets as a proportion of the B ell population are shown before (blue) and after (red) NAC for $CD27^{+}$ (**B**), $CD24^{hi}CD27^{+}$ (**C**), $CD5^{hi}$ (**D**), $CD5^{+}CD1d^{hi}$ (**E**), $CD24^{hi}CD38^{hi}$ (**F**), $CD71^{+}CD73^{-}$ (**G**) and $CD25^{+}$ (**H**) are shown.

4.2.10.3. Changes in B cell subsets for non-allergic controls in the absence of allergen challenge

To control for diurnal variation on the NAC day, which could account for the changes in B cell subsets attributed to nasal allergen, 7 normal controls were recruited for blood samples at 0 and 8 hours on an unchallenged control day (Figure 42). Donors differed from the cross-sectional study but were equally screened for allergic rhinitic symptoms and aeroallergen sensitisation. Proportions of B cell subsets did not differ throughout the day (Figure 42 B), with the exception of $CD1d^{hi}$ $CD5^{+}$ B cells which were significantly decreased at 8 hours compared to baseline. No proportions of B cell subsets examined were increased at 8 hours, as they were in the NAC day, relative to the whole B cell population. However, overall B cell numbers were significantly increased at 8 hours compared to baseline (p=0.016) (Figure 42 A), which resulted in numbers of $CD5^{hi}$, CD24hi $CD38^{hi}$ and $CD71^{+}$ $CD73^{-}$ B cells to be significantly increased (Appendix Figure 14).

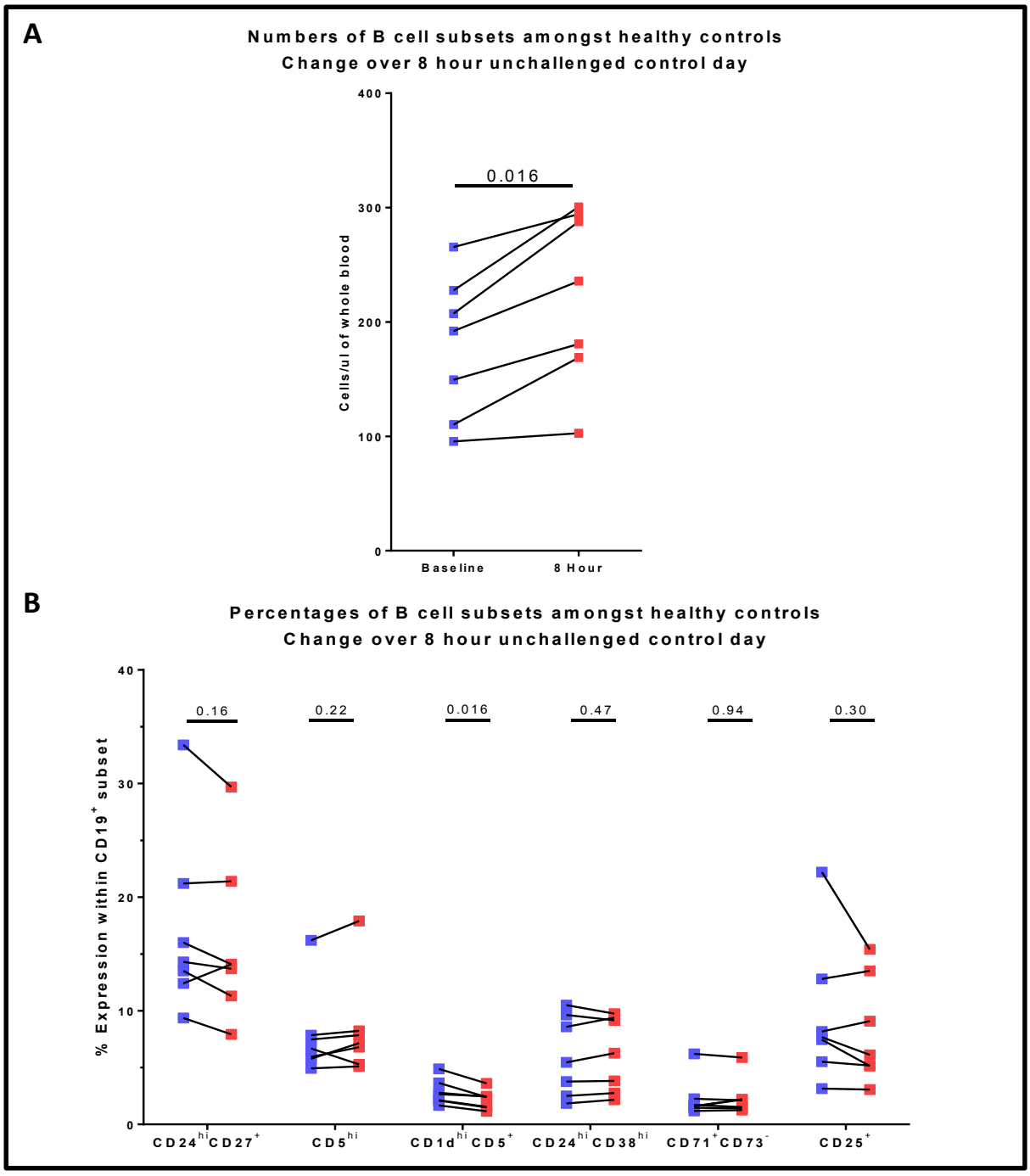


Figure 42 – Change in B cell subsets for non-allergic patients on an unchallenged day.

Non-allergic participants (n=7) donated blood at 0 (blue) and 8 hours (red) of an unchallenged control day to assess changes in B cell subsets. Whole blood was stained for flow cytometry and run in the presence of cell counting beads to assess absolute numbers of cells. Numbers of $CD19^{+}$ B cells per µL blood are shown (**A**). The percentages of $CD27^{+}$, $CD24^{hi}CD27^{+}$, $CD5^{hi}$, $CD5^{+}CD1d^{hi}$, $CD24^{hi}CD38^{hi}$, $CD71^{+}CD73^{-}$ and $CD25^{+}$ cells within the $CD19^{+}$ B cell population are also compared at 0 and 8 hours (**B**). Wilcoxon matched-pairs signed-rank p values are shown.

4.3. Examination of IL-10-producing B cells by histology

4.3.1. Immunohistochemistry staining of tonsil sections

Initially tonsil sections from historical donors undergoing routine tonsillectomy, who were not clinically phenotyped as to their allergic status, were used in order to validate staining methodology (detailed in section 2.11 on p61). Initially these sections were stained toward CD19, CD20, CD138 and IL-10 alone. CD19 antibodies did not stain B cells within the tissue, instead showed only non-specific staining of the epithelium (data not shown). CD20, CD138 and IL-10 showed positive staining, not observed in matched sections with negative antibody nor PBS controls (data not shown). All immunostaining was accompanied by DAPI (a nuclear stain) in order to localise individual cells within the sections (Figure 43 – A and Figure 44 – A). Combined staining of tonsil sections with CD20 and IL-10 (Figure 43) or CD138 and IL-10 (Figure 44) showed clearly detectable dual staining of both surface markers with IL-10. High densities of B cells and good numbers of plasma cells, typically less densely arranged, were observed in these sample tonsil sections. As staining of other features was not carried out it is difficult to discriminate exacting location of these cells. $CD20^+$ cells appeared to be within a region dense with B cells and $CD4^+$ T cells (stained during optimisation), suggesting these cells are likely follicular B cells. IL-10-expressing B cells were not distinctive by location, given their sparsity in some sections. $CD138^+$ cells were much larger and scattered throughout the extra-follicular spaces, with IL-10-expressing plasma cells also unremarkable by location. Dual staining for both CD20 and CD138 positive cells co-stained for intracellular IL-10 are identified by arrows and appear to be greater than background for IL-10. Areas containing these dual-stained cells are digitally zoomed for both CD20 and IL-10 (Figure 43 E and F) and CD138 and IL-10 dual staining (Figure 44 B), with an example of CD138 staining in the absence of IL-10 shown (Figure 44 C).

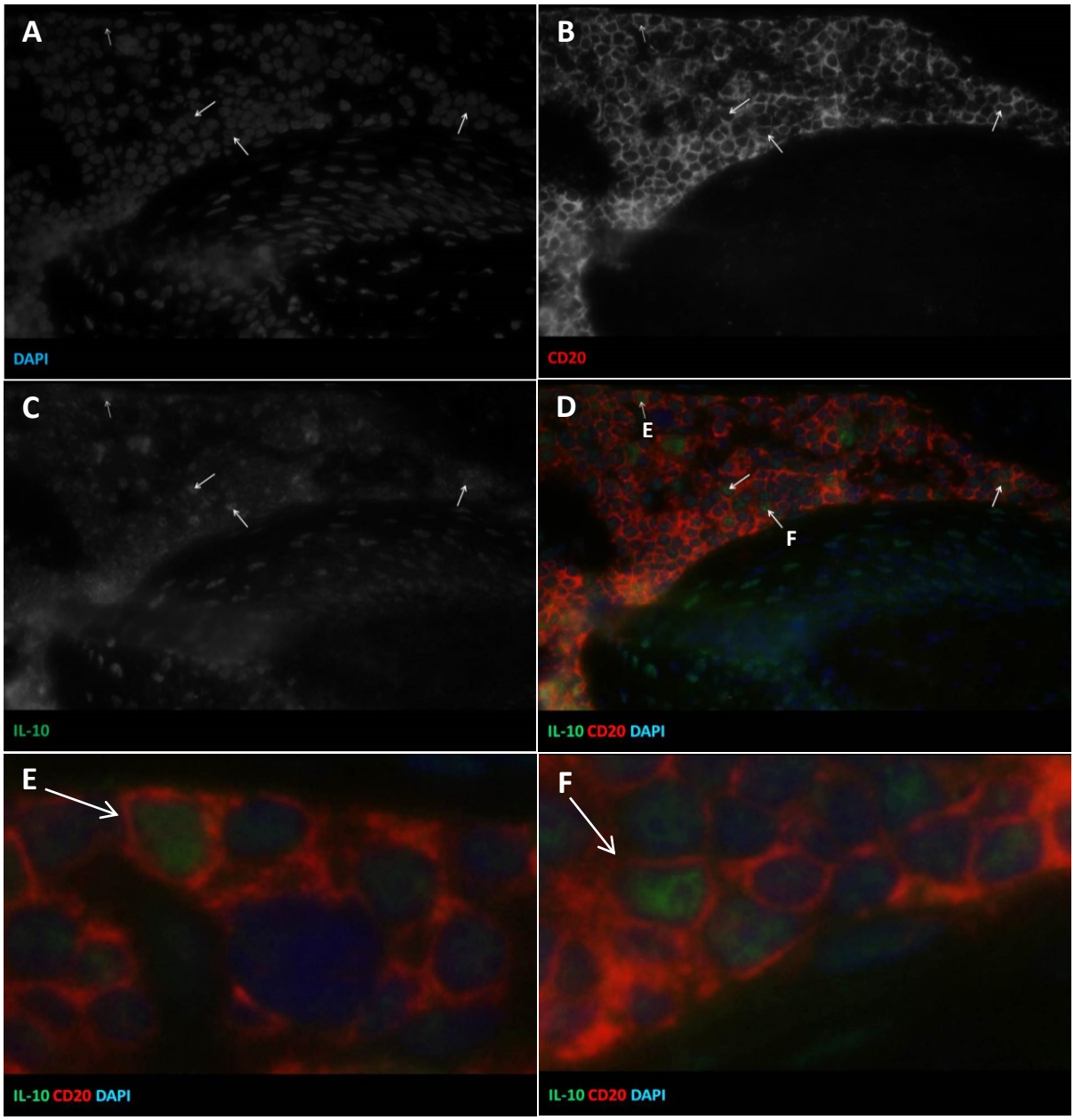


Figure 43 – Tonsil staining of IL-10 and CD20.

Sections of historical tonsil were stained with antibodies towards IL-10 (green) and CD20 (red) by 3-step immunohistochemistry. Cell nuclei are stained with DAPI (blue). Each colour image capture is shown with arrows in fixed positions for DAPI (**A**), CD20 (**B**), and IL-10 (**C**) staining alone or composite image (**D**). Digitally zoomed sections are also shown (**E** and **F**), identified by arrows in (**D**).

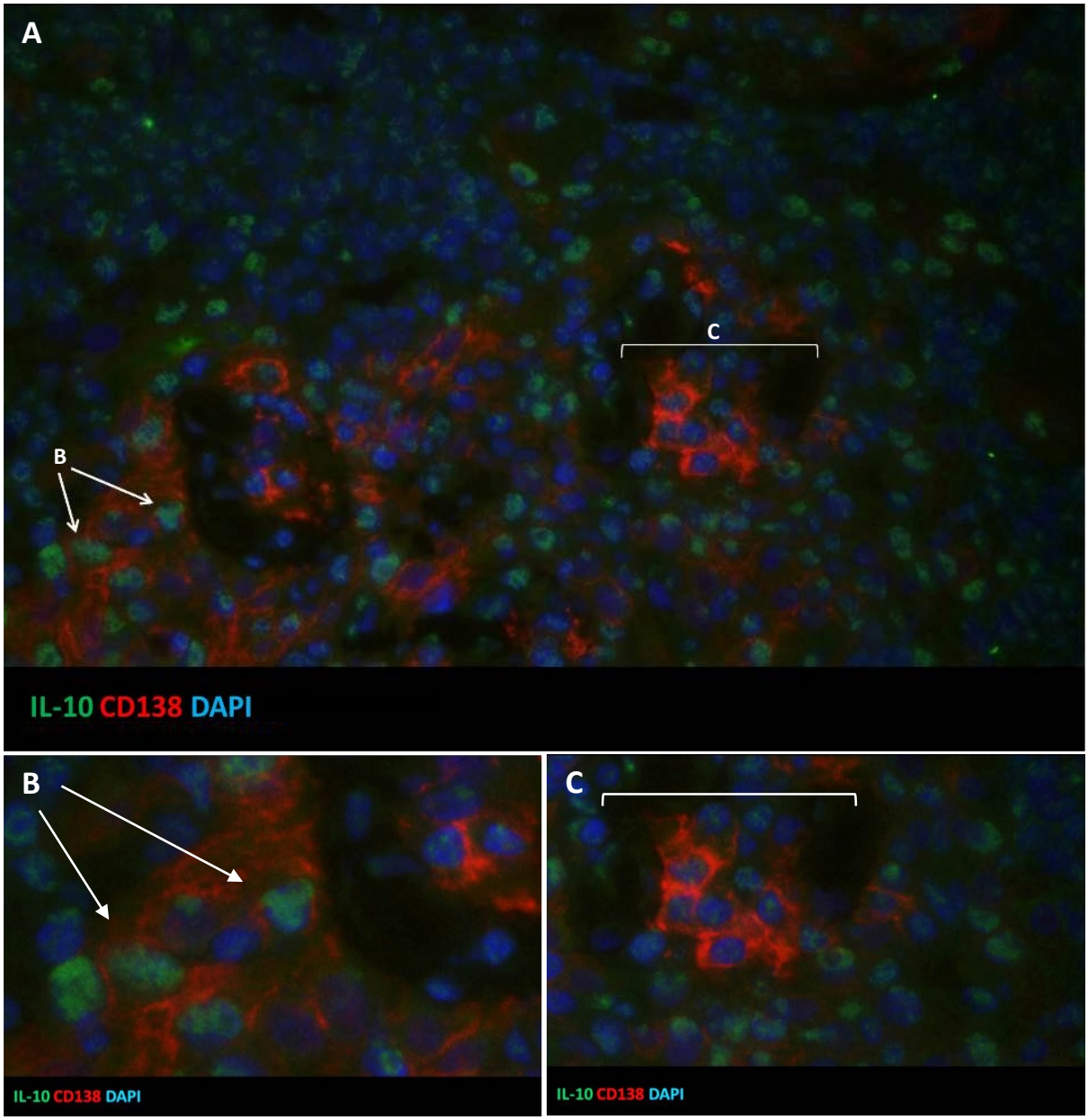


Figure 44 – Tonsil staining of IL-10 and CD138.

Sections of historical tonsil were stained with antibodies towards IL-10 (green) and CD138 (red) by 3-step immunohistochemistry. Cell nuclei are stained with DAPI (blue). Main image shows triple immunofluorescence composite image (**A**). Digitally zoomed sections of (**A**) identified by arrows and brackets are also shown (**B** and **C**).

4.3.2. Immunohistochemistry staining of nasal sections

Having validated the antibodies and staining in human tonsillar tissue for IL-10 and dual staining for B cells (CD20) and plasma cells (CD138), nasal sections were then stained both by light and florescence microscopy. CD20 and CD138 positive cells were identified in nasal sections by both light microscopy (Figure 45 A and B, respectively) and fluorescence microscopy (Figure 45 C and D, respectively), with negative staining observed amongst matched sections using the same concentration of isotype control antibody (Figure 45 E). Arrows identify the CD20-stained cells which are harder to make out by light microscope, but remain clear by fluorescence microscopy. Titration of IL-10 also confirmed that positive staining was observed with IL-10 antibody used at 1 in 5 and 1 in 10 (Figure 46 A and B), but with higher background observed at these higher concentrations, including non-specific epithelial staining. Greater dilution of IL-10 did not convincingly reveal positively stained cells (Figure 46 C and D). In order to confirm efficiency of the protocol an anti-CD3 antibody from the same species and also a polyclonal antibody was used to demonstrate positive staining (Figure 46 E). Negative control antibody did not show any positive staining (Figure 46 F), nor did PBS used in place of IL-10 antibody (data not shown).

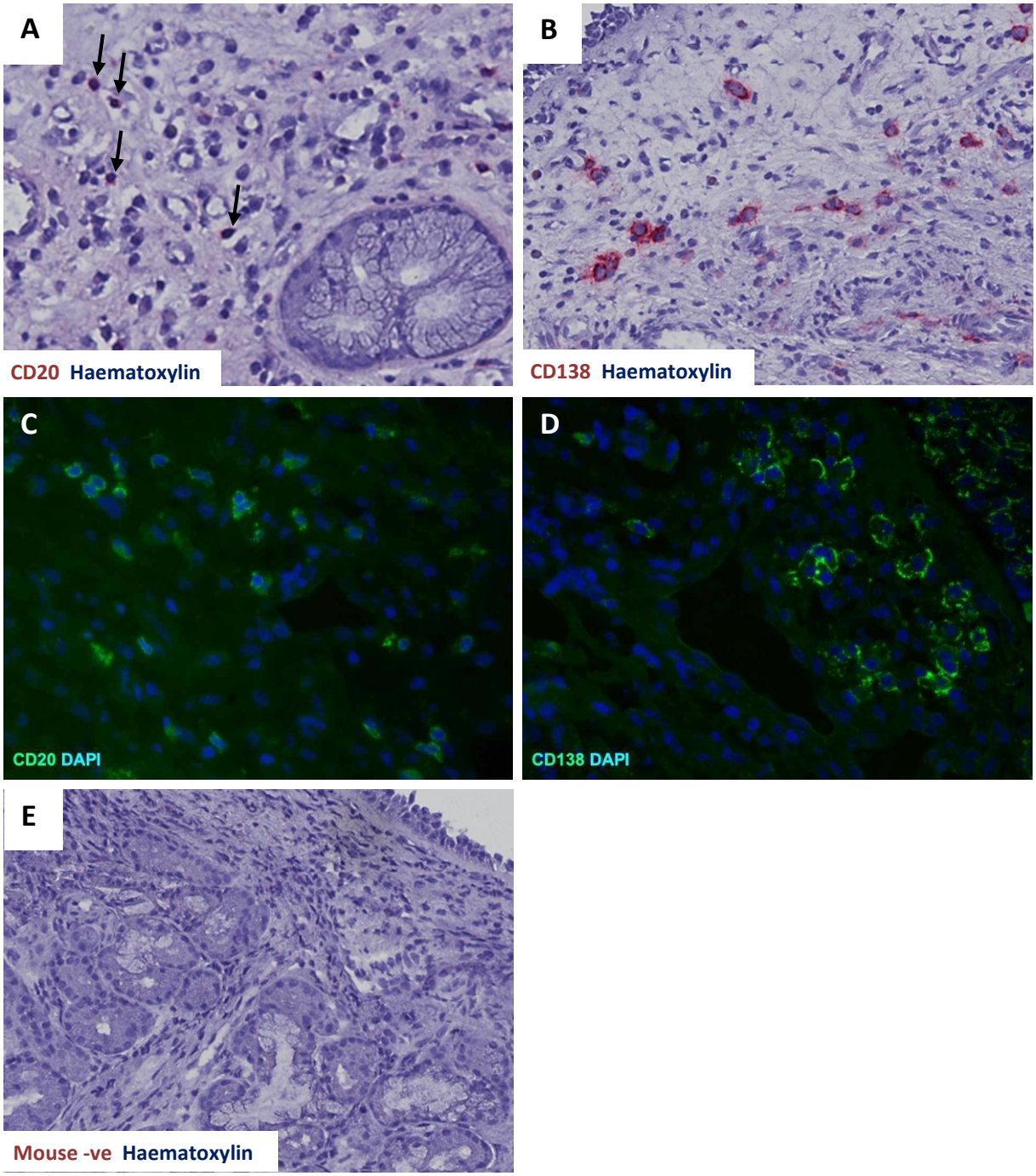


Figure 45 - Nasal staining of CD20 and CD138.

Sections of nasal tissue were used to validate staining with antibodies towards CD20, CD138 and mouse negative control antibodies by fast red with counter-staining using haematoxylin for nuclei staining (**A**, **B** and **E**, respectively). Immunofluorescent staining for CD20 and CD138 was also carried out using Alexa fluor 488 (green) to visualise, cell nuclei were stained with DAPI (blue) (**C** and **D**).

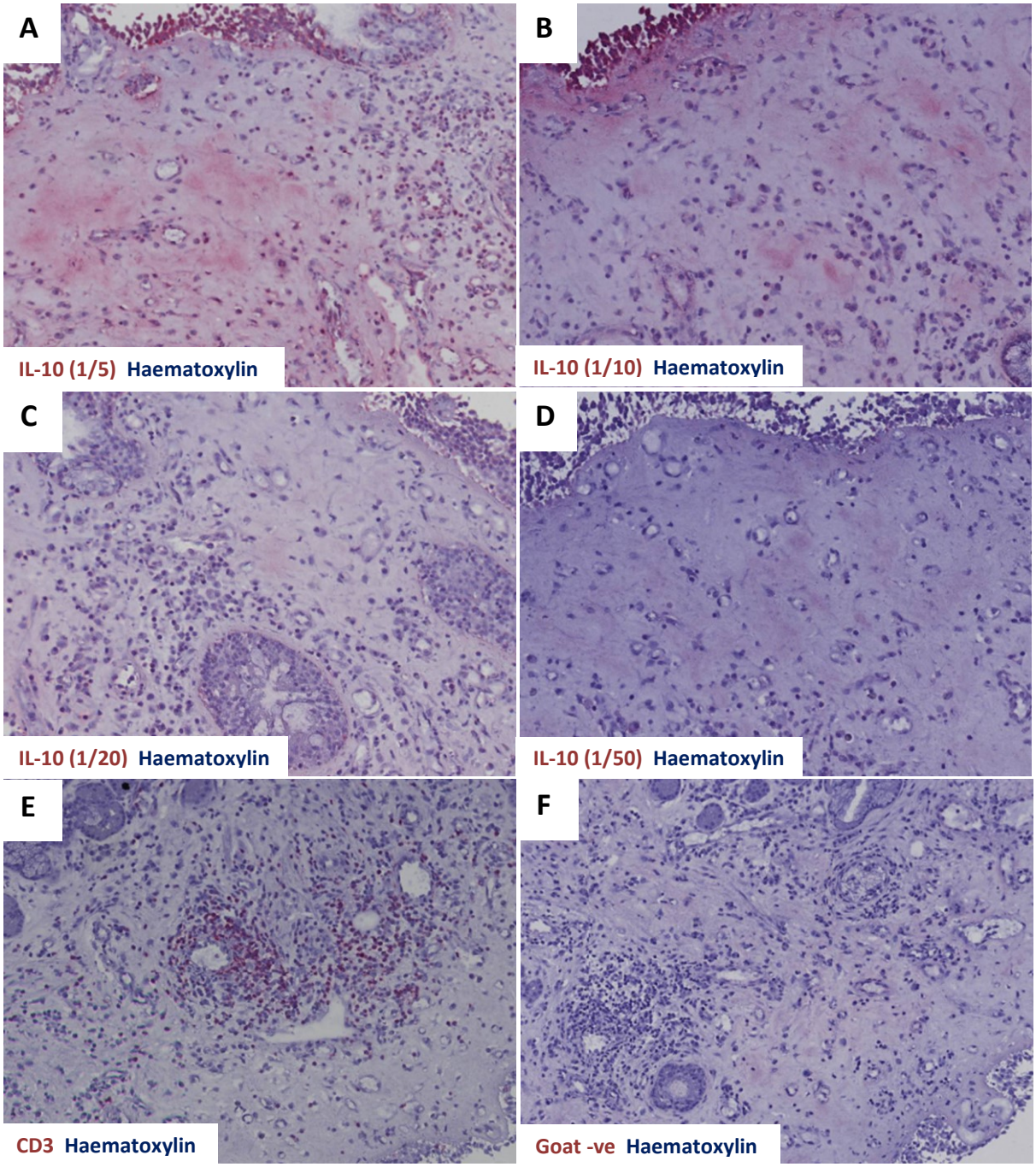


Figure 46 – Optimisation of IL-10 staining of nasal sections.

Sections of nasal tissue were used to validate staining with IL-10 antibodies, with CD3 and goat negative control antibodies used as controls. Fast red development was used and counter-staining with haematoxylin identified nuclei (**A**, **B** and **E**, respectively). IL-10 was used in decreasing concentrations, as shown (**A-D**), with CD3 used at 1/20 (**E**) or goat negative control (**F**) used at the highest concentration for IL-10 shown.

Dual staining of nasal sections was initially carried out with CD4 (green) and CD20 (red) to confirm both efficacious and discrete staining of these markers. CD4 is raised in goat as a polyclonal antibody, comparable to the antibody used for IL-10 staining, whilst $CD4^+$ cells are known to be present and easier to detect. Discrete cellular staining of $CD4^+$ and $CD20^+$ cells was observed in nasal tissue (Figure 47 A). However, staining using the same concentration of goat negative control (green) and antibody towards CD138 (red) showed non-specific IL-10 staining in these tissues (Figure 47 B, arrowed). This was also observed with CD20 in the place of CD138 antibody or in the absence of other antibodies (data not shown). However, closer examination of the green staining (digitally zoomed in Figure 47 C) shows no background staining with weakly staining green cells. The reverse is shown in staining with IL-10 antibody (green) and mouse negative control (red), in which clear IL-10 staining can be observed (Figure 47 D, arrow 1) with weak non-specific staining in red (Figure 47 D, arrows labelled 2). These non-specific staining cells appear to be eosinophils, with a granular cytoplasm and multi-lobed nuclei, which have previously been found to show weak non-specific fluorescence under UV with both filters (personal communication, Mikila Jacobson). Dual IL-10 and CD20 staining showed specific cells staining for both targets (Figure 47 E); however no cells were co-stained for both CD20 and IL-10 in these sections. Co-staining of IL-10 and CD138 showed one cell dual positive for both (Figure 47 F, arrow), with clear staining separately of other cells.

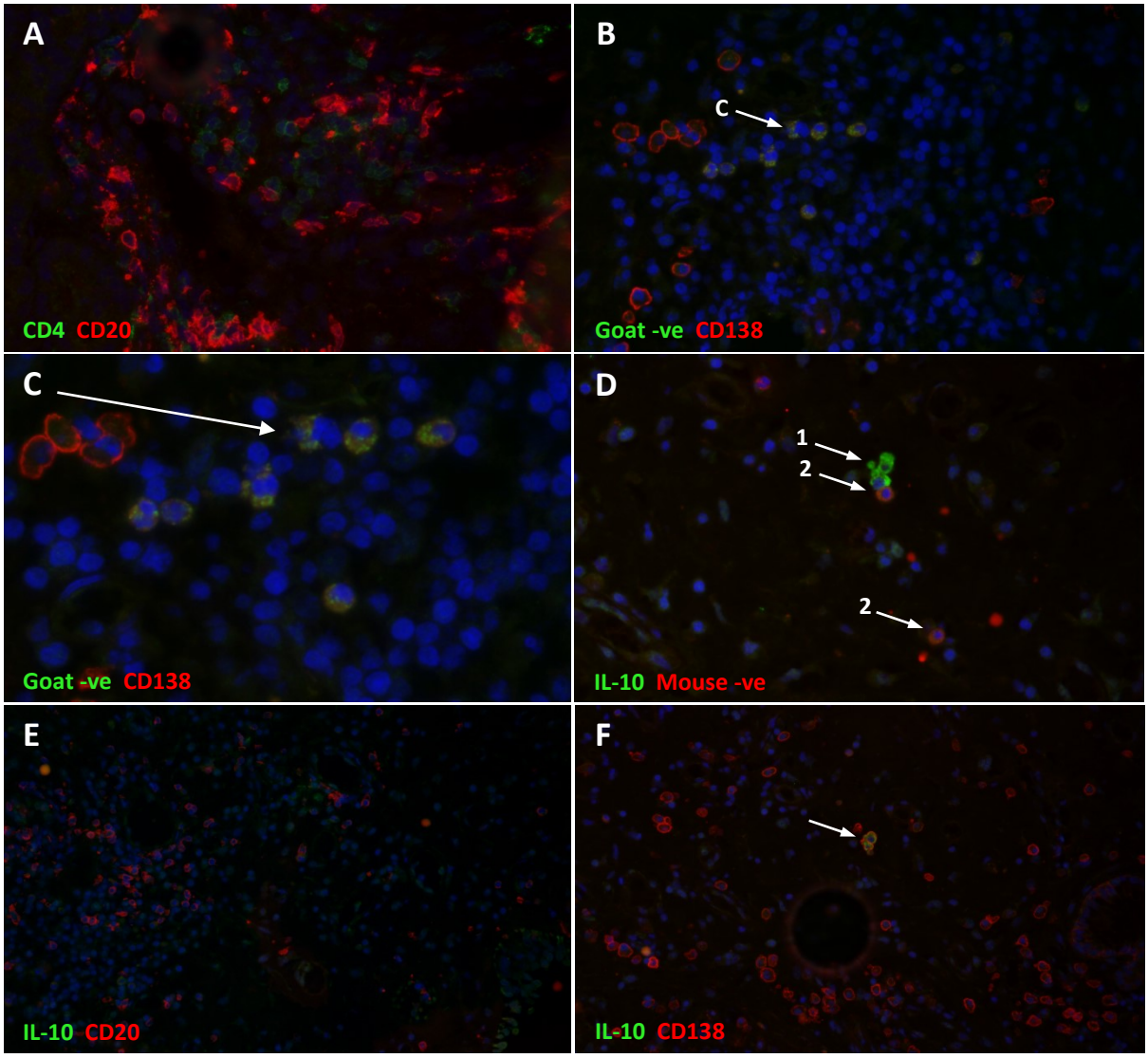


Figure 47 – Dual IL-10 and CD20 or CD138 staining in nasal sections.

Sections of nasal tissue were used to validate staining with antibodies towards CD20 (red), CD4 (green), CD138 (red), IL-10 (green) or using mouse (red) or goat (green) negative control antibodies by 3-step immunofluorescence staining. Cell nuclei were stained with DAPI (blue). Dual Staining of CD4 and CD20 (**A**), goat negative control and CD138 (**B**, digitally zoomed on arrow **C**), IL-10 and mouse negative control (**D**), IL-10 and CD20 (**E**) and IL-10 and CD138 (**F**) are shown.

4.4. Discussion

4.4.1. Pilot comparison of allergic and non-allergic responsiveness and identification of IL-10 detection by FluoroSpot

Initial examination of stimulated PBMCs by flow cytometry suggested that there was a reduced frequency of IL-10-producing B cells in allergic individuals, compared to non-allergic controls. However, this observation was not replicated with isolated B cells, stimulated with either CpG or LPS, by flow cytometry. This finding supports the hypothesis, described in the discussion to the previous chapter (section 3.3.2, on p97), that putative increases in B cell IL-10 responses amongst stimulated PBMCs is likely to be due to cells other than B cells within these PBMC cultures.

FluoroSpot was the technique subsequently used to detect IL-10-producing B cells in response to CpG, allergen alone, or in combination. FluoroSpot appeared to offer the greatest sensitivity to detect an intrinsic defect in B cell IL-10 capacity between clinical groups, without requiring either PMA and Ionomycin re-stimulation or the presence of other PBMCs in culture. Whilst it is of interest that B cells from allergic donors showed an IL-10 deficit when cultured amongst PBMCs for flow cytometry compared to non-allergic donors, this was not replicated by isolated B cells by flow cytometry and thus may not be attributed to B cells in isolation. Subsequent studies therefore employed isolated B cells, rather than B cells amongst PBMCs, in order to explore the differences in B cells between allergics and non-allergic participants and to study the influence of allergen immunotherapy on these differences.

4.4.2. Cross sectional study

In a clinical cross sectional study of non-allergic volunteers, untreated grass pollen allergic individuals and seasonal grass allergic donors, who had received allergen immunotherapy, proportions of regulatory B cells subsets and B cell IL-10 capacity was examined as a putative model for loss of tolerance amongst the allergic individuals and induction of tolerance following successful AIT. Previous studies of AIT have demonstrated a significant increase in total IL-10 production from PBMCs (Akdis et al., 1998, Jutel et al., 2003, Francis et al., 2003, Francis et al., 2008), this has previously been associated with an increased IL-10 capacity amongst T cells and monocytes (Akdis et al., 1998, Nouri-Aria et al., 2004, Francis et al., 2003). Akdis and colleagues have twice demonstrated increased B cell IL-10 capacity following bee venom AIT (van de Veen et al., 2013, Akdis et al., 1998), whereas this has not previously been explored following AIT for inhalant allergies and in particular seasonal pollinosis.

4.4.2.1. Participants

Allergic participants in the cross-sectional study collectively had significantly greater total and specific IgE with a large range compared to normal controls and no significant difference between untreated-allergic and AIT-treated participant groups. The duration of AIT varied with SCIT patients having received significantly fewer months of AIT (a median of 6) compared with SLIT-treated patients (median of 34 months). It is possible that the differing treatment duration and or varied cumulative dose of immunotherapy received between SCIT and SLIT may possibly have accounted for some of the observed differences.

Clinical outcomes in this small study showed that AIT patients reported significantly reduced pollen seasonal symptoms compared to untreated allergics; however, SCIT patients also had significantly reduced seasonal symptoms compared to SLIT patients, despite the shorter course of AIT received. Although adequately controlled, head to head studies are lacking, modelling studies of outcomes based on meta-analysis suggests a greater suppression of symptoms amongst SCIT-treated, compared to SLIT-treated, individuals (Di Bona et al., 2012, Meadows et al., 2013). Three small head-to-head studies of SCIT vs SLIT vs placebo did not show statistically significant differences (Khinchi et al., 2004, Yukselen et al., 2012, Aasbjerg et al., 2014). However, they were inadequately powered to detect a difference if one existed and so are of doubtful clinical relevance.

Within this thesis, self-reported clinical outcomes were similar for SCIT and SLIT treatment. Similarly, the early and late responses to intradermal skin challenge, as a surrogate objective clinical marker, showed a marked reduction amongst the AIT group compared to untreated controls, including both SCIT and SLIT-treated participants, with no differences being observed in the reductions for either early or late phase skin responses between SCIT and SLIT-treated patients compared to controls.

4.4.2.2. IL-10 responses of B cells from cross sectional patients

4.4.2.2.1. Allergen-induced responses

B cells stimulated with CpG in addition to allergen had a significantly greater number of IL-10 spots than CpG alone across all donors (Figure 25), which, although non-significant by clinical phenotype, was likely to be largely attributed to the AIT-treated group (Figure 26). This suggests that this stimulus identifies a further subset of IL-10-producing B cells, beyond CpG alone. This should be considered with a later figure (Figure 29), that showed numbers of IL-10 spots following the addition of allergen alone. A trend for greater numbers of IL-10 spots were observed amongst normal control or AIT-treated B cells relative to grass allergics, although the few numbers studied has limited the ability to detect or not a statistical significance, as the limited numbers of isolated B cells were prioritised for CpG stimulation. Allergen alone induced IL-10 amongst few B cells, showing a small population of allergen-specific IL-10^+ B cells may be disproportionately detected within the peripheral blood of non-allergic or AIT-treated individuals. Together this data suggests an altered allergic B cell phenotype following AIT, with an increased non-specific B cell IL-10 capacity, accompanied by a recovery of allergen-specific IL-10 responses. A study of naturally tolerant beekeepers and bee venom AIT-treated patients showed comparable results, with greater IL-10 induced from PLA-specific B cells exposed amongst non-allergic (tolerant) beekeepers or AIT-treated, but not untreated, allergic individuals compared to the non-specific PLA^- B cell population in the same individuals (van de Veen et al., 2013).

4.4.2.2.2. CpG-induced IL-10 responses

Stimulation of isolated B cells with CpG or with or without allergen, showed an increase in proportions of IL-10 producing B cells amongst AIT-treated patients compared to untreated allergics (Figure 27 and Figure 28, respectively). This was largely attributed to B cells derived from SLIT patients, rather than SCIT- treated subjects. Proportions of IL-10-producing B cells between non-allergic controls and untreated grass allergic patients was not significantly different, suggesting there is not a loss of regulatory B cell tolerance amongst grass allergic individuals. Little comparable work exists, which has examined regulatory B cell IL-10 responses amongst allergics or non-allergic controls. A study of asthmatics also showed that IL-10-producing B cells following CpG did not differ between allergic asthmatics and non-allergic controls (van der Vlugt et al., 2014), however, LPS stimulation, despite poor induction of B cell IL-10, showed significantly fewer IL-10-producing B cells amongst allergic asthmatics compared to non-allergic controls.

Assessment of IL-10 mRNA 5 hours following CpG stimulation showed gene induction in all B cells (Table 7); however a trend towards significantly reduced IL-10 mRNA induced amongst grass allergic patients compared to non-allergic controls (Figure 30, p121). If a significantly reduced induction of IL-10 mRNA could be shown amongst allergics, this may suggest that rapidly inducible IL-10 responses amongst B cells (at 5 hours) are depressed in allergics compared to non-allergics, despite comparable proportions of IL-10-producing B cells and IL-10 protein at 48 hours. Tedder et al., have coined the term 'B10' cells to describe B cells which produced IL-10 after 5 hours, as opposed to 'B10-pro' cells which are IL-10 producers by 48 hours of stimulation (Iwata et al., 2011). The potential defect in allergic IL-10 gene induction at 5 hours may therefore be defined as a reduced B10 capacity, rather than B10-pro B cells which may be comparable to non-allergics. No change in IL-10 mRNA was observed compared to or amongst AIT-treated groups.

Relative IL-10 concentrations in the supernatant of CpG-stimulated B cell cultures reproduce the trends and significant changes observed between clinical groups with respect to proportions of IL-10-producing B cell spots (Figure 32, on p125). Although the numbers studied are small the relative proportions of IL-10 competent B cells and the concentration of IL-10 produced are enhanced largely amongst the SLIT-treated patients. The IL-10-producing B cell bias observed for AIT donors is strengthened by the repetition of the bias in protein concentration and the concordance observed between proportions of IL-10 spots, protein concentration and IL-10 mRNA (Figure 33, on p126). Closer correlations were observed between proportions of IL-10-producing B cells and IL-10 concentration, suggesting that these two factors are more strongly inter-related, also reflected by the demonstration of an AIT bias. It is therefore likely that increased IL-10 protein concentration amongst SLIT patients is a result of greater relative numbers of B cells capable of producing IL-10. The weakest correlation was observed between proportions of IL-10-producing B cells (at 48 hours) and IL-10 mRNA induction (at 5 hours), suggesting that immediate message induction may be less predictive of 48 hour IL-10 capacity.

4.4.2.3. IL-10, IL-6 and TNFα detected in culture supernatants

As explored in the introduction, B cells are capable of producing a range of cytokines (section 1, on p40). For this reason a 25-plex ELISA was carried out on CpG-stimulated B cell supernatant from all clinical groups, as expected IL-10 protein was significantly greater than medium, however both IL-6 and TNFα were also significantly induced (Figure 32, on p125). The other 22 cytokines examined showed no significant protein following CpG stimulation (Appendix Table 4). Although IL-10 protein concentrations were significantly greater amongst SLIT-treated B cells compared to those from SCIT

or non-allergic donors, this was not evident for the concentrations IL-6 or TNFα amongst these supernatants, suggesting a possible IL-10-specific augmentation of B cells in SLIT-treated patients.

IL-6 was detected at 4x greater concentrations than IL-10, whilst TNFα was present 1.5x greater than IL-10 (Table 8). In view of the substantial amounts of cytokine detected, B cell produced IL-6 and TNFα warrant further investigation *in vitro* and *in vivo*. This data is consistent with levels of IL-10, IL-6 and TNFα detected following CpG stimulation of isolated B cells by others (Ziegler et al., 2014, Thibult et al., 2013, Iwata et al., 2012). IL-10, IL-6 and TNFα have also been shown to be produced following stimulation with the self-antigen, thyroglobulin, by B cells in healthy human donors (Langkjaer et al., 2012). Human B cells also showed a dose-response relationship to the hormone leptin with respect to IL-10, IL-6 and TNFα *in vitro* (Agrawal et al., 2011), with leptin-induced cytokine production increased by age (Gupta et al., 2013).

Although B cell IL-10 production and capacity is the focus of this thesis, it appears somewhat contradictory to find the pro-inflammatory cytokines IL-6 and TNFα highly represented amongst B cells in the context of IL-10. Unfortunately it is unclear from the data collected here whether individual cells expressing IL-10 co-express either or both of IL-6 or TNFα in addition to IL-10. IL-6-secreting B cells, induced by R848 (a TLR7 and TLR8 stimuli) show a subset of IL-10-co-expressing B cells (Liu et al., 2014a). Indeed, whilst B cells were stained for in isolation, IL-10 was always co-expressed with IL-6. It is not clear whether IL-10-producing CpG-stimulated B cells also all co-express IL-6, although it is likely that the IL-6-producing B cells outnumber those producing IL-10, given the concentrations of IL-6 detected in this thesis and others. In a separate study, the combined CpG and CD40L stimulation showed that a subset of IL-10 expressing B cells also co-expressed TNFα (Cherukuri et al., 2014), although both TNFα and IL-10 were also detected in isolation from B cells. These two studies imply that most IL-10-producing B cells are also capable of producing TNFα or IL-6 along with IL-10 (and potentially all three), suggesting a complex phenotype. Neither of these studies was able to define a pure B cell that produced IL-10 alone.

4.4.2.4. Beyond IL-10 – mRNA at Baseline and following CpG

Due to limitations in cell numbers, protein expression of TLR4 and TLR9 was not examined between donor groups. Given the reliance on TLR9 signalling within this thesis by the use of CpG, TLR4 and TLR9 mRNA was examined as a compromise, to exclude relative changes between clinical groups as a causal factor on IL-10 responses (Figure 31, on p123). TLR4 expression was also determined (by mRNA) to identify any change in B cell TLR4 expression between groups, as observed amongst allergic asthmatics by protein (van der Vlugt et al., 2014). Neither TLR9 nor TLR4 mRNA differed by clinical phenotype,

suggesting that differing B cell IL-10 responses following CpG are not driven by differing TLR9 or TLR4 translation, but a downstream capacity to produce an IL-10-specific response.

Examination of baseline immunoglobulin mRNA (Figure 31, on p123) amongst freshly isolated B cells, showed significantly greater IgG4 mRNA amongst AIT-treated donors compared with non-allergic controls. The SLIT group (who have received the longest course of AIT) appeared to have slightly greater proportions of detectable IgG4 mRNA; however, the limited sample size prevents statistical analysis. Neither total IgG nor IgA or IgE mRNA differed between clinical groups, with a large spread detected in IgE for all groups.

Previous data has suggested that serum IgG4 increases early during AIT treatment, typically after 6 months for SCIT (Francis et al., 2008). The median duration of AIT received for SCIT donors in this thesis was just 6 months, which suggests they may be expected to have lower levels of IgG4 than SLIT patients, who had received a median of 34 months treatment. Measures of immunoglobulin mRNA are a surrogate for protein and is not as robust, but indicates a trend consistent with the induction of allergen-specific IgG4, typically detected during AIT. It should be noted however that this analysis of $CD19^{+}$ B cells in the periphery does not account for switched plasma cells, likely to reside within the bone marrow, spleen or tissue.

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4.4.2.5. Examination of confounders in detecting increased capacity for B cell IL-10

4.4.2.5.1. AIT duration

As mentioned, SLIT-treated B cells showed greater concentrations of IL-10 and greater proportions of IL-10-producers, but had also received a longer course of AIT. AIT duration of treatment showed a strong trend toward correlation with respect to proportions of IL-10-producers following CpG with and without allergen (Figure 34, on p127), but not allergen alone or with CpG-induced IL-10 protein concentration. Within the AIT group alone, few numbers limited the ability to achieve significant correlates in this group alone. It is clear, however, that there is a relationship between treatment duration and CpG-induced IL-10 spots. Together, this raises the possibility that a longer duration AIT, rather than use of SLIT or SCIT, could enhance the global proportions of IL-10-capable B cells, whilst a more immediate response may be observed by enhanced proportions of allergen-specific IL-10-producing B cells. It is not clear from these data whether the route of administration rather than the duration of AIT has the greatest influence on IL-10-producing B cells.

4.4.2.5.2. Gender of participants

Given all SLIT donors were female and also showed both greater proportions of IL-10-producing B cells and greater concentrations of IL-10 from B cell supernatants compared to SCIT-treated or untreated donors, this led to the hypothesis that the gender of participants contributed to a confounding variable when examining B cell IL-10 responses (Figure 27, Figure 28 and Figure 32).

This was examined in Figure 35 (p129), which showed both the proportions of IL-10^{+} B cell spots and IL-10 concentration by donor gender. When all donors were considered, IL-10 supernatant concentration is significantly greater amongst female donors, whereas a trend was observed for proportions of IL-10-producing B cells being greater amongst female donors. In order to further determine the influence of gender AIT samples were excluded, given AIT is known to induce peripheral tolerance. Exclusion of AIT subjects did not significantly alter the female bias observed for IL-10 supernatant protein concentration observed, whilst the weak trend toward significance amongst IL-10 spots was lost. This suggests that female donor B cells do indeed produce significantly greater concentrations of IL-10 than males. However, proportions of IL-10-producing B cells not being significantly altered by gender, especially with the exclusion of AIT donors, suggests the possibility that proportions of IL-10-producing B cells are less influenced by gender than IL-10 protein concentration. This suggests that the increased proportions of IL-10-producing B cells observed amongst SLIT-treated donors is unlikely to be explained by them being an entirely female group.

Previously reported data has suggested that IL-10 responses are biased by gender and age. Antigen-specific stimulation of PBMCs has shown significantly fewer IL-10-producing cells amongst males (Haralambieva et al., 2013) and polyclonal stimuli have shown significantly lower concentrations of IL-10 protein in supernatants from males (Giron-Gonzalez et al., 2000). Additionally, TLR4-mediated IL-10 responses are also greater amongst female-derived PBMCs than male (Ono et al., 2005, Asai et al., 2001). Although, conversely, TLR9 induced IL-10 has been shown to be produced at higher concentrations amongst PBMCs from male rather than female donors (Torcia et al., 2012). Female donors appear to account for the predominant gender bias of IL-10 capacity reported. Murine studies have suggested that oestrogen is capable of increasing proportions of IL-10-producing B cells (Bodhankar et al., 2011, Subramanian et al., 2011). To my knowledge there are no reports of AIT efficacy differing by gender, although one recent study that examined time taken to achieve maintenance dosing (which may be taken as a surrogate for immunological tolerance of high dose AIT) showed significantly shorter escalation times amongst females (Jourdy and Reisacher, 2012).

4.4.2.5.3. Donor age

Age was equally distributed across donor phenotype, so unlikely to skew outcomes between clinical phenotypes. IL-10-producing B cells have been shown to be influenced by donor age, a study of CpG-induced IL-10-producing B cells showed older subjects have fewer IL-10-producing B cells, and produced lower concentrations of IL-10 (Duggal et al., 2013). This data is in agreement with the findings reported here, that shows a trend towards increasing age of the participants associated with both fewer IL-10-producing B cells and lower concentrations of IL-10 in culture supernatants.

4.4.2.6. Relationships between clinical outcomes and IL-10$^+$ B cells

Intradermal allergen challenge responses correlated with proportions of CpG-induced IL-10-producing spots. Whilst CpG-induced IL-10 spots and protein concentration correlated with early phase wheal responses only, CpG and allergen-induced IL-10 spots correlated with both early and late responses (Figure 36, on p131). Together this data suggests that CpG-induced IL-10 responding B cells are more related to suppression of early phase responses, whilst the addition of allergen allows the detection of IL-10-producing spots, B cells which relate close to late phase responses. It is these additionally antigen-responsive IL-10-producing B cells which may act on T cells, which mediate the late phase response. It is of interest that the addition of allergen to CpG enhanced the significant correlation between IL-10-producing B cells and the size of the late phase responses. No subjective clinical measures of rhinitis symptoms related to proportions of IL-10-producing B cells. Given the small size of this study, it may be more likely that associations between *ex vivo* measures of tolerance and *physiological* responses, rather than subjective symptom scores, are likely to be more readily achieved. A larger study may allow for the breadth of subjective scores to correlate to a degree with a greater proportion of regulatory B cells, if a relationship exists. However, subjective experience of rhinitis during a pollen season are influenced by a multitude of factors such as relative sensitivity to allergen, variable subjective perception of severity, overlapping viral or alternate-allergen-derived rhinitic symptoms, variable exposure to pollen due to geography or habit (for example time spend in/outdoors) etc.

4.4.3. Nasal allergen challenge

4.4.3.1. B cell distributions at baseline

The use of nasal allergen challenge permitted the examination of B cell subsets between clinical phenotypes both at baseline and compared to after an experimental allergen challenge in the target organ. Baseline characterisation showed fewer overall numbers of B cells amongst normal controls compared to allergic rhinitic donors; both treated and untreated, although this did not reach significance following treatment (Figure 39, p134). This suggests that allergic donors have greater numbers of circulating B cells.

This surprising finding suggests raised numbers of B cells overall in the blood of allergic donors may account for differences observed in the proportions of IL-10-producing B cells. Donors with reduced proportions of IL-10-producing B cells may have greater overall numbers of B cells in circulation and thus a similar number of IL-10 competent B cells. By applying the numbers of B cells from matched patients to the proportions of IL-10-producing B cells detected by FluoroSpot, differences between clinical phenotypes remained consistent (Appendix Figure 12, p204). Increased numbers of B cells amongst allergic donors (treated and untreated) did not affect the observations with respect to proportions of IL-10-producing B cells, which were increased for SLIT-treated donors compared to SCIT-treated or untreated controls, when *numbers* of IL-10-producing B cells are calculated using these data (Appendix Figure 12). This back-calculation does lend some robustness to the notion that the SLIT-treated B cells have a greater capacity to produce an IL-10 response, compared to un-treated allergics in this study. Confirmation will require a prospective controlled study of grass pollen SLIT.

Proportions of B cell subsets at baseline showed marginal differences by phenotype. A significantly reduced proportion of $CD5^{+}CD1d^{hi}$ B cells were observed amongst AIT-treated B cells, although this was not reflected in the withdrawal group. Few other subsets showed significant differences, with the exception of the $CD71^{+}CD73^{-}$ subset, demonstrated recently by Akdis and colleagues (van de Veen et al., 2013). These cells trended towards greater proportions amongst AIT-treated compared to un-treated allergics, which was significant for the 4 AIT-completed donors alone and all AIT-treated donors when the completed donors were included (Figure 40, on p135).

These baseline comparisons suggest few differences between proportions of regulatory B cell populations in the peripheral compartment between the groups studied. Several investigators have reported proportions of regulatory B cell subsets in diseased populations, such as vasculitis, Sjögren's

syndrome, RA and SLE, in order to demonstrate a regulatory B cell deficits (Todd et al., 2014, Lepse et al., 2014, Furuzawa-Carballeda et al., 2013, Ma et al., 2013). With respect to allergy, few differences within the peripheral B cell compartment have been reported. A study of allergic asthmatics has shown proportions of $CD24^{hi}CD38^{hi}$ B cells to be greater, whilst $CD24^{hi}CD27^{+}$ B cells fewer, in proportion and absolute count, relative to healthy controls (van der Vlugt et al., 2014). In addition, increased proportions of $CD5^{+}$ B cells (and $CD24^{hi/+}CD38^{hi/+}$) in early life have been suggested to be predictive of later development of allergic disease (Lundell et al., 2014).

The data in this thesis, acknowledging the limitations of the small sample studied, support that there is no clear deficit within Breg subsets amongst allergic individuals compared to non-allergic controls, nor was there clear evidence of greater proportions of Breg subsets amongst AIT-treated allergics as a result of treatment, when these subsets are considered at baseline and outside of the pollen season.

4.4.3.2. Comparison of B cells before and after allergen challenge

NAC demonstrated a significant physiological response to allergen, as shown by significant nasal blockage amongst allergic patients compared to non-allergic controls. The AIT groups showed a reduction in nasal blockage following NAC compared to untreated allergic controls, however when examined separately SLIT donors did not show a significant reduction in nasal blockage compared to untreated allergics (whereas SCIT remain significant), likely due to spread of the data and only 6 repeats.

The numbers and percentages of B cell subsets were examined by flow cytometry at baseline before nasal challenge and compared with matched samples taken following allergen challenge (Figure 41, on p138).

Much like the data from the NAC day, the non-challenge control day showed that *numbers* within regulatory B cell subsets increased throughout the day, suggesting this change was due to diurnal variation. However, the *percentages* of B cell subsets amongst non-allergic donors were not affected by diurnal variation on the non-challenge day. This suggests that the increased percentages of Breg subsets recorded on the NAC day may indeed occur as a consequence of allergen challenge. Only the proportions of $CD24^{hi}CD38^{hi}$ B cells (as a percentage of total B cells) were significantly increased following NAC for both non-allergics and AIT-treated allergic donors, but not in untreated allergic patients. This may suggest a challenge-induced mobilisation of a subset of regulatory B cells amongst

this transitional subset in order to suppress allergen-driven inflammation. It is unclear to where these increased proportions of circulating transitional cells are migrating; further work is required to establish to where these cells localise following NAC. Surprisingly, $CD24^{hi}CD27^{+}$ and $CD25^{+}$ B cell subsets were significantly increased, whilst $CD5^{hi}$ B cells trended towards significance following NAC, amongst non-allergic donors alone. Non-allergic donors within this study are naturally allergen tolerant, with no positive skin responses to any common aeroallergens or allergic clinical history. It is possible that these individuals may have more developed mechanisms of tolerance than those induced by AIT.

The numbers of total B cells amongst clinical phenotypes at baseline were significantly increased in AIT-treated donors, with a strong trend amongst normal controls and AIT-completed donors, compared to strikingly no change amongst grass allergic donors. This was shown to be likely due to diurnal variation (Figure 42, on p140), not NAC, as a separate group of non-allergics, followed in the absence of allergen challenge, also demonstrated a significant increase in total B cell numbers. A rise in B cell numbers throughout the day, specifically absent amongst untreated-allergics compared to AIT-treated allergics or non-allergic controls, is surprising and suggests B cells are regulated by a mechanism not observed amongst the grass allergic populations. Little recent data exists to confirm the diurnal relationship of B cells numbers throughout the day, however this has been examined in previous decades (Abo et al., 1981, Petitto et al., 1993), and shows significantly increased PBMC and B cell absolute numbers between 8 am and 4pm, as observed in this study. An inverse relationship between the absolute number of lymphocytes and serum cortisol was observed, which may suggest that allergic lymphocytes are insensitive to glucocorticoid suppression. This may additionally explain the higher baseline absolute count observed in this thesis. Glucocorticoid insensitivity to exogenous steroid treatment has been well described amongst poorly controlled asthmatics (Chan et al., 1998), although smoking is often a contributing factor, which was not the case with the population recruited for this study.

4.4.4. Histological evidence of IL-10-producing B cells in vivo

Dual immunofluorescence staining of tonsils from patients with unknown allergic status showed both IL-10-producing $CD20^{+}$ B cells and $CD138^{+}$ plasma cells. Whilst there was a greater abundance of both B cells and plasma cells detected amongst the sections from tonsils, none the less staining for IL-10, CD20 and CD138 was successful in nasal samples. False positive staining was observed for both colours, principally labelling eosinophils, although the intensity and pattern of staining was distinct

from the true positive staining that was co-localised to B cells. No examples of dual staining for CD20 and IL-10 were found amongst nasal examples, whereas dual staining for CD138 and IL-10 was achieved. This may suggest that outside of secondary lymphoid organs, plasma cells are the dominant or only IL-10-producing B lineage-derived cells resident in tissues. Recent evidence offers IL-10-producing plasma cells would support this in vivo finding, showing that IL-10-producing B cells mature to become IgM- or IgG-secreting plasma cells (Heine et al., 2014, Neves et al., 2010). The paucity of dual stained cells meant that apart from a qualitative confirmation of dual staining in the human tonsil and nasal mucosa, it was not possible to undertake a quantitative assessment of the number of cells detectable in tissue derived from the whole population studied. None the less, analysis of the whole population for single stained B cells and plasma cells could be performed.

4.4.5. Future work

In view of discrepancies between the 'pilot' and 'cross sectional' studies of isolated B cells, it is clear that a much larger blinded study would be required to fully dissect any minor deficiency in B cell IL-10 capacity in allergic compared to non-allergic donors. A comparison of IL-10-producing B cells from allergic and non-allergic groups, both in and out of the pollen season, might have identified any variance due to natural allergen exposure. This cross sectional study was carried out before the pollen season and as such allergics were non-symptomatic at the time IL-10-producing B cells were examined. Within the cross sectional study there was a random mismatching for both gender and duration of AIT of participants which confounded the interpretation of the data. Further work should control for gender, as this has a clear impact on the concentration of IL-10 produced by B cells, and matched for duration of intervention received. This study did, however, provide evidence for an enrichment of IL-10-producing B cells in the AIT-treated cohort. A prospective, blinded, study of both SLIT and SCIT relative to placebo would identify both the time course and degree of IL-10-competent B cells induced as a result of AIT and these studies are currently in progress. Studies which have examined the effect of exacerbations and remission during autoimmune disease have demonstrated that this approach may be relevant to identify immunological changes in regulatory populations. For example patients with an acute exacerbation of vasculitis show fewer IL-10-producing B cells compared to patients in remission or to healthy control individuals (Lepse et al., 2014, Todd et al., 2014, Wilde et al., 2013). Similarly, in the context of allergy, IL-10-producing T cells have been shown to be increased amongst allergen-tolerant beekeepers during the beekeeping season (Meiler et al., 2008). In addition, a prospective study of AIT would permit the examination of both Treg and Breg compartments, in order to determine whether expansion of one compartment might precede the other during the evolution of allergen-specific tolerance.

Examination of IL-10 expression by B cells in non-allergic individuals during the pollen season, compared to allergics, would test whether the regulatory B cell compartment may be critical for the development of natural allergen tolerance, such as through mobilisation of a greater variety of regulatory B cell subsets. AIT-induced tolerance, however, may be a separate immunological mechanism involving IL-10-producing regulatory B cells to a greater extent. The non-allergic control group in this study were additionally non-atopic, with no skin prick response nor significant specific IgE towards any aeroallergen. It would have been of interest to include a group of asymptomatic but allergen-sensitised participants, with specific IgE and skin prick responses matched to allergics. Inclusion of such a subset would assess immunological tolerance in a group who are likely to have genetic predisposition towards sensitisation but who have maintained a natural immunological tolerance.

Allergen-induced B cell IL-10 requires further investigation, especially with respect to the phenotype of B cells which produce IL-10 in response to allergen, compared to CpG alone or in combination, as well as the clinical phenotypes producing allergen-induced B cell IL-10. If these cells can be isolated by IL-10-capture and cell sorting, this could provide an ideal experiment for measurement of mRNA by microarray in order to determine the differential subgroups of IL-10-producing B cells. This has been reported by two groups in recent years, both isolating B cells by IL-10 surface capture of actively IL-10-producing isolated B cells following CpG stimulation (Lin et al., 2014, van de Veen et al., 2013), identifying IL-10^{+} Bregs as germinal centre B cells or $CD25^{+}CD71^{+}CD73^{-}$ B cells, respectively. As part of this thesis microarray was attempted with IL-10+/- sorted B cells by IL-10-capture at 48 hours following stimulation (Appendix Figure 1, p190 and Appendix Table 1, p191), however as IL-10 was not returned as significantly up-regulated amongst the IL-10-sorted population further optimisation is required and was not therefore included in the main body of this thesis. In this preliminary work, both CD71 and lymphotoxin alpha were returned as up-regulated amongst IL-10^{+} B cells, which is consistent with the previously cited studies.

Unfortunately, IL-6 and TNFα responses to allergen were not examined alongside IL-10. This would also have provided valuable contribution concerning a likely allergen-specific B cell phenotype. B cell FluoroSpot allowed for detection of IL-10-secreting B cells, which are unlikely to be captured by flow cytometry as re-stimulation with PMA and Ionomycin may dilute out any allergen-specific signal. Further use of dual or triple colour FluoroSpot, would allow the detection of B cells secreting IL-10, IL-6 and/or TNFα, in isolation or combination, in response to a range of stimuli including CpG and allergen.

This study used baseline B cell mRNA expression to determine TLR4, TLR9 and immunoglobulin expression amongst the clinical phenotypes. Future work should examine protein expression of these molecules on B cells by western blot and flow cytometry between clinical groups, but also between IL-10-expressing and non-expressing B cells. Comparison of serum total and allergen-specific IgG1, IgG4 and IgA1 and IgA2 should be used to draw correlation between B cell IL-10 capacity and induction following AIT. This serum antibody data was not available at the time of writing this thesis.

Although there was no clear difference in the proportions of regulatory B cell subsets observed at baseline amongst allergics (or recovered in AIT - Figure 40, p135), this may be due to the relatively small numbers of participants per group, or due these patients being non-symptomatic at the time of assessment. Further work may be able to identify whether the proportions of any Breg subsets differ at baseline, by comparison of grass pollen seasonal allergic patients in and out of pollen seasons, or with the use of perennial symptomatic allergics, but certainly with a larger subset of patients in each group.

More work is needed to examine and explain the possible loss of diurnal variation of B cell *numbers* throughout the day amongst untreated allergic donors that was observed on both unchallenged and challenge days amongst non-allergic individuals. As a non-challenge day was not carried for untreated allergic or AIT-treated allergic donors, it is unclear whether the increase in B cell numbers observed amongst AIT donors, not observed for allergics, was truly a diurnal change as for non-allergics, or an effect of NAC. Future work, should initially aim to repeat the non-challenge day amongst allergic patients (both treated and untreated), to fully determine whether there is a defect in the diurnal regulation of B cell numbers amongst untreated allergic individuals, which is restored amongst AIT-treated patients. Further work would then be required to understand the mechanism underlying diurnal regulation of B cell numbers, and how this has been recovered amongst AIT-treated allergic donors. However, the functional relevance of this finding is as yet unclear. Further work may be able to examine whether IL-10-producing B cells are stable as a proportion of the B cell population throughout the day and throughout the pollen season. One possible explanation for a loss of diurnal regulation of global B cell numbers in allergic individuals could be a reduced sensitivity to endogenous cortisol, although this hypothesis would require investigation

The increased *proportions* of $CD24^{hi}CD38^{hi}$ B cells (relative to the whole B cell population) observed following allergen challenge amongst non-allergics or tolerant AIT-treated allergics, but not untreated allergics, is of potential interest. Ideally this could be reconfirmed in relation to natural pollen exposure by the taking of blood samples before, during and following the grass pollen season. If $CD24^{hi}CD38^{hi}$ B cells are indeed proportionally increased during the pollen season amongst naturally

or AIT-tolerised donors compared to outside the pollen season, and in contrast to non-tolerant allergic donors, this subset may be proposed as an *in vivo* mechanism of tolerance. Whether these cells are being mobilised in order to migrate to the nasal mucosa, draining lymph or other secondary lymphoid disuse is unclear and requires further exploration.

Histological evaluation of nasal samples from the cross sectional study was limited by the paucity of dual-stained cells that could be detected within the nasal mucosa. Sensitivity could likely be increased by detection of IL-10 mRNA rather than protein in B cells by the use of dual in situ hybridisation and immunohistochemistry, as previously shown for detection of IL-10-producing T cells during AIT (Nouri-Aria et al., 2004). Such an approach is planned in relation to a prospective trial of sublingual grass pollen immunotherapy.

The demonstration that CpG combined with grass pollen enhances the proportions of IL-10-producing B cells is in line with data showing CpG as an adjuvant in both murine (Hessenberger et al., 2013, Huang et al., 2007, Tighe et al., 2000) and feline (Reinero et al., 2008) models of allergen immunotherapy. In these models the addition of CpG was more effective in inducing tolerance to allergen than administration of high-dose allergen alone. In human studies the focus has largely been on the potential Th1 polarising effect rather than induction of IL-10 production by CpG adjuvants. A series of studies have assessed safety and efficacy of CpG-conjugated to ragweed allergen for subcutaneous immunotherapy. Both suppression of immunological responses and symptom scores was observed compared to placebo treatment (Simons et al., 2004, Tulic et al., 2004, Creticos et al., 2006).CpG has shown promise in early clinical studies as an adjuvant for house dust mite immunotherapy (Senti et al., 2009), or as a therapy alone in the absence of allergen (Klimek et al., 2011). None of these studies have compared AIT with allergen alone to AIT carried out with allergen conjugated to CpG in order to appreciate whether CpG provides an additive effect nor have these studies examined the B cell compartment or IL-10 synthesis in detail following CpG-conjugated immunotherapy. This fast-developing area of allergen immunotherapy would do well to explore the comparative immunotype provided by the addition of CpG adjuvants as well as the potential CpG-conjugated allergen given by alternative routes such as sublingual AIT.

5. Summary and concluding remarks

Regulatory B cells (Bregs) that are able to regulate inflammatory responses, have largely been defined by their capacity to produce IL-10. Bregs have been shown to interact with and induce Tregs, the master regulators. The dysregulation of Bregs amongst individuals with autoimmune diseases suggest that these cells contribute to regulation of self-tolerance. Research on Bregs has largely explored diseases such as autoimmunity, cancer, graft versus host disease and infection, with little reported about the role of Bregs in allergic disease.

A large body of evidence now exists to suggest that IL-10-producing Bregs are detected amongst many B cell phenotypes. A recent study described two clear subsets for IL-10-Bregs, the transitional $CD24^{hi}CD38^{hi}$ subset as well as the $IgM^{+}CD27^{+}$ memory population (Khoder et al., 2014), which may condense some of the differing subsets of IL-10-Bregs reported. The identification of Bregs at the transitional stage of B cell development and within the B cell memory pool suggests that Bregs exist at checkpoints of B cell inflammatory progression. For example, transitional or memory Bregs may provide a regulatory threshold prior to the development of a GC response or prior to the activation of a memory response, respectively. Furthermore, murine evidence that IL-10-producing plasma cells can be detected *in vivo* (Shen et al., 2014, Neves et al., 2010) or that amongst mice and humans IL-10-producing B cells can develop into plasma cells (Maseda et al., 2012, van de Veen et al., 2013) suggests that IL-10-Bregs are present throughout the B cell lifecycle and may be relevant in all diseases in which humoral immunity is dysregulated.

The first results chapter of this thesis (section 3, p66 onwards) set out to explore methods of identifying the greatest proportions of IL-10-producing B cells, and following this, explore the surface markers of B cells which were $IL\text{-}10^{+}$ and $IL\text{-}10^{-}$ under the same stimulatory conditions. Following on from this, IL-10-producing B cells were to be used in T cell co-cultures to show B cell mediated suppression of allergic inflammation *in vitro*. The second results chapter (section 4, p107 onwards) aimed to use allergic rhinitis as a model for a loss of immunological tolerance, with Bregs hypothesised to be dysregulated in order to permit chronic allergic responses to innocuous environmental antigens. Allergic rhinitic donors treated with allergen immunotherapy were used as a model of induced immunological tolerance, within which it was hypothesised Bregs may be induced.

Similar to previous reports, the TLR9 ligand CpG showed the greatest capacity to increase the proportions of IL-10-producing B cells beyond those detected in medium alone. Whilst some investigators have shown a similar response with the TLR4 ligand LPS by human B cells, this study showed an LPS-induced IL-10 response amongst B cells cultured with PBMCs but not in isolation.

Whilst CpG demonstrated greater proportions of IL-10-producing B cells compared to medium alone, these were highly correlated, suggesting CpG is able to enhance the proportions observed in medium and demonstrate the greatest potential B cell IL-10 capacity. The greatest proportions of IL-10-producing B cells were detected within the $CD24^{hi}CD38^{hi}$ and $CD5^{hi}$ subsets. The $CD27^{+}$ subset did not show greater proportions of B cells producing IL-10 compared to the whole population, suggesting here that naïve B cells are a more likely regulatory B cell subset. The identification of IL-10^{+} B cells as naïve was supported by their chemokine receptor expression compared to IL-10^{-} B cells. The chapter concludes by demonstrating that CpG-primed B cells suppress both polyclonal and allergen-stimulated T cells in co-culture, as compared with non-primed B cells. Suppression of both proliferation and pro-inflammatory supernatant cytokine concentration was observed, which could not be attributed to altered proportions of $FoxP3^{+}$ Tregs or T cell death. The addition of blocking antibodies throughout co-culture demonstrated that IL-10 or TGF-β, or the expression of PD-L1, by CpG-primed B cells may be factors able to drive T cell suppression in this context.

The chapter exploring Bregs *ex vivo* (p107 onwards) used CpG stimulation as a method of detecting the greatest proportion of B cells capable of IL-10 production. B cells from grass pollen allergic donors were stimulated and proportions of IL-10-producing B cells compared to non-allergic controls and AIT-treated allergic donors. CpG-induced IL-10-Bregs were not significantly altered amongst the allergic group compared to non-allergic controls, nor were Breg subsets altered at baseline when examined prior to nasal allergen challenge. However, trends were observed towards reduced IL-10 mRNA and allergen-induced proportions of IL-10-Bregs amongst allergic donors compared to non-allergic controls. This suggests that B cell transcriptional control of IL-10 or allergen-specific IL-10 responsiveness may be dysregulated in allergic rhinitis, although further work is required to confirm this. A report examining B cells amongst beekeepers and bee sting-AIT-treated patients also showed B cell IL-10 responses to be allergen-specific (van de Veen et al., 2013). AIT in this study showed a suppression of subjective and objective clinical measures, with both SCIT and SLIT demonstrating similar clinical tolerance. IL-10-Bregs were increased amongst the AIT population compared to untreated AR, particularly for the SLIT-treated donors. The sublingual route of administration may be able to induce differing immunological tolerance, although due to the confounding effects of gender and duration of treatment in the small cohort studied, it was not possible to confirm this. None-the-less, the proportions of IL-10-Bregs amongst treated and untreated AR groups showed significant correlation with clinical surrogate endpoints, namely the early and late responses to intradermal allergen challenge, that were inhibited after AIT. This provides support for the concept that AIT-induced IL-10-Bregs may play a role *in vivo* in the induction of allergen-induced tolerance. Further work is required to explore the relationship between Tregs, IgG4 and Bregs in larger prospective

controlled trials of AIT by both sublingual and subcutaneous routes. Exploration of the effect of a nasal allergen challenge on Breg subsets in peripheral blood showed that whereas proportions of several Breg subsets increased amongst non-allergic donors, only $CD24^{hi}CD38^{hi}$ B cells increased additionally amongst AIT-treated allergic donors, occurring in similar proportions as observed in untreated allergics. No other Breg subset was altered by proportion amongst allergic donor groups. Further work will be required to determine whether these cells were mobilised in response to nasal allergen to migrate to the site of inflammation. B cells were also explored by immunohistochemistry of human tonsil as well as in nasal biopsies following allergen challenge. The presence of $IL\text{-}10^{+}$ B cells and plasma cells was confirmed in tonsillar tissue. Dual IL-10 and CD20 staining was not observed in nasal sections, despite evidence of plasma cells co-staining with IL-10. Further studies in humans, simultaneously exploring blood, nasal tissue and bone marrow might identify niches in which various IL-10-Breg subsets and, possibly, IL-10-plasma cells might exist *in vivo* and their relative abundance.

Overall, the data presented here demonstrates that B cells are able to regulate allergen-specific T cell responses from allergic individuals, even at very low relative numbers. This population may be preferentially induced following AIT, particularly during long-term SLIT. This work supports the concept that strategies which aim to modify conventional allergen immunotherapy to target the expansion of an allergen-specific or bystander Breg populations may be a rational approach to optimise the induction of long term tolerance in allergic disease.

6. References

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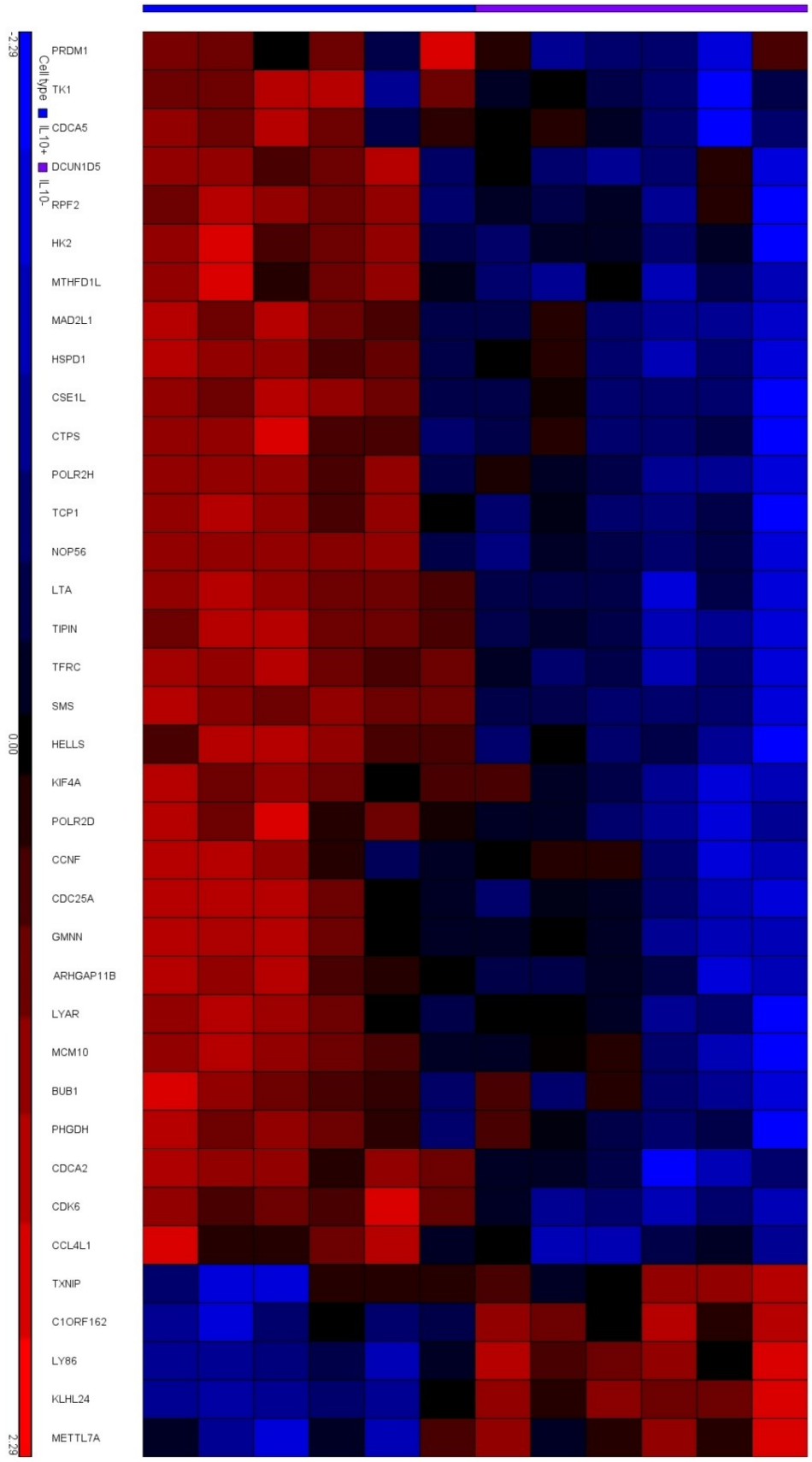
7. Appendix



Appendix Figure 1 – Heatmap of 37 significantly up or down regulated genes between IL-10$^+$ and IL-10- B cells sorted for RNA microarray.

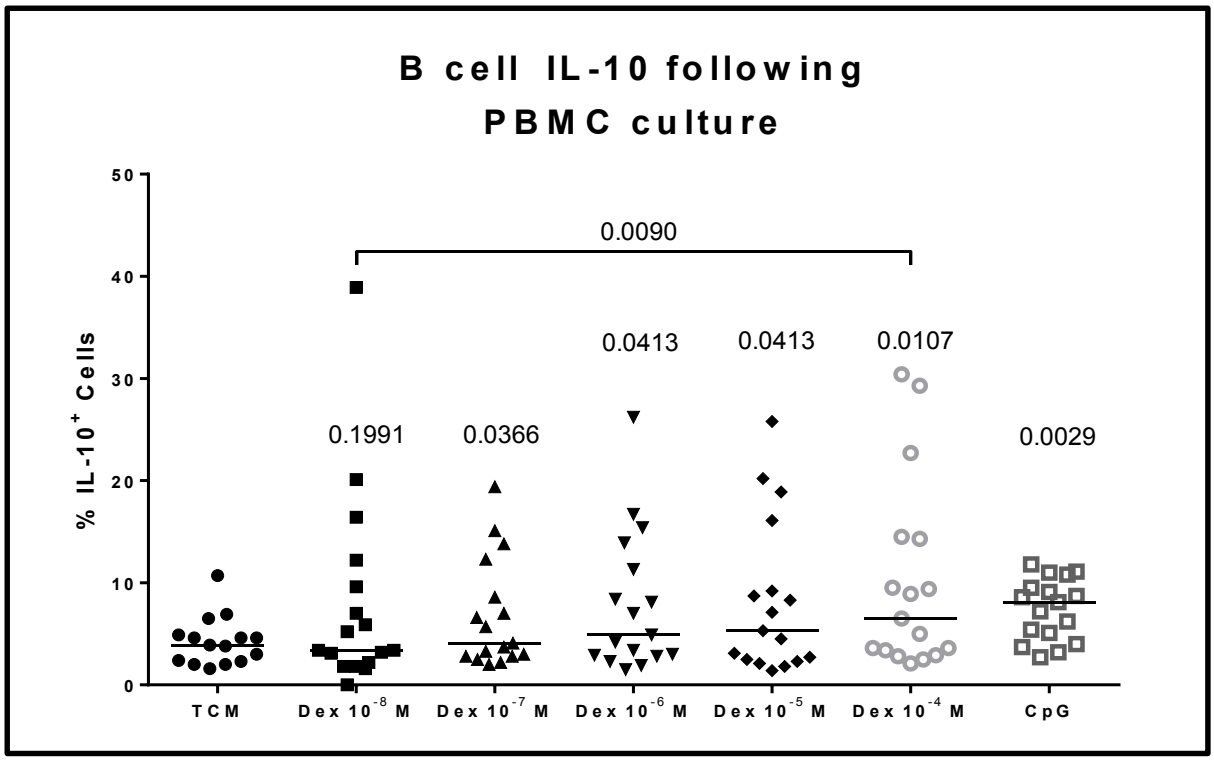
This is included for completeness as the experiment was carried out, showing gene changes supported by the literature. However, IL-10 was not returned as significantly up regulated amongst IL-10$^+$ B cells, so further optimisation is required, which was outside the time constraints for this thesis.

Gene	Common Name (Relevance)	p-value	Fold Change	Direction of change
CCL4L1		0.000138758	2.39496	IL10- down vs IL10$^+$
TFRC	**CD71**	**0.000126122**	**2.17668**	**IL10- down vs IL10$^+$**
LTA	**Lymphotoxin alpha (TNFβ)**	**0.000195827**	**2.0813**	**IL10- down vs IL10$^+$**
MCM10		0.000328083	1.94421	IL10- down vs IL10$^+$
PHGDH		0.000236381	1.78204	IL10- down vs IL10$^+$
CDC25A		7.99E-06	1.68794	IL10- down vs IL10$^+$
CDCA5		0.000235005	1.67023	IL10- down vs IL10$^+$
GMNN		2.12E-06	1.66839	IL10- down vs IL10$^+$
LYAR	Ly 1 antibody reactive	0.000217046	1.66726	IL10- down vs IL10$^+$
ARHGAP11B	Rho GTPase activating protein 11B	0.00026561	1.66062	IL10- down vs IL10$^+$
MAD2L1		0.000263515	1.6515	IL10- down vs IL10$^+$
NOP56		3.91E-06	1.64958	IL10- down vs IL10$^+$
CDK6		0.000368062	1.63493	IL10- down vs IL10$^+$
CTPS		1.73E-05	1.61842	IL10- down vs IL10$^+$
HSPD1	Heat shock 60kDa protein 1	0.000101394	1.60104	IL10- down vs IL10$^+$
DCUN1D5		0.000161499	1.58341	IL10- down vs IL10$^+$
SMS	spermine synthase	0.000170161	1.56941	IL10- down vs IL10$^+$
BUB1		6.30E-05	1.56863	IL10- down vs IL10$^+$
HELLS	helicase, lymphoid specific	0.000126154	1.56671	IL10- down vs IL10$^+$
KIF4A	kinesin family member 4	0.000204483	1.55789	IL10- down vs IL10$^+$
PRDM1	**BLIMP-1**	**8.98E-05**	**1.54996**	**IL10- down vs IL10$^+$**
TIPIN	TIMELESS interacting protein	4.39E-05	1.54108	IL10- down vs IL10$^+$
RPF2		0.000222218	1.54103	IL10- down vs IL10$^+$
CDCA2		0.000327091	1.53225	IL10- down vs IL10$^+$
POLR2H	polymerase (RNA) II (DNA directed) polypeptide H	0.000156648	-1.52933	IL10- down vs IL10$^+$
CCNF	Cyclin F	6.04E-05	-1.52456	IL10- down vs IL10$^+$
TCP1	t-complex 1	0.000110687	-1.52099	IL10- down vs IL10$^+$
HK2	hexokinase 2	0.000341117	-1.52044	IL10- down vs IL10$^+$
CSE1L	chromosome segregation 1-like (yeast)	1.03E-05	-1.51793	IL10- down vs IL10$^+$
TK1	Thymidine kinase 1, soluble	0.000271782	-1.51573	IL10- down vs IL10$^+$
POLR2D	polymerase (RNA) II (DNA directed) polypeptide D	0.000229616	-1.50867	IL10- down vs IL10$^+$
MTHFD1L		5.15E-05	-1.50302	IL10- down vs IL10$^+$
LY86	(RP105-associated (TLR2/4))	0.000141767	1.57105	IL10- up vs IL10$^+$
METTL7A	Methyltransferase Like 7A	1.84E-05	1.59445	IL10- up vs IL10$^+$
TXNIP	Thioredoxin Interacting Protein	0.000245581	1.71844	IL10- up vs IL10$^+$
KLHL24	Kelch-Like Family Member 241	9.35E-05	1.76759	IL10- up vs IL10$^+$
C1orf162	chromosome 1 open reading frame 162	0.000256972	1.79032	IL10- up vs IL10$^+$

Appendix Table 1 – Spreadsheet of 37 significantly up (red) or down (blue) regulated genes between IL-10$^+$ and IL-10- B cells sorted for RNA microarray.

RNA affymetrix microarray was carried out following a sort and lysis of 6 IL-10$^+$/- B cells from n=4 non-allergic and n=2 allergic donors. Two-way ANOVA was carried out against donor (as a random effect) and cell type (IL-10$^+$ or IL-10-). Genes shown are greater than 1.5 fold changes (shown), with a p value <0.05 following a false discovery rate (FDR) exclusion. Common gene names are shown where relevant. Rows in bold are highlighted for interest.

This figure is not included in the main body due to optimisation required, as stated in appendix fig. 1, due to the lack of significant changes in IL-10 mRNA detected and time constraints to fully optimise.



Appendix Figure 2 – Increasing concentrations of Dexamethasone increase proportions of IL-10-producing B cells from PBMC cultures.

PBMCs (n=17, NA=6, AR=9, AIT=2) were stimulated for 48 hours in the conditions shown, with proportions of IL-10-producing B cells detected by flow cytometry. Wilcoxon matched-pairs signed-rank test was used to compare differences following significant Friedman test ($p<0.0001$).

Analyte	T cells unstimulated	T cells Stimulated	Stimulated T cells + un-primed B cells	Stimulated T cells + CpG-primed B cells	P value (un-primed v.s. CpG- co-culture)
GM-CSF	0.89	1605.95	3674.01	891.91	**0.0156**
IFN-g	0.07	1070.54	1522.14	398.14	**0.0156**
MDC	42.03	245.01	11665.76	4185.32	**0.0156**
IL-13	0.30	477.22	4879.70	1277.86	**0.0156**
sIL-2Ra	1.85	510.01	2555.98	634.41	**0.0313**
IP-10	n.d.	453.16	7209.08	1639.17	**0.0156**
MIP-1a	5.00	1582.37	2124.81	1498.84	0.2188
MIP-1b	7.03	1042.23	1785.94	1874.19	0.8125
RANTES	20.74	1474.67	4541.93	1011.07	**0.0156**
IL-2	1.08	1.62	30.24	4.27	**0.0156**
IL-8	14.96	261.61	860.70	725.13	0.8125
TNF-a	1.16	262.14	1460.47	205.98	**0.0156**
TNF-b	0.18	32.42	849.85	20.63	**0.0156**
Fractalkine	11.06	33.63	116.64	78.38	0.4375
IL-10	1.20	31.13	111.91	177.16	0.0781
IL-12p40	1.76	16.92	138.97	45.52	**0.0156**
PDGF-AA	5.02	27.07	125.34	129.52	0.9375
sCD40L	n.d.	232.21	283.27	119.52	**0.0156**
IL-9	0.38	2.89	126.82	7.28	**0.0156**
IL-5	0.22	40.64	583.90	163.24	**0.0313**
IL-6	2.21	2.43	33.54	28.06	**0.0156**
MCP-1	44.61	27.52	36.48	75.34	0.4688
VEGF	n.d.	60.61	54.19	46.87	0.1250
PDGF-BB	n.d.	32.90	37.19	19.28	0.2500
G-CSF	4.27	27.51	46.86	40.97	0.2969
Flt-3L	1.33	21.55	56.47	34.51	**0.0313**
IL-3	0.21	10.48	58.93	15.42	**0.0156**
IL-9	0.38	2.89	126.82	7.28	**0.0156**
IL-17a	0.22	22.52	38.46	46.12	0.2969
EGF	5.40	9.96	25.35	17.65	**0.0156**
FGF-2	7.05	1.88	26.05	12.22	0.3125
TGF-a	n.d.	0.61	0.86	2.11	Too few pairs
IFN-a2	2.04	10.07	28.38	17.57	**0.0313**
GRO	5.27	10.94	29.47	13.27	**0.0156**
MCP-3	5.28	6.47	11.71	10.43	0.3750
IL-12p70	0.17	1.25	1.04	0.39	>0.9
IL-15	0.19	0.98	2.56	1.61	**0.0313**
IL-1ra	2.69	8.38	26.38	16.59	**0.0469**
IL-1a	0.33	5.61	43.25	12.22	**0.0156**
IL-1b	0.29	0.58	2.97	1.46	0.1406
IL-4	1.23	6.20	13.67	7.71	**0.0313**
IL-7	n.d.	7.05	16.46	10.35	**0.0313**

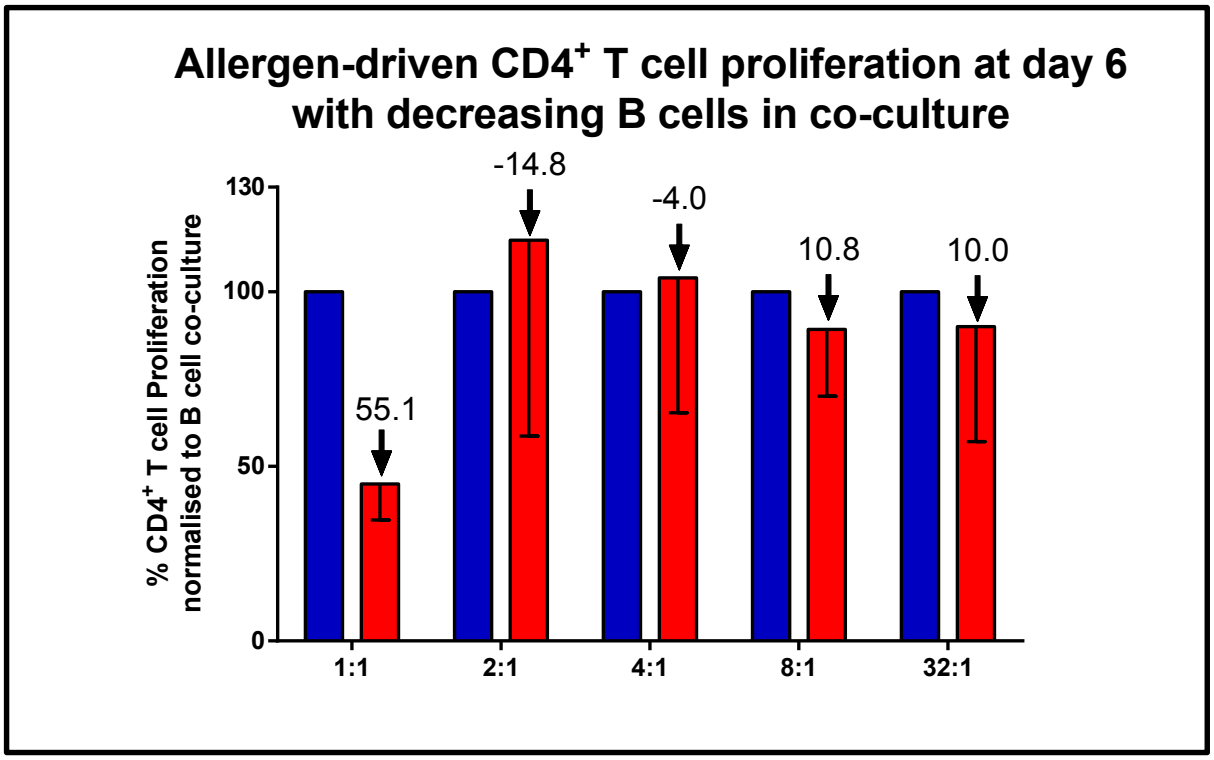
Appendix Table 2 – Median cytokine concentrations from polyclonally stimulated co-culture (pg/mL).

Detailed cytokine results (n=7: NA=4 AR=3) of T and B cell cultures (as described). 42plex multiplex ELISA was carried out, not all wells showed detectable cytokine; these were ignored for median concentrations above (pg/mL). Some conditions show no detectable cytokines at all (n.d.). P values are the result of Wilcoxon matched-pairs signed-rank test.

Analyte	T cells $^{+}$ APC unstimulated	T cells $^{+}$ APC Stimulated	Stimulated T cells $^{+}$ APC $^{+}$ un-primed B cells	Stimulated T cells $^{+}$ APC $^{+}$ CpG-primed B cells	P value (un-primed v.s. CpG- co-culture)
IL-17F	0.19	0.06	0.09	0.11	0.8203
GM-CSF	4.81	2.91	5.61	4.92	**0.0002**
IFNg	1055.71	332.68	2320.42	338.09	**<0.0001**
IL-10	21.93	16.07	68.66	125.04	**0.0092**
CCL20/MIP3a	67.67	16.50	28.43	12.23	**0.0420**
IL-12p70	4.06	2.66	4.33	3.08	**0.0443**
IL-13	2821.17	1500.62	5377.81	2154.09	**0.0003**
IL-15	17.22	15.79	8.76	9.94	**0.0479**
IL-17A	161.45	54.67	16.39	21.88	0.4887
IL-22	0.06	0.03	0.14	0.16	**0.0322**
IL-9	54.11	65.26	66.60	24.54	**0.0155**
IL-1b	2.91	6.31	3.35	1.98	0.6240
IL-33	2.04	1.73	1.58	1.43	0.9375
IL-2	16.09	5.64	39.43	23.17	**0.0034**
IL-21	26.89	20.52	24.93	14.82	**0.0322**
IL-4	0.10	0.19	0.08	0.06	**0.0256**
IL-23	0.26	0.24	0.17	0.10	0.1602
IL-5	261.92	262.16	485.85	208.80	**0.0027**
IL-6	26.55	16.64	85.65	82.14	0.5614
IL-25	n.d.	n.d.	n.d.	n.d.	-
IL-27	0.10	0.10	0.28	0.21	0.1230
IL-31	0.00	0.01	0.01	0.02	0.6250
TNFa	336.77	227.42	418.04	248.60	**0.0020**
TNFb	0.33	0.17	0.33	0.20	**0.0029**
IL-28A	0.02	0.01	0.02	0.03	>0.9

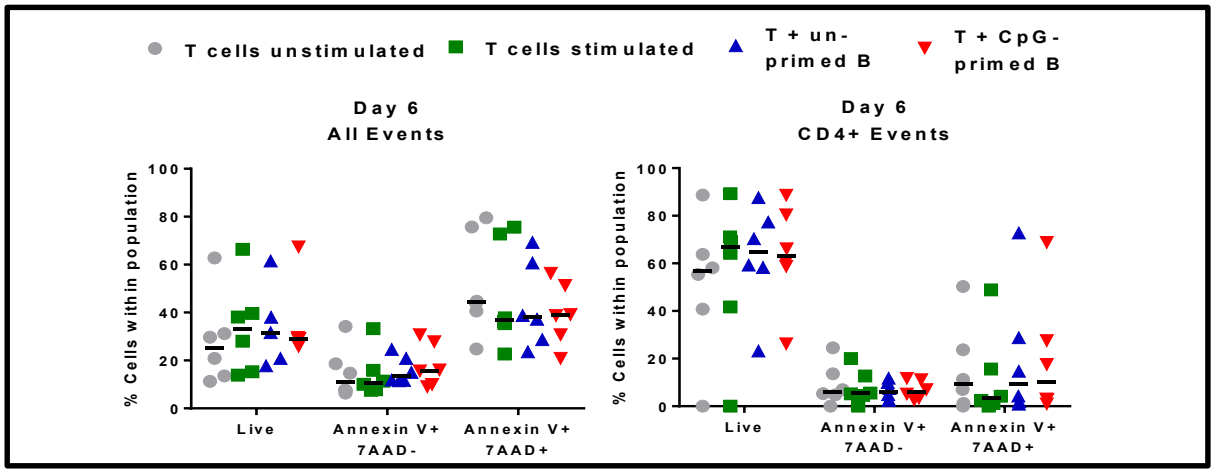
Appendix Table 3 – Median cytokine concentrations from allergen-stimulated co-cultures (pg/mL).

Detailed cytokine results (all NA, n=11 for all 25, n=16 for GM-CSF, IFNg, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-9, IL-2, IL-4, IL-5, IL-6, TNFa, TNFb from 42plex plate) of T and B cell cultures (as described), with irradiated non-CD4^{+} PBMCs used to present allergen. 25plex multiplex ELISA was carried out, not all wells showed detectable levels of cytokines, these were ignored to show median concentrations of wells above (pg/mL). If no wells showed detectable cytokines none detected is shown (n.d.). P values are the result of Wilcoxon matched-pairs signed-rank test.



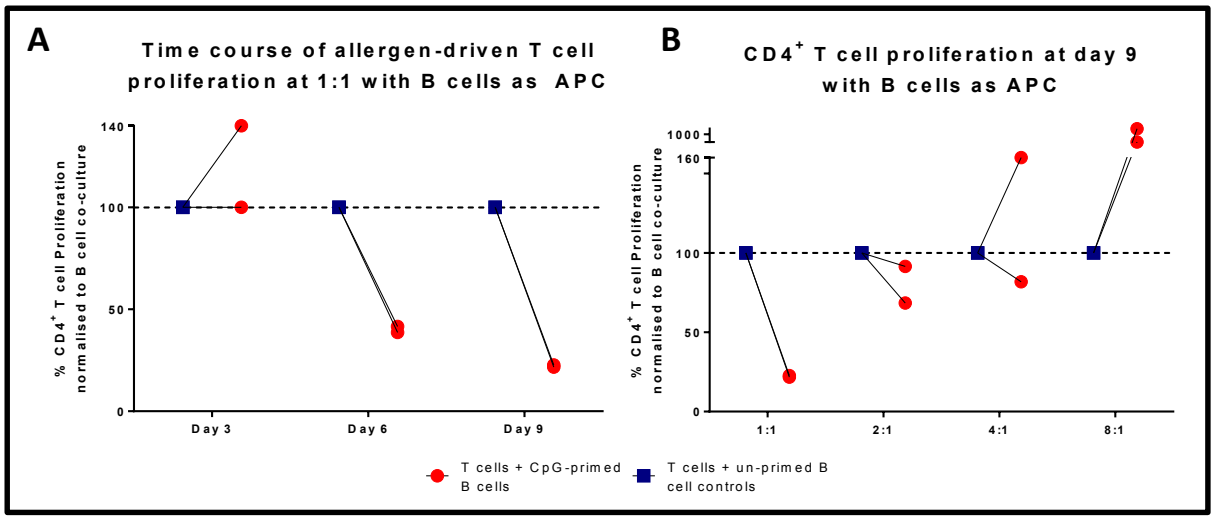
Appendix Figure 4 – Day six (6) CD4⁺ T cell allergen-driven proliferation in co-culture with B cells.

Percentages of Phl P stimulated T cells proliferating (with allergen presented by irradiated non-CD4⁺ PBMCs) following co-culture with un-primed (blue) or CpG-primed (red) B cells at day 6. T cell proliferation with un-primed B cells is shown as 100%, with relative proliferation of T cells with CpG-primed B cells shown as a percentage thereof (n=8 for all, AR) with ratios of T cells to B cells (T:B = 1:1, 2:1, 4:1, 8:1 and 32:1) shown.



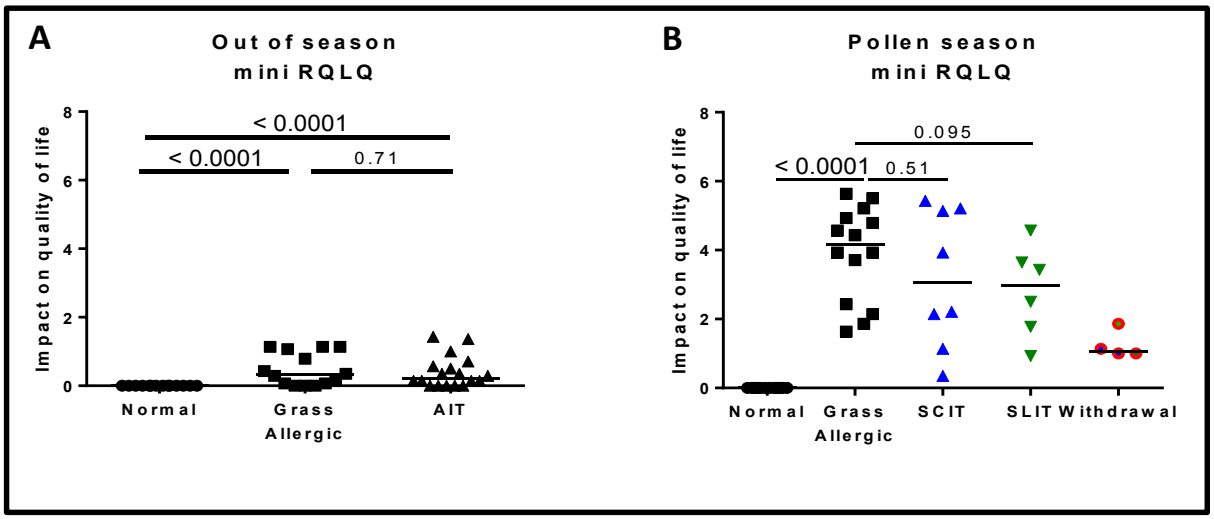
Appendix Figure 3 – Day six (6) cell death in T and B cell co-cultures, as measured by Annexin V and 7AAD.

T cells remained unstimulated (grey), stimulated with Phl P (green) or stimulated in the presence of un-primed B cells (blue) or CpG-primed B cells (red). Following 6 days of co-culture staining with 7AAD and Annexin V identifies live, dead and dying cells. This is quantified for all events (left) and for CD4⁺ events only (right).



Appendix Figure 6 - Allergen stimulated T cells co-culture with B cells win the absence of APCs.

PBMCs were isolated from allergic rhinitic donors (n=2) and T and B cells were isolated separately. T cells were preserved in culture unstimulated for 48 hours, whilst B cells remained in medium alone (blue) or stimulated with 1µg/mL CpG (red). B cells were washed and co-cultured with CFSE-labelled T in the presence of 5µg/mL of grass pollen allergen (Phleum pratense), APCs were not used to present antigen in this experiment. At days 3, 6 and 9 of co-culture cells were stained with antibodies towards CD4 and assessed for proliferation (**A**). Percentage suppression of proliferating T cells in co-culture with CpG-primed compared with un-primed B cells is shown. B cells were titrated compared to T cells (T:B cell ratios shown) and co-cultured for 9 days (**B**).



Appendix Figure 5 – Self-assessment quality of life data of cross-sectional study participants.

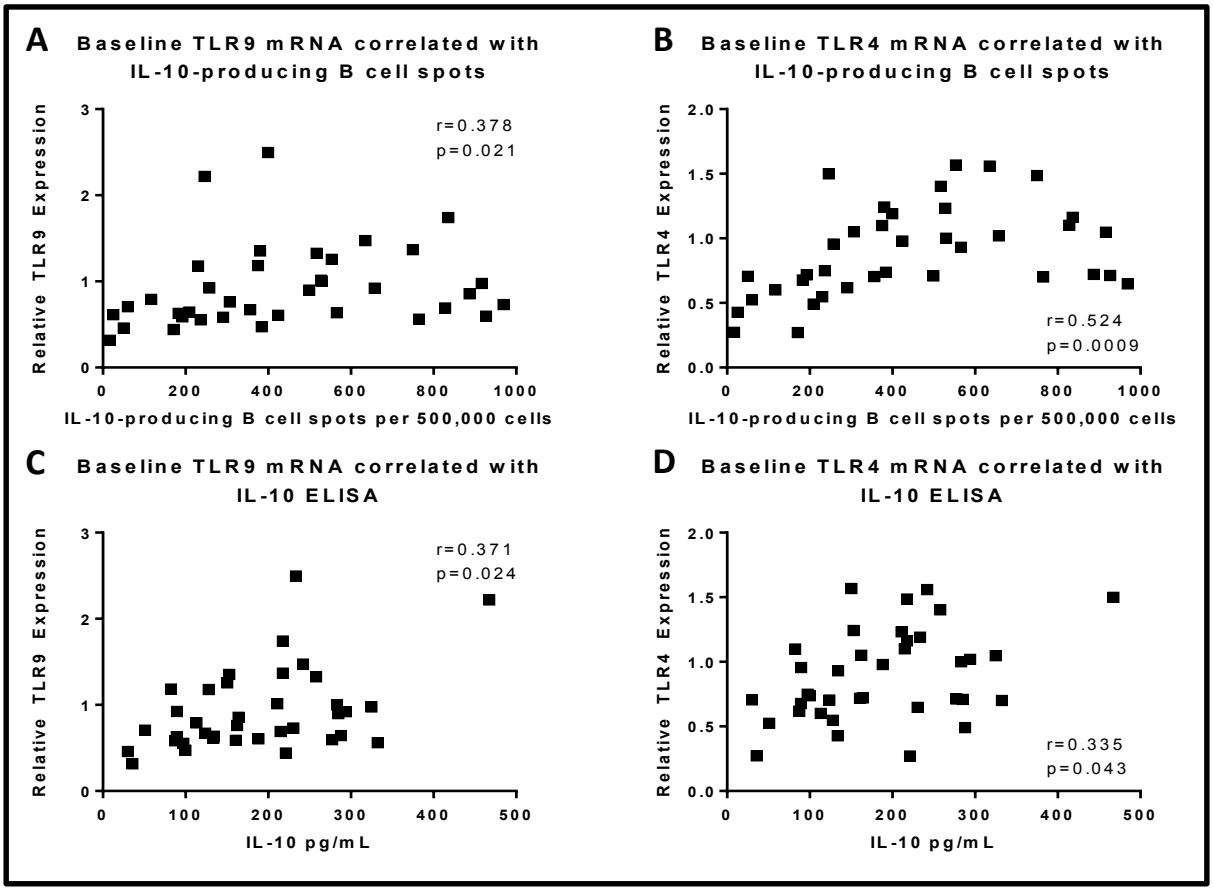
NA, AR and AIT donors (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT). Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) was used to assess patients' experience of symptoms both during (**D**) and outside (**C**) the pollen season (after treatment for AIT donors). Results of Mann-Whitney tests are shown.

	All samples	Normal controls	Grass Allergic	SCIT	SLIT	AIT Withdrawal	P Value
Number	45	14	13	8	6	4	N/A
GM-CSF	0.02 (0.016-0.025)	0.02 (0.014-0.031)	0.02 (0.016-0.025)	0.02 (0.016-0.026)	0.02 (0.016-0.026)	0.02 (0.013-0.029)	0.87
IFNγ	2.18 (1.56-3.12)	2.26 (1.56-3.16)	1.87 (1.56-2.96)	2.18 (1.41-2.49)	2.03 (1.18-3.28)	2.03 (1.41-2.89)	0.97
CCL20	2.86 (2.53-3.19)	2.98 (2.59-3.51)	2.78 (2.53-3.10)	2.65 (2.46-2.88)	2.96 (2.34-3.21)	3.00 (2.43-3.47)	0.55
IL-12p70	1.94 (1.46-2.66)	1.78 (1.39-2.62)	2.26 (1.46-3.23)	2.18 (1.70-2.58)	1.94 (1.66-2.62)	2.35 (1.35-3.85)	0.96
IL-13	2.83 (1.62-3.78)	3.31 (2.03-4.62)	2.43 (1.41-3.97)	2.83 (1.83-3.50)	2.22 (0.76-3.87)	3.00 (0.75-4.80)	0.85
IL-15	0.03 (0.015-0.278)	0.278 (0.0-0.339)	0.030 (0.0-0.278)	0.030 (0.030-0.216)	0.40 (0.023-0.584)	0.154 (0.030-	0.65
IL-17A	1.15 (1.04-1.21)	1.15 (1.04-1.18)	1.15 (1.04-1.21)	1.04 (0.92-1.24)	1.15 (1.12-1.30)	1.04 (0.95-1.21)	0.57
IL-22	0.011 (0.009-0.013)	0.012 (0.009-0.013)	0.010 (0.006-0.013)	0.010 (0.008-0.012)	0.010 (0.009-0.013)	0.013 (0.012-0.013)	0.31
IL-9	2.60 (2.31-2.79)	2.61 (2.33-2.87)	2.60 (2.28-2.72)	2.53 (2.38-2.77)	2.55 (2.14-2.92)	2.44 (2.13-2.80)	0.91

	All samples	Normal controls	Grass Allergic	SCIT	SLIT	AIT Withdrawal	P Value
IL-1β	Not	Detectable					
IL-33	0.635 (0.635-0.749)	0.749 (0.635-0.778)	0.635 (0.578-0.778)	0.635 (0.635-0.749)	0.635 (0.635-0.764)	0.749 (0.578-0.807)	0.37
IL-2	0.137 (0.0-0.42)	0.137 (0.103-0.418)	0.137 (0.0-0.347)	0.137 (0.034-0.348)	0.069 (0.0-0.418)	0.137 (0.137-0.559)	0.91
IL-21	6.78 (5.00-8.75)	6.19 (5.00-9.53)	7.17 (5.00-8.35)	6.38 (4.90-8.45)	7.37 (4.90-9.34)	5.59 (3.80-8.84)	0.93
IL-4	Not	Detectable					
IL-23	0.047 (0.039-0.047)	0.047 (0.045-0.047)	0.039 (0.037-0.056)	0.039 (0.039-0.047)	0.047 (0.039-0.054)	0.043 (0.039-0.060)	0.65
IL-5	1.28 (1.20-1.36)	1.28 (1.26-1.36)	1.20 (1.20-1.32)	1.24 (1.20-1.28)	1.28 (1.26-1.38)	1.28 (1.17-1.39)	0.47
IL-17e	Not	Detectable					
IL-27	0.10 (0.06-0.14)	0.08 (0.06-0.13)	0.09 (0.02-0.12)	0.09 (0.04-0.16)	0.14 (0.09-0.16)	0.12 (0.10-0.14)	0.43
IL-31	0.005 (0.005-0.006)	0.005 (0.004-0.006)	0.005 (0.004-0.007)	0.005 (0.004-0.006)	0.006 (0.005-0.008)	0.006 (0.005-0.007)	0.45
IL-17F	Not	Detectable					
IL-28A	Not	Detectable					

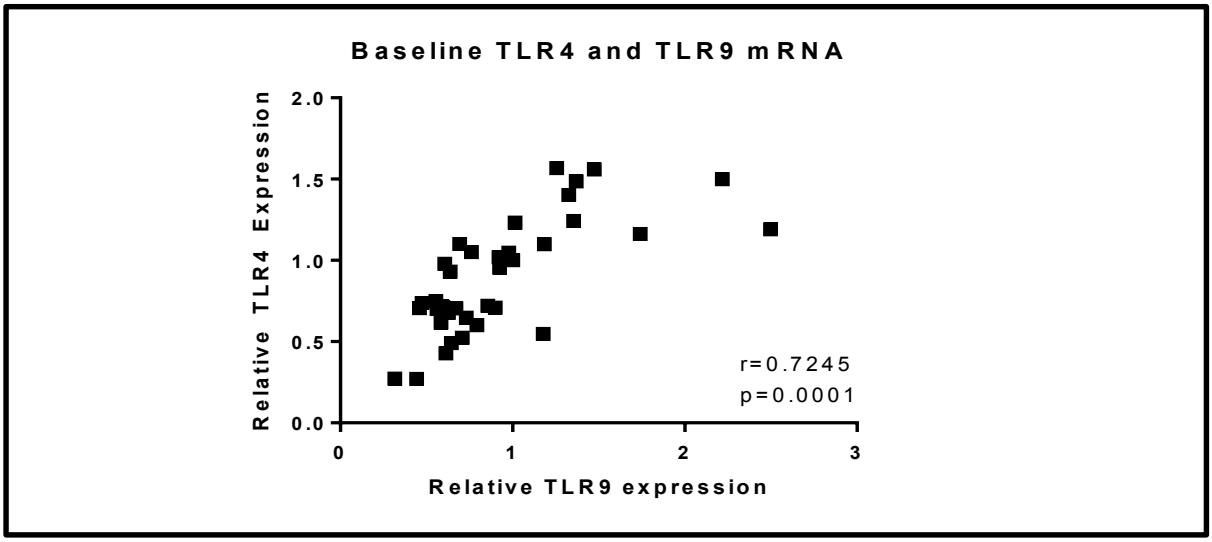
Appendix Table 4 – Additional cytokine concentrations data from CpG-stimulated B cells derived from cross sectional study of AIT.

25plex ELISA was carried out to determine the supernatant protein concentrations following CpG stimulation of isolated B cells between clinical groups in the cross sectional study. Median (IQR) are shown, P values represent Kruskal-Wallis tests.



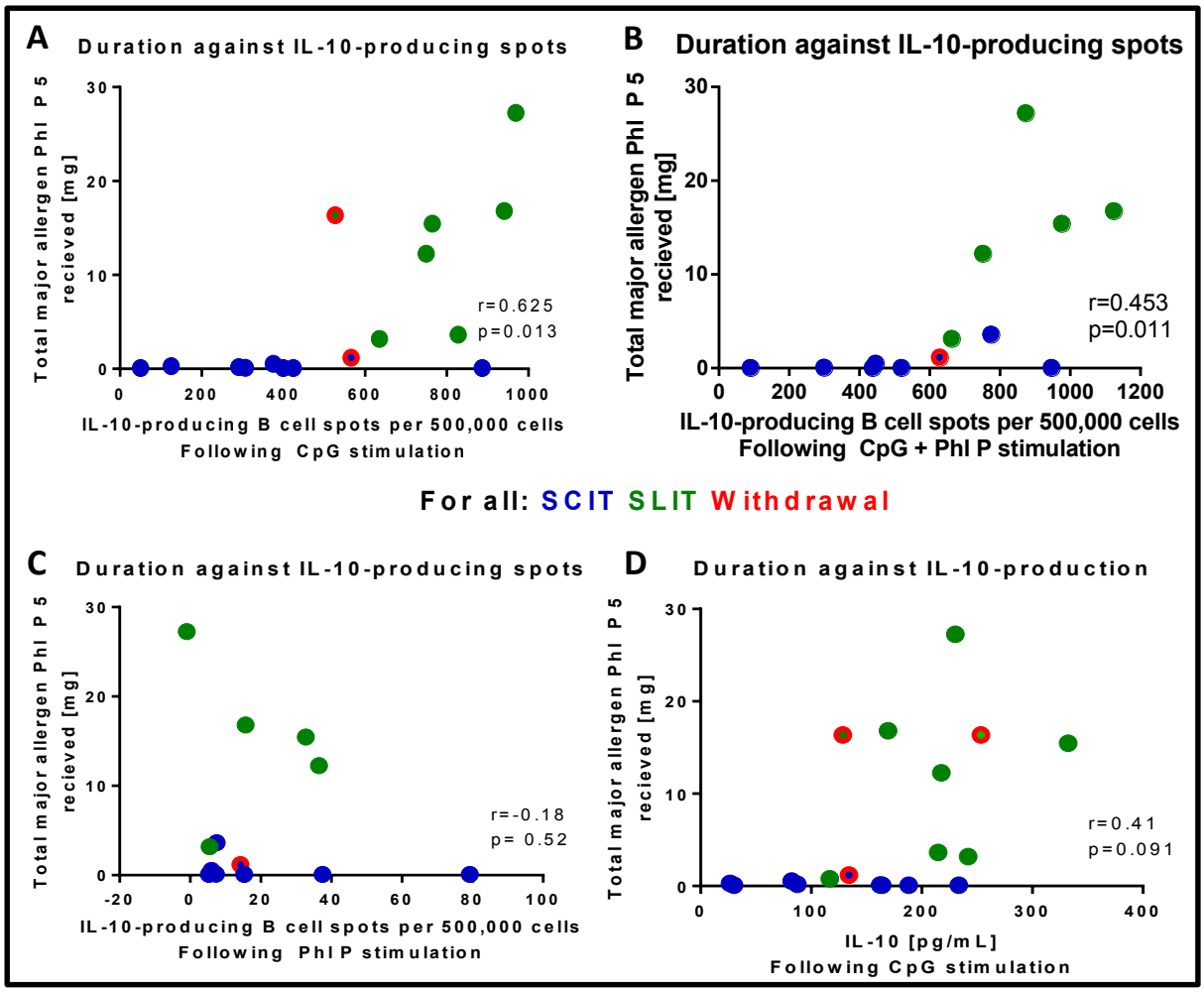
Appendix Figure 8 – Correlation of IL-10 FluoroSpot and ELISA to relative expression of mRNA at baseline.

Cross-sectional participants (n=37, NA=12 AR=12 SCIT=7 SLIT=5 withdrawal=1 –SCIT). B cells were isolated and lysed immediately or cultured for with CpG. IL-10-producing B cells were assessed by IL-10 FluoroSpot and supernatant IL-10 protein concentration established by ELISA. CpG-induced IL-10 spots are shown against relative expression of mRNA towards TLR9 (**A**) and TLR4 (**B**), whilst IL-10 concentration is also shown correlated to TLR9 (**C**) and TLR4 (**D**). Spearmen correlation is shown for each.



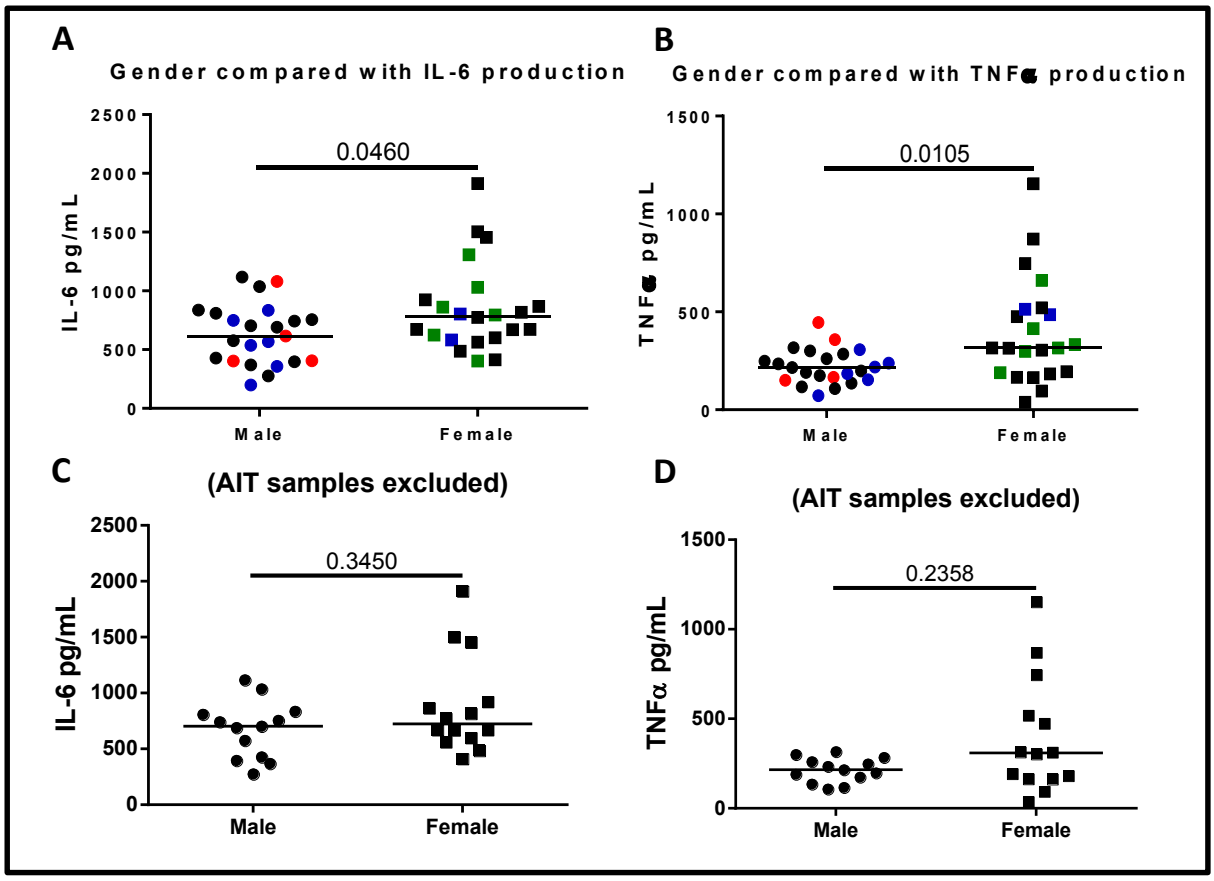
Appendix Figure 7 – Correlation of relative mRNA towards TLR4 and TLR9 in freshly isolated B cells.

Isolated B cells from cross-sectional donors were assessed for relative expression of mRNA towards TLR4 and TLR9; spearman rank correlation p and r values are shown.



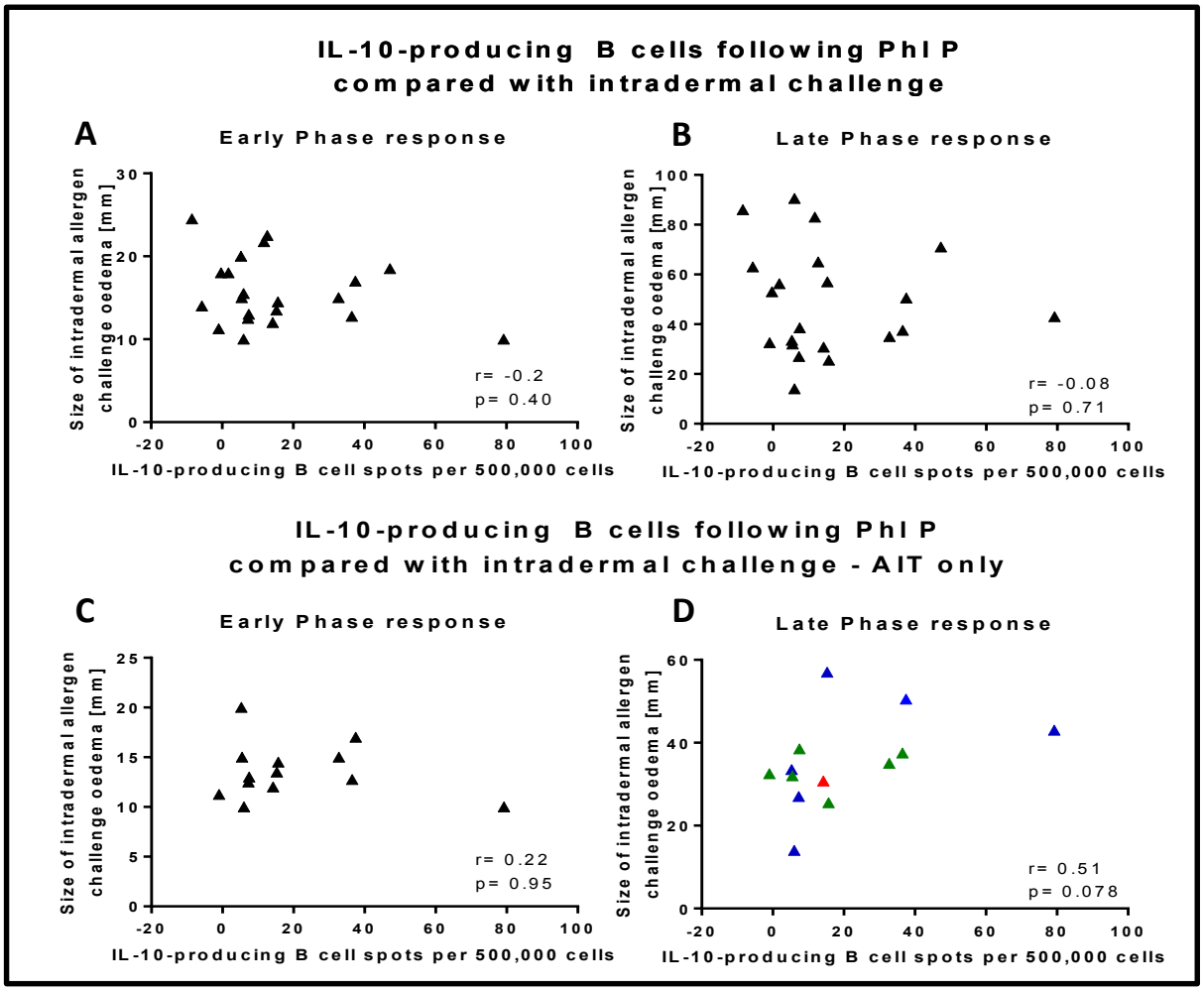
Appendix Figure 9 – Approximate cumulative dose of allergen received by AIT patients against proportions of IL-10-producing B cells and IL-10 concentration.

Participants received SCIT (blue), SLIT (green) or had completed a course of AIT (withdrawal - red). B cells were isolated and cultured for 42 or 48 hours with 1µg/mL CpG or for 42 hours with 5 µg/mL Phl P $^{+}$/- 1µg/mL CpG. B cells to be cultured for 42 hours were plated at 5x10^{5} cells per well on IL-10 FluoroSpot plates and IL-10 spots were counted. 48-hour cultured B cells had supernatant removed and IL-10 protein concentration was quantified. Numbers of IL-10 B cell spots were correlated with approximate total treatment cumulative allergen dose received against B cells stimulated with CpG alone (**A**), CpG $^{+}$ Phl P (**B**) or Phl P alone (**C**). IL-10 protein concentration following CpG stimulation is also correlated with cumulative allergen received (**D**).



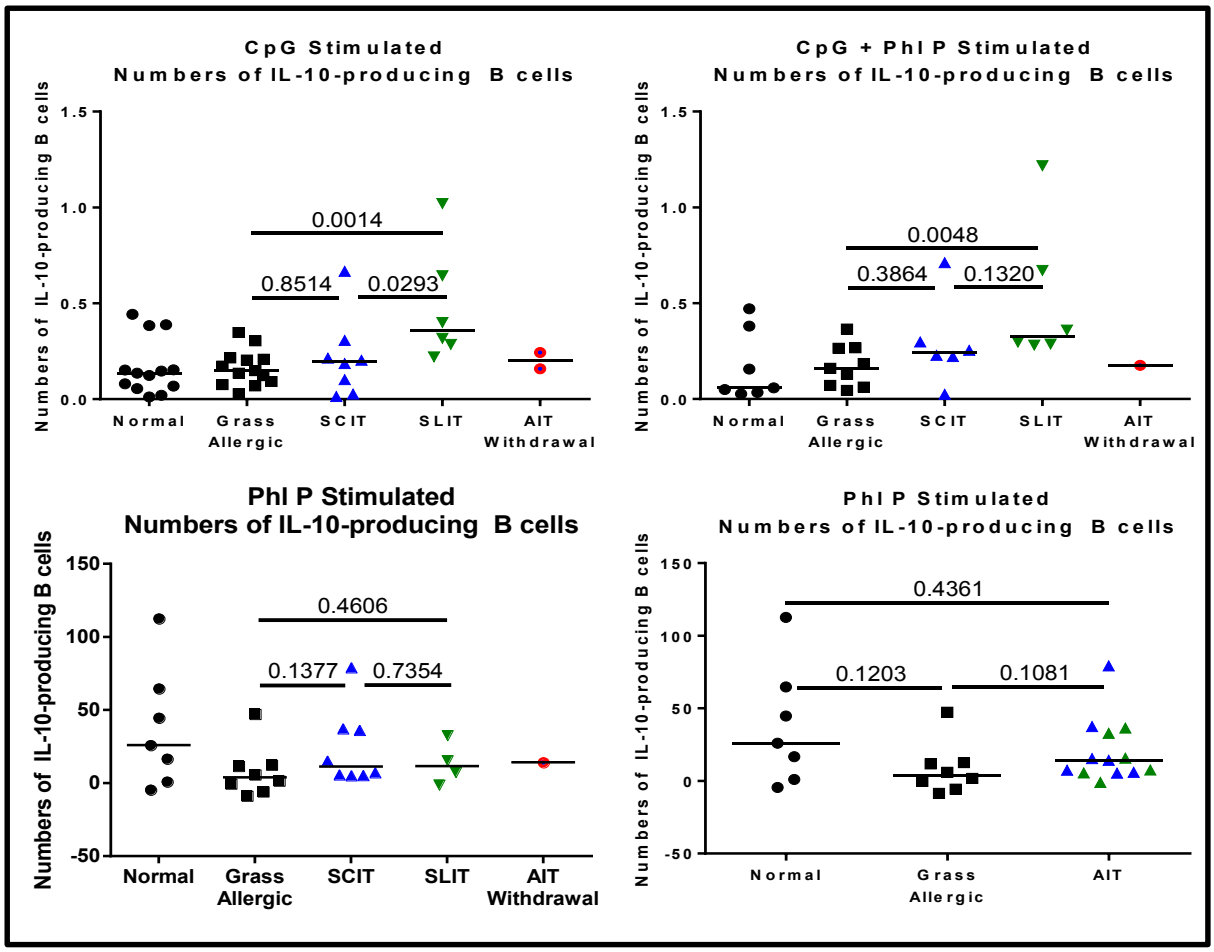
Appendix Figure 10 – Concentrations of IL-6 and TNFα in CpG-stimulated B cell supernatants by gender.

Concentrations of IL-6 (**A** and **C**) and TNFα (**B** and **D**) were established from isolated, CpG-stimulated, B cell supernatants by multiplex ELISA. These are stratified for all donors by gender (**A** and **B**) and also with AIT donors removed from analysis (**C** and **D**). Values shown are the resulting p values of a Man-Whitney U test between the groups.



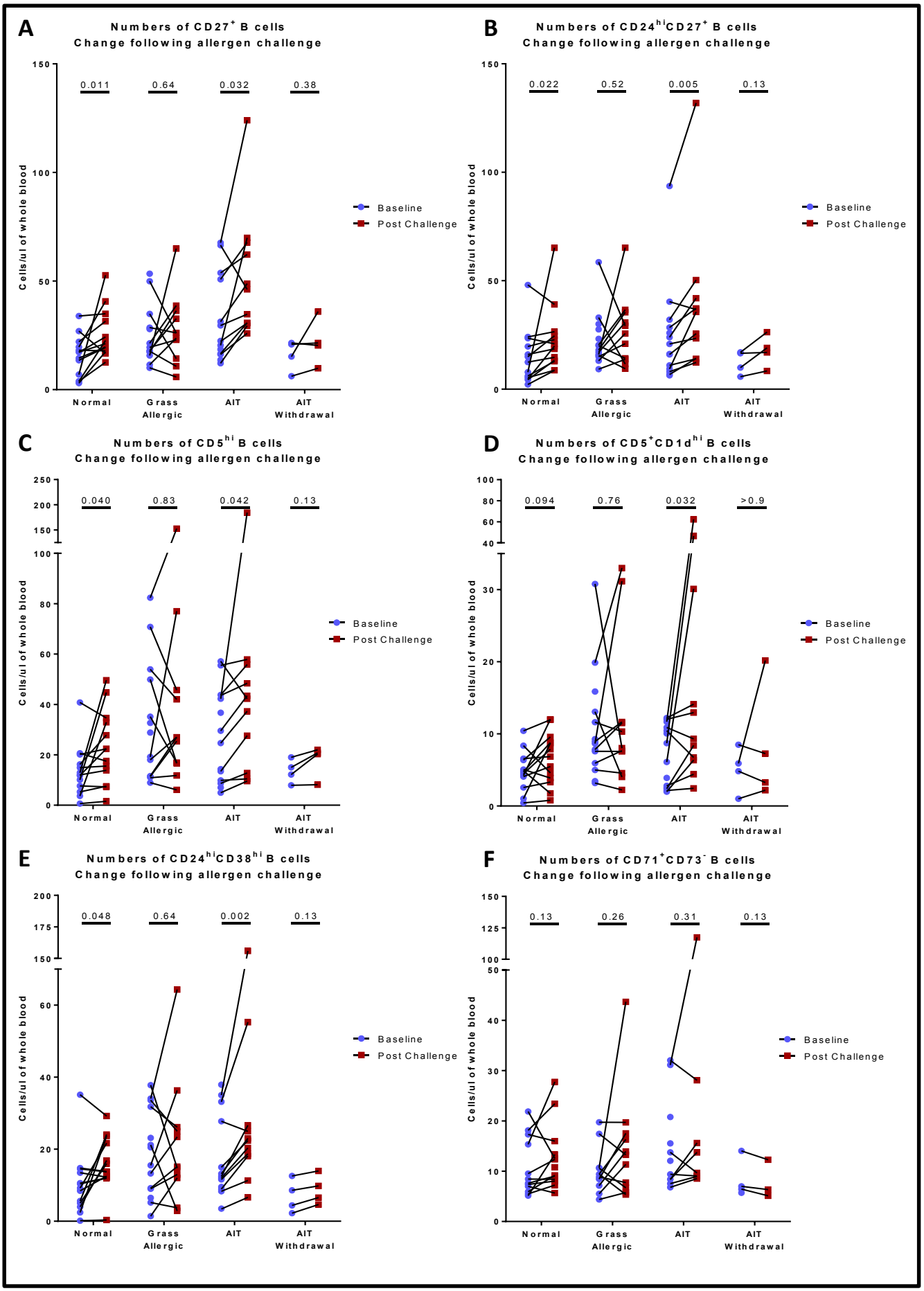
Appendix Figure 11 – Early and late phase responses correlated to allergen-induced IL-10 spots.

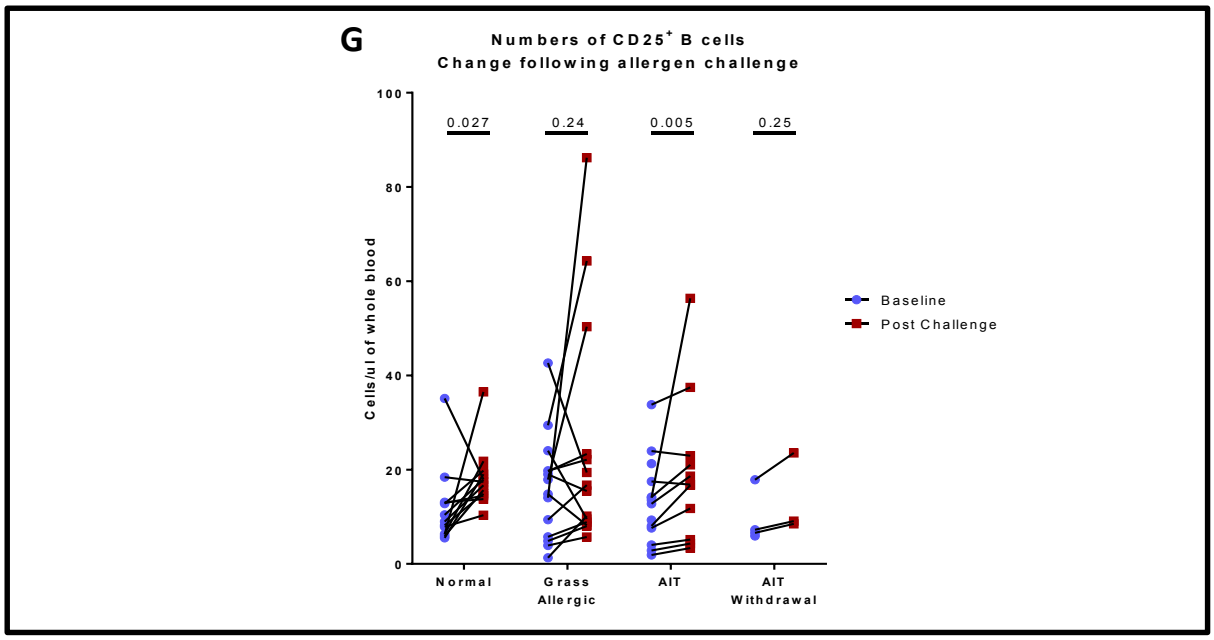
Wheal size (diameter, mm) following intradermal allergen challenge of 1BU at early (15 minutes, **A** and **C**) and late (8 hours, **B** and **D**) phase of responses amongst all allergics (A and B) or AIT-treated donors alone (**C** and **D**) is correlated to the proportions of IL-10-producing B cells in response to allergen stimulation alone. Spearman rank correlation p and r values are shown.



Appendix Figure 12 – Proportions of IL-10-producing B cells by FluoroSpot, adjusted by baseline numbers of peripheral B cells.

NA, AR, SCIT, SLIT or AIT completed (withdrawal) donor B cells were isolated for IL-10 capture FluoroSpot (n=43, NA=14, AR=13, SCIT=8, SLIT=6, withdrawal=2 – of which 1 had received SLIT and 1 SCIT – for CpG, fewer as shown). B cells were stimulated as shown and numbers of IL-10 B cell spots per 500,000 cells were calculated at 48 hours. This was then compared to matched whole blood staining for CD19, using counting beads, in which numbers of B cells per µl of blood had been calculated. Data shown is the numbers of B cells producing IL-10 per µl of blood, based on the numbers of B cells at baseline.


A
Numbers of CD27+ B cells
Change following allergen challenge
Cells/ul of whole blood
0.011
0.64
0.032
0.38
Normal
Grass Allergic
AIT
AIT Withdrawal
Baseline
Post Challenge
B
Numbers of CD24hiCD27+ B cells
Change following allergen challenge
0.022
0.52
0.005
0.13
C
Numbers of CD5hi B cells
Change following allergen challenge
0.040
0.83
0.042
0.13
D
Numbers of CD5+CD1dhi B cells
Change following allergen challenge
0.094
0.76
0.032
>0.9
E
Numbers of CD24hiCD38hi B cells
Change following allergen challenge
0.048
0.64
0.002
0.13
F
Numbers of CD71+CD73- B cells
Change following allergen challenge
0.13
0.26
0.31
0.13



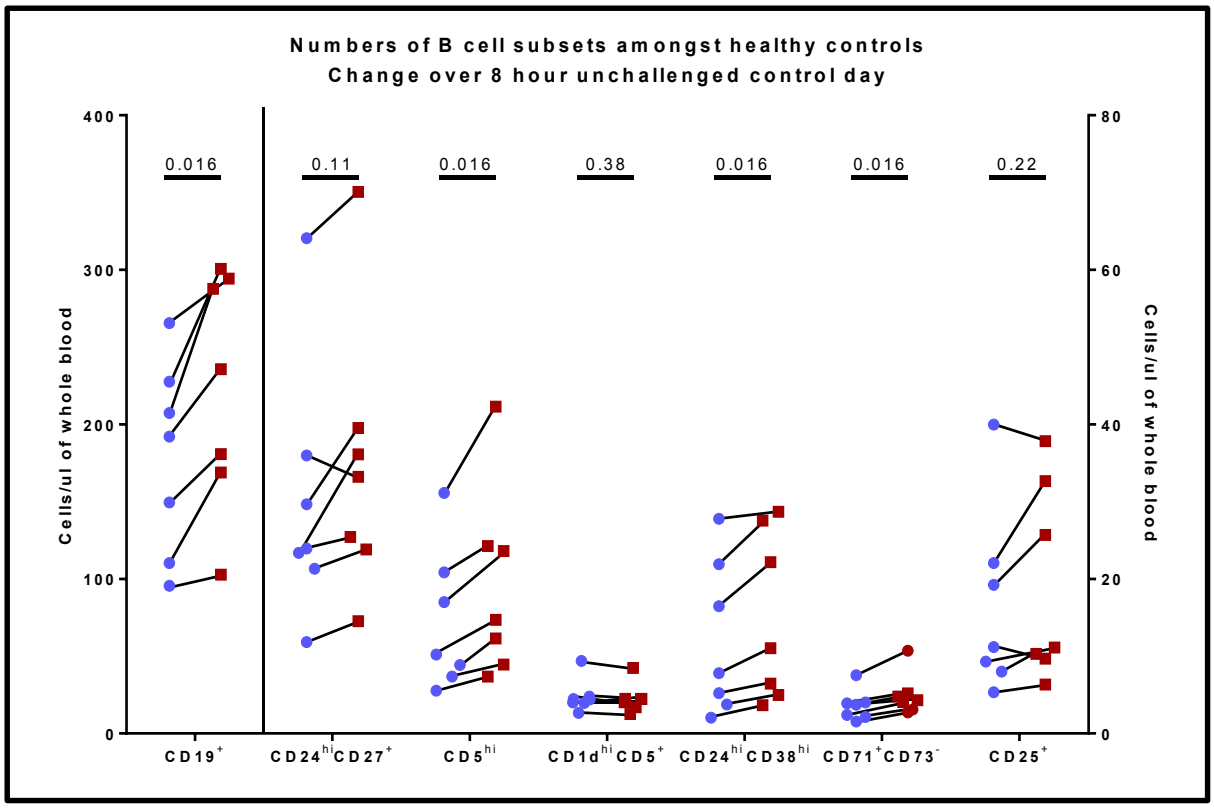
Appendix Figure 13 – Changes in B cell subsets after nasal allergen challenge.

NA, AR, SCIT, SLIT and AIT completed (withdrawal) (n=46, NA=14, AR=14, SCIT=8, SLIT=6, withdrawal=4 – of which 2 had received SLIT and 2 SCIT) volunteers underwent grass-pollen NAC. Blood was taken before and 8 hours following NAC. Whole blood was stained for flow cytometry and run with cell counting beads to quantify cell numbers relative to the amount of blood used. Numbers B cells within subsets are shown before (blue) and after (red) NAC. Numbers of $CD27^{+}$ (**A**), $CD24^{hi}CD27^{+}$ (**B**), $CD5^{hi}$ (**C**), $CD5^{+}CD1d^{hi}$ (**D**), $CD24^{hi}CD38^{hi}$ (**E**), $CD71^{+}CD73$ (**F**)$^{-}$ and $CD25^{+}$ (**G**) are shown per µl of whole blood.

Area under the curve for:	Normal controls	Grass Allergics	SCIT	SLIT	Withdrawal	p value
EPR – Change in peak nasal inspiratory flow (PNIF) area under the curve (AOC)	5.833 (-13.3-32.6)	-102.3 (-121.8--58.0)	-49.69 (-58.9--20.9)	-66.04 (-90.7--26.5)	-11.88 (-39.3-5.7)	<0.0001
LPR – change in PNIF, AOC	6.607 (-13.5-27.68)	-32.50 (-65.5- -8.5)	-16.61 (-21.6-25.0)	-20.00 (-49.6-2.05)	20.36 (6.2-33.8)	0.0048
VAS EPR+LPR – equally weighted	16.50 (-25.2-63.0)	-145.4 (-162.5--64.6)	-67.37 (-77.8-3.7)	-83.18 (-140.3--28.7)	8.482 (-31.1-37.4)	<0.0001
PNIF EPR+LPR Mann-Whitney p value compared to Grass Allergic	<0.0001	-	0.008	0.27	0.005	-
			SCIT + SLIT 0.016			

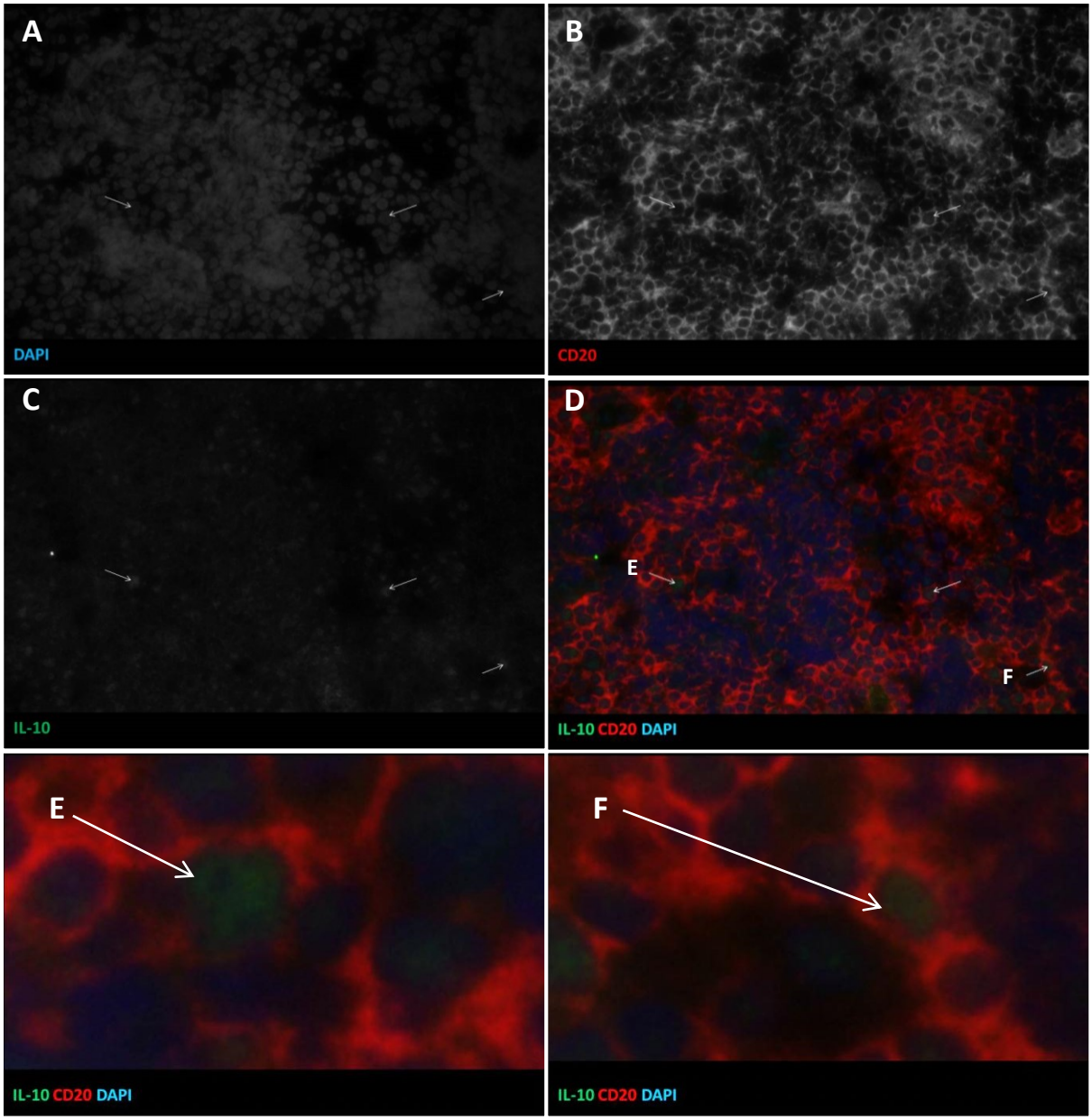
Appendix Table 5 – Changes in peak nasal inspiratory flow following nasal allergen challenge.

Patients were monitored before and throughout nasal allergen challenge for nasal obstruction by measuring peak nasal inspiratory flow (PNIF). Data shows change in PNIF from baseline over the first hour (EPR), during hours 1-8 (LPR) or both (equally weighted EPR+LPR). P value column shows Kruskal-Wallis test for data within the row. Mann-Whitney test p value is shown in the bottom row. Data courtesy of Dr Guy Scadding (Scadding, G.W. et al., unpublished data).



Appendix Figure 14 - Change in B cell subsets for non-allergic patients on an unchallenged day.

Non-allergic participants (n=7) donated blood at 0 (blue) and 8 hours (red) of an unchallenged control day to assess changes in B cell subsets. Whole blood was stained for flow cytometry and run in the presence of cell counting beads to assess absolute numbers of cells. Numbers of $CD19^{+}$ B cells per µL blood are shown along with numbers of $CD27^{+}$, $CD24^{hi}CD27^{+}$, $CD5^{hi}$, $CD5^{+}CD1d^{hi}$, $CD24^{hi}CD38^{hi}$, $CD71^{+}CD73^{-}$ and $CD25^{+}$ cells within the $CD19^{+}$ B cell population (**A**). Wilcoxon matched-pairs signed-rank p values are shown.



Appendix Figure 15 – Tonsil staining of IL-10 and CD20.

Sections of historical tonsil were stained with antibodies towards IL-10 (green) and CD20 (red) by 3-step immunohistochemistry. Cell nuclei are stained with DAPI (blue). Each colour image capture is shown with arrows in fixed positions for DAPI (**A**), CD20 (**B**), and IL-10 (**C**) staining alone or composite image (**D**). Digitally zoomed sections are also shown (**E** and **F**), identified by arrows in (**D**).