The Role of Surfactant Protein D in the Control of Human Helminth Infections

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Declaration

I, Zoe Baker, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree, in this or any other university.

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Zoe Baker

Feb 2020

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Abbreviations

°C	Degrees Celsius
AAM (M2)	Alternatively activated macrophages
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
APC	Antigen-presenting cell
ATII	Alveolar type II cell
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Centres for disease control
CLR	C-Type lectin receptor
CRTH2	Chemoattractant receptor-homologous molecule
CRD	Carbohydrate recognition domain
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescently activated cellular sorting
FBS	Fetal bovine serum
FMO	Fluorescence minus one
FITC	Fluorescein isothiocyanate
GIT	Gastrointestinal tract
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
H. polygyrus (Hp)	*Heligmosomoides polygyrus*
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IL-33R	Interleukin-33 receptor
IL-4rα	Interleukin-4 receptor alpha
ILC	Innate lymphoid cell
ILC2	Type 2 innate lymphoid cell
iNOS	Nitric oxide synthase
KO	Knockout
Lin	Lineage
MFI	Median Fluorescent intensity
MHC	Major histocompatibility complex
MR	Mannose receptor
N. americanus	*Necator americanus*
N. brasiliensis	*Nippostrongylus* brasiliensis
NET	Neutrophil extracellular traps
NK	Natural killer cells
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

PRR	Pattern recognition receptor
SAFFA	South African food allergy study
S. mansoni	*Schistosoma mansoni*
STAT	Signal transducer and activator of transcription
STH	Soil-transmitted helminth
rfhSP-D	Recombinant fragment human surfactant protein D
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCORAD	SCORing Atopic Dermatitis
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
TB	Tuberculosis
Th2	T helper 2 cell
TLR	Toll-like receptor
TGFβ	Transforming growth factor beta
T. muris	*Trichuris muris*
TSLP	Thymic stromal lymphopoietin
T. spiralis	*Trichinella spiralis*
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T-cell
W. bancrofti	*Wuchereria bancrofti*
WHO	World Health Organization
WT	Wild-type

Abstract

Lung produced surfactant protein D (SP-D) is essential for both homeostasis and as an innate immune opsonin. In the project presented here, we aimed to translate data recently published by our group, which demonstrated that SP-D contributes to protection against murine parasitic nematode infections, to human work. In the first part of this study, we determined whether individuals exposed to helminths have altered serum SP-D in comparison to unexposed individuals, through analysis (ELISA and Western Blot) of bio banked samples in 2 clinical cohorts from South Africa. Secondly, we aimed to identify if SP-D influences the magnitude of anti-nematode responses in human immune cells (type 2 innate lymphoid cells, monocytes and macrophages) through *in vitro* cell work and flow cytometry. Our findings indicated an association between serum SP-D and exposure to helminths that have a lung migration stage as part of their life cycle (*Ascaris* spp and *Toxocara* spp). Furthermore, *in vitro* analysis demonstrated that human immune cells primed with SP-D might have an altered response to helminth antigen. These findings point toward the need for further investigation into the novel role of SP-D in the control of human helminth infections in the context of immune physiology, as a biomarker and eventually treatment option.

Project rationale

In this study we use human serum samples to assess if there is an association between surfactant protein D (SP-D) and helminth infections in order to determine whether it could be used as a novel anti-helminthic treatment. Immunity is often modified with repeated exposure to various pathogens and in many instances, this leads to immune regulation, which allows commensal micro-organisms to avoid destruction by host immunity. This also appears to be the case for some helminth infections; long-lived infections are associated with potent parasite induced regulatory responses. Recent pre-clinical studies from our laboratory have linked the immunity and modulation of helminth infections with SP-D, a pulmonary collectin that interacts with mucosal immune effectors, most notably in the lung. SP-D is well understood as being able to both opsonize certain pathogens or to maintain tissue homeostasis depending on the immune environment. The SP-D induced modulation of helminth infection identified by the aforementioned study is a previously undescribed mechanism for pulmonary parasite control and showed raised SP-D levels enhances lung homeostasis and protects from immune pathology. In this study we explore the prospect of SP-D as a novel clinical treatment for soil transmitted helminth infections. This project focuses on determining whether an association between SP-D and immune markers of helminth infection (specific antibody isotypes) exists and what the exact mechanistic role of certain immune effector cells is and their potential interaction with SP-D, not only in the context of helminth infection but also in the broader terms of allergic responses.

1 Introduction

1.1 The immune system

Vertebrates live under the constant threat of invasion by harmful microorganisms and have evolved a system of immune defence that protects and eliminates pathogens from the body. Aside from the nervous system, it is the most complex system in the human body. Made up of different components variant in complexity, it functions in a few significant ways; pathogen neutralization, recognition of harmful substances, and autoimmunity towards the body's cells that change due to illness (i.e. cancers) [1].

The mammalian immune system comprises of two main branches: innate and acquired/adaptive immunity. **Innate immunity** is our first line of host defence and is mediated by phagocytes including macrophages and dendritic cells. **Acquired/adaptive immunity** is involved in the elimination of pathogens at a later stage of infections and is responsible for immunological memory [1]. Immune function is highly dependent on how these haematopoietic cells of the innate and adaptive arms of immunity interact in their microenvironments with non-haematopoietic stromal cells. Stromal cells comprise of a range of tissue forming cells (epithelial, endothelial, fibroblasts, nerve, etc.) that create the architecture and molecular cues for haematopoietic cell-cell interactions determining growth, differentiation and survival. In other words, the stromal network organization imposes order on innate and adaptive immunity driving efficient responses to pathogens [2].

In this body of work, we examine how our immune system, through the crosstalk of innate and adaptive arms interacts with parasitic nematode infections. To understand this complex interaction, we will first introduce the fundamental aspects of immunity and cellular crosstalk.

1.1.1 Innate immunity

The innate arm of immunity refers to non-specific host defences that are activated within hours of pathogen entry into the body. Discrimination between self and various pathogens happens through a limited number of pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) and mannose receptors; that is a type of C-type lectin

receptor, which recognizes conserved pathogen-associated molecular patterns (PAMPs) [3]. Recognition via PRRs leads to inflammatory pathway activation through both haematopoietic and non-haematopoietic cells of the innate arm. This, in turn, leads to the secretion of both cytokines (messenger molecules) and more specialized chemokines (a type of cytokine that directs specific cells to the site of the immune response). Innate immunity has various arms of functionality; physical barriers (skin, gastrointestinal tract, respiratory tract, cilia, and other body hair), defence mechanisms (secretions such as mucous, bile, gastric acid, saliva, tears, and sweat), and generalized immune responses (inflammation, complement, and non-specific cellular responses). The strength of innate immunity is in its non-specific short start-up phase in response to pathogens. This broad effect means its non-specificity limits its capabilities. Innate immunity's complex nature is woven into how humans have evolved over millennia. Here we broke down its functionality based on the major immune cell types at play:

Macrophages are large agranulocytes phagocytic cells found in the tissues that congregate at the site of infection. Depending on the environmental stimuli macrophages fall into two broad activation states; Classically activated macrophages are associated with Th1 responses which primarily engulf and destroy intracellular bacteria and viruses dependent upon inducible nitric oxide synthesis (iNOS) [4]. Alternatively, activated macrophages (AAMs) are rapidly recruited to the sites of helminth infection during Th2 immune response [5-7] triggered by IL-4, IL-13 and IL-10 [4, 8]. Their primary functions during helminth infection involve the down-regulation of Th1 response through IL-10 [9] [7] and wound healing by clearance of debris, growth and angiogenic factor release [10] which when combined provides a critical defence against invasive infections.

Monocytes are stored in the spleen and circulate throughout the blood. These large leukocytes function to replenish resident macrophages under normal homeostatic conditions [11] as well as acting as responders to inflammatory signals and migrating to infection sites to differentiate into both macrophages and dendritic cells [11]. Migration is initiated by the inflammatory milieu and pattern-recognition receptor stimulation. Monocytes have emerged as facilitators of divergent immune functions during infectious disease with Turner *et al.* [12] suggesting both pro-inflammatory and anti-inflammatory states for the monocyte, not unlike macrophage classification.

Dendritic cells are antigen-presenting cells that play a crucial role in exposing antigen to T-cells that initiate immune the response. They are a link between innate and adaptive arms, promoting immunity to foreign antigen or tolerance to self-antigens [13]. They are present in tissues in contact with the external environment like epithelium, mucosa, lungs, gastrointestinal tract etc. They induce particular phenotypes in T-cells: Th1, Th17 and Treg – this is well established, but their role in inducing Th2 phenotypes is unclear [14].

Neutrophils are spherical granulocytes, phagocytic in nature, that are attracted to infected areas via chemotaxis in the bloodstream. The granulocyte contains an array of vasoactive mediators such as histamine, which establishes its role during fungal and extracellular bacterial infections not only through the production of pro-inflammatory cytokines but also through phagocytosis, reactive oxygen and nitrogen species (ROS/RNS) and neutrophil extracellular trap (NET) formation [15]. Recently, there has been a shift in studies focusing on more non-traditional and detrimental positions for neutrophils during disease, specifically inflammatory conditions that range from seasonal allergies to diabetes, viral infections and effects during cancer [16]

Eosinophils are granulocytes containing a bi-lobed nucleus and large cytoplasmic granules. Expressing a broad array of ligand receptors that play roles in cell growth, chemotaxis, adhesion and degranulation the eosinophil activates complement via both classical and alternative pathways, synthesizes, stores and secretes cytokines, chemokines and growth factors. They stimulate T-cells via antigen presentation and promote humoral responses with B-cell interactions. Their presence amongst dead/dying parasites has proven them to be critical in defence against helminth infection as an end-effector cell. Eosinophils contain specific granule proteins that have potent cytotoxic effects that not only damage parasitic pathogens but in the process, the surrounding host tissue [17]. The granule proteins play a role in defence mechanisms not only against helminth infections but also viral and bacterial infections. The interactions of eosinophils with parasites and other pathogens are now recognized to be much more complex than previously thought [17].

Mast cells are granulocytes, widely distributed throughout vascularised tissues (particularly skin, airways and gastrointestinal tract), containing large amounts of histamine and heparin [18, 19]. Along with dendritic cells, they are on the frontline of interaction with environmental antigens, toxins and invading pathogens. Mast cells express a high-affinity receptor for IgE, which makes them critical effector cells in IgE-associated allergic disorders and parasitic infection responses [20]. Mast cell activation is very microenvironment and signal dependant. They play a prominent role in driving pathology associated with allergic disease and anaphylaxis but can also contribute to host defence by enhancing resistance to pathogens. In certain circumstances, mast cells can also limit inflammation and tissue injury by promoting host resistance [21], limiting pathology during some immune responses to environmental antigens [22], and act almost like adjuvants in developing protection against pathogens. This suggests that mast cells play a critical bridging role between innate and acquired immunity where, microenvironment dependant, they function on both sides of inflammatory homeostasis that can either promote health or contribute to disease.

Basophils are closely related to mast cells, being a granulated cell containing histamine and heparin expressing high-affinity IgE receptor [23]. Basophils develop from a different haematopoetic lineage and leave the bone marrow already matured. They are the least abundant of all granulocytes and poorly understood due to the low abundance, short life span and lack of specific surface markers [23]. What is known is that they are critically involved in the development of T helper type 2 immunity both during infection with helminth and allergic responses.

The complement system is part of the innate arm of immunity, comprising of a group of over 30 proteins and fragments that circulate throughout blood and tissue fluids. These molecules function through the stimulation of phagocytes which clear foreign and or damaged material, promote inflammation (enhancing immune cell recruitment) and attack cell membranes [24]. In response to the recognition of molecular components of a pathogen, they become activated in enzymatic cascade via three distinct pathways. Each pathway causes activation of C3 that is cleaved to C3a, which promotes inflammation, and C3b, which acts as an opsonin, binding to pathogen surfaces aiding in phagocytosis. Activated C3 also triggers lytic pathway damaged cellular membranes. C5a is also produced through the enzymatic cascade and attracts

macrophages, neutrophils and mast cells [24]. Classical pathway activation is a highly specific response, requiring antigen-antibody complexes for activation. In contrast, the alternative pathway is a non-specific response, where C3 hydrolysis is activated by allergens, pathogens or damaged cells. The mannose-binding lectin pathway is also non-specific and can be activated by spontaneous hydrolysis or antigens without antibodies present [24].

Type 2 innate lymphoid cells (ILC2s), are a recently identified set of mononuclear hematopoietic cells with lymphoid morphology but distinguished from classification as a T or B cell due to absence of somatically rearranged antigen-specific receptors. They lie at the cusp of innate and adaptive immunity, falling within the innate arm of immunity but understood to provide critical linkage to adaptive defence cells such as dendritic cells and T-cells [25]. ILC2s are activated through extracellular parasites and endothelial damage responding by the release type-2 cytokines IL-13, IL-5, IL-9 under the transcriptional control of GATA3 and RORα [26]. The majority of ILC2 effector function comes from a considerable release of IL-13, which leads to the initial name given to the population, the Nuocyte – nu is Latin for "13" [25].

1.1.2 Adaptive immunity

The adaptive arm of immunity refers to pathogen-specific (antigen-specific) immune responses. In comparison to innate immunity, adaptive immunity has added layers of complexity whereby mounting an immune response can only occur once the antigen has gone through identification, processing and recognition. When recognition is complete, an army of immune cells is released, mounting a response to that specific antigen. The adaptive arm also provides a memory response, which allows for fast recognition of future infections by the same pathogens. The central cells of the adaptive immune system are T and B-lymphocytes. They are both derived from the same lineage of pluripotent haematopoetic stem cells but upon activation distinguish into 2 cell types with different functionality.

T-cells play a central role in cell-mediated immunity. Maturation occurs in the thymus where they develop their unique T-cell receptor, which is responsible for low-affinity recognition of antigens bound to major histocompatibility complex (MHC molecules). They are split into effector (helper and cytotoxic subsets) and memory populations [27]

Based on their expression of CD3 combined with either CD4 or CD8. Effector populations respond immediately to stimuli, whereas memory cells are long-lived and target future infections. Helper T cells (CD4+ T-cells), as the name suggests, assist other immune cells, for example, B-cells (maturation into plasma and memory cells) and activation of both cytotoxic T cells and macrophages [27]. Their activation comes from being presented antigen by MHC class II to molecules on APCs, they then differentiate to a particular subtype of helper cell that secretes specific cytokine profiles. CD4+ T-cells are subdivided into Th1 (promotes inflammation), Th2 (helps B-cells), Th9 (pathogen immunity and allergic responses), Th17 (promotes inflammation) and Treg (regulates immunosuppressive responses) groups.

On the other hand, cytotoxic T cells (CD8+ T-cells), specifically destroy virus-infected cells and tumour cells. They bind to MHC class I molecules which are present on all cells containing a nucleus. They can also be inactivated to a dormant state that prevents reaction to self-antigen and ultimately autoimmune disease [28]. T-cells are antigen naïve until they are presented with their linked antigen (through MHC molecule on the surface of APC) that then allows them to differentiate and expand into memory and effector cells. Memory T-cells develop as long-lived cells that quickly expand on re-exposure to linked antigen [28].

B-cells and antibodies function on the humoral arm of immunity. The B-cells primary function involves binding to an antigen and receiving activation in either a T cell (antigen linked) dependant manner or independent manner. Stimulation leads to differentiation into a plasma cell that secretes a large amount of antibody [29]. B-cells also present antigen and secrete cytokines [30]. Antibodies can occur in two forms; soluble form that is secreted from the cell circulating in blood, and membrane form that attaches to the surface of B-cell becoming a receptor (BCR). The activation of the BCR determines the fate of the B-cell as plasma cells (antibody factories) or memory B-cells. Antibody genes can also reorder that changes the heavy chain fragment, creating a new isotype of the same antibody; this process is known as class switching. It is critical to allow a single antibody the ability to be expressed in different parts of the immune system. There are several other antibody classes IgA, IgD, IgE, IgG and IgM. The subclasses are characterized by biological properties, location of function and ability to bind to different antigens [31].

1.1.3 The other arms

Non-haematopoetic stromal cells. Immunity does not just arise from its cellular components and their functions but also the microenvironments in which they exist. This microenvironment is determined by interactions between haematopoetic cells (immune cells) and non-haematopoetic stromal cells; which consists of a group of tissue forming cells (fibroblasts, endothelial, epithelial, nerve cells etc.), which are collectively known as the immune stroma [32, 33]. This environment makes up a landscape governing haematopoetic cell growth, survival, functionality and positioning; a level of organization enforces order upon innate and adaptive immune cells [33].

1.2 Helminth infections

1.2.1 Impact of helminth infections, lifecycles, symptoms and treatment

Helminth is a general term meaning parasitic worm. These are eukaryotic invertebrates characterized by elongated, flat or round bodies. This body of work focussed on Nematodes (roundworms) with 3 major human species, two of which mature in humans and one which does not.

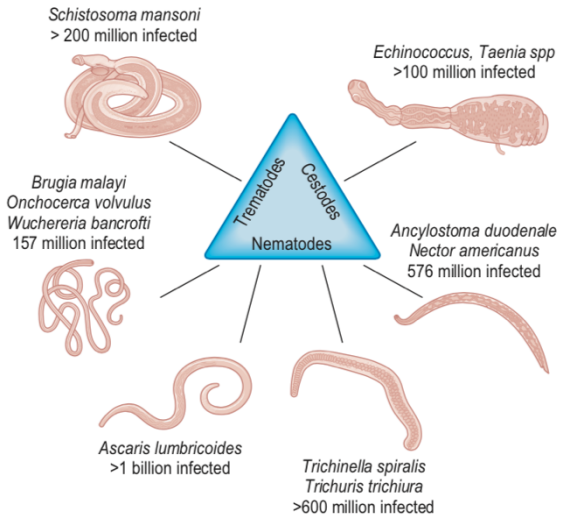

Figure 1.1: Medically relevant helminths. Common medically relevant human helminth infections and their associated global prevalence separated by phyla—Diagram is sourced from Clinical Immunology (5th edition) [24].

Worldwide a large proportion of the human population (1.5 billion/20%) [34] is infected with one or multiple soil-transmitted helminths (STHs) (see figure 1.2); these are parasitic worms that are transmitted through contaminated soil [35]. There are 3

significant subgroups of STHs that account for the immense burden of disease; roundworm (*Ascaris*) (807-1,121 million), hookworm (576-740 million) and whipworm (604-795 million). Figure 1.2 (world map) shows that these parasites are most prevalent in tropical and sub-tropical regions of developing countries. The reason for this is 2-fold; firstly the warm and moist climate is favourable for growth and survival. Secondly, in developing nations, there is limited access to clean water, sanitation and particularly efficient sewage disposal (STH life cycles rely heavily on human exposure to sewage) [36] [37].

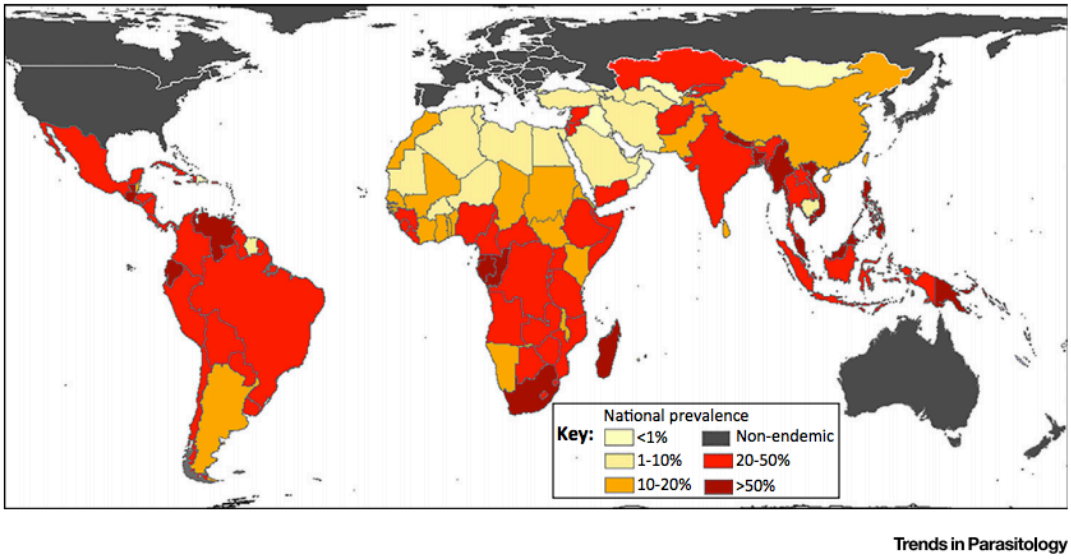

Figure 1.2: Global distribution of soil-transmitted helminths. Data from 2010 global atlas of helminth infection sourced to show global estimates of *Ascaris lumbricoides*, *Trichuris trichuria*, *Necator americanus*, and *Ancylostoma duodenale*. Trends in parasitology, Jan 2016 [34].

Helminth life cycle. Soil-transmitted helminth infections colonize in the intestine (area depends on species), with their eggs being passed through the faeces of an infected individual. Both *Ascaris* and hookworm eggs mature in soil and become infective [38]. In places with poor sewage management, human-faeces contact is increased, leaving the eggs with a higher chance of being transferred (consumed orally) to another individual through direct contact, water supply contamination, fertilizer mismanagement or consumption of unwashed food [38]. Hookworm eggs are slightly different and do not mature to become infective in soil, instead, they hatch in soil releasing larvae that mature to a form which penetrates human skin through direct contact, for example, walking barefoot over the contaminated area.

In terms of sub-Saharan Africa, The research centred around two STH's that have the most considerable impact for the burden of disease: *Ascaris lumbricoides* (Figure 1.3 A) and *Trichuris trichuria* (Figure 1.3 B) [39]. For mechanistic comparison, a helminth infection that turns humans into a dead-end host was included, with a slightly more variable life cycle, *Toxocara spp* (Figure 1.3 C).

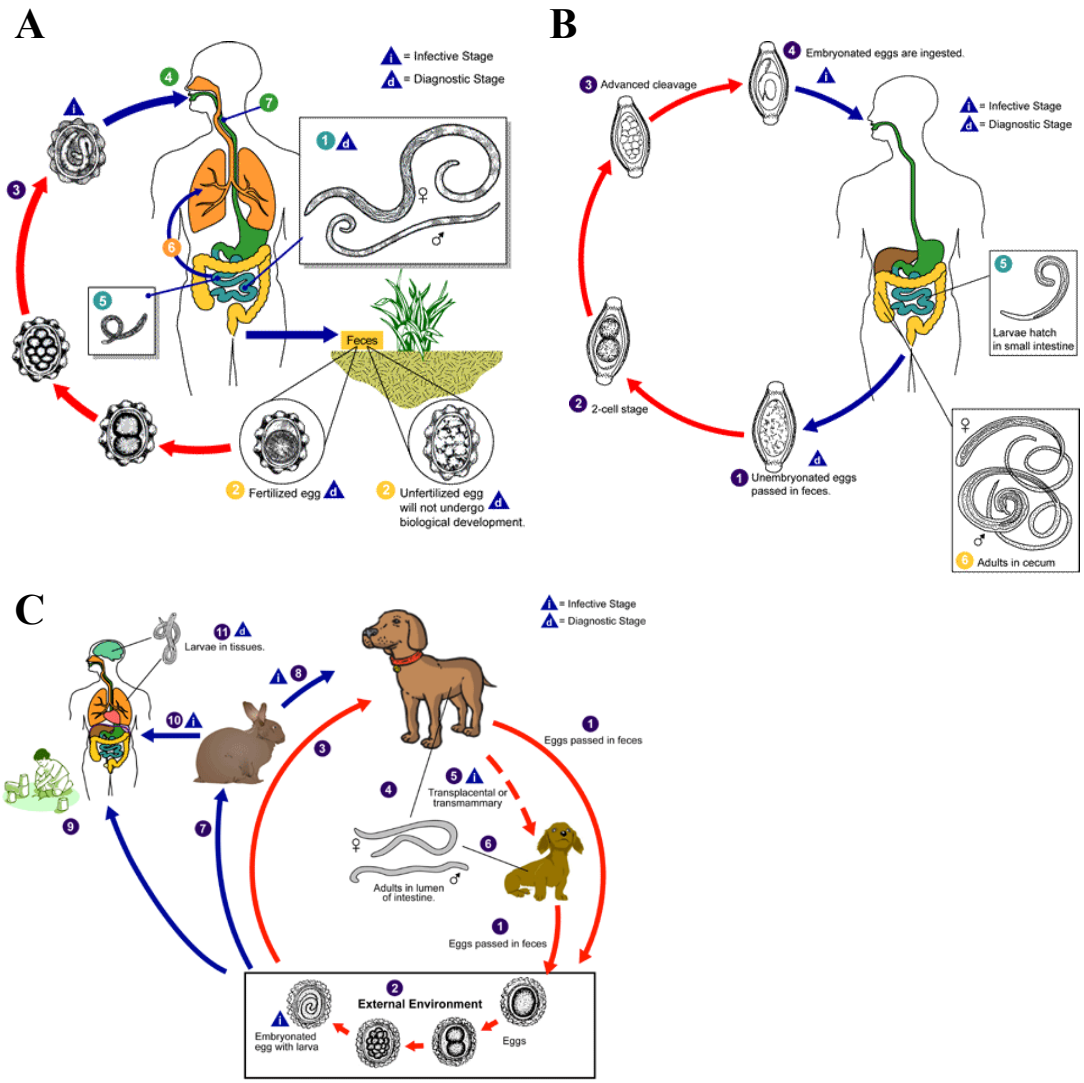

Figure 1.3: Life cycles of helminths. (A) *Ascaris*, (B) *Trichuris* and (C) *Toxocara*. Lifecycle diagrams courtesy of Centers for Disease Control [24].

Symptoms. The importance of soil-transmitted helminth infections may not lie in the sheer volume of people infected but in the demographics of those affected. According to the WHO, roughly 267 million preschool-age children and 568 million school-age children live in areas where these parasites are frequently transmitted and require treatment and preventive interventions. An STH infection can range from light

infection, with the individual being totally asymptomatic, to heavy infections causing a range of health problems. Although outward helminth morbidity is rare, immune impairment generated from infections affects bacterial and viral clearance, such that the WHO has indicated that eradication of helminthic infections may be essential for successful vaccination against life-threatening diseases like human immunodeficiency virus (HIV) and tuberculosis (TB) [40]. Morbidity and symptoms are centred around the gut due to the STH lifecycle. Infections with more considerable morbidity (more worms) will cause intestinal issues such as diarrhoea and abdominal pain, malnutrition, malaise and weakness, eventually causing impairments in development and growth. The helminth infection has various mechanisms by which it impairs growth; worms feeding directly on host tissues leading to loss of iron and protein, intestinal malabsorption (including roundworm competition for vitamin A [41]), hookworms cause chronic intestinal blood loss leading to anaemia, and the loss of appetite that usually accompanies any GIT illness and therefore reduction in nutritional intake. In the case of *Ascaris*, larval migration, through direct mechanical force, can cause substantial pulmonary damage with type 2 pathology resembling allergic airway disease (Figure 1.3). As previously mentioned helminth infections generally centre around more impoverished communities of the tropics and subtropics, with the struggle of poor socioeconomic circumstances, the added burden of a chronic and debilitating, but curable, disease inflicts unnecessary burden. This perpetuates a brutal cycle of poor health, whereby STHs result from poor socioeconomic conditions and contribute to further poor health through there long term effect on public health.

Treatment. The current public health control strategies in place for helminth infection control include chemotherapeutics and vector control strategies. The chemotherapy uses medications such as lbendazole, mebendazole, levamisole, and pyrantel pamoate, all of which target the mature worm through metabolic starvation or paralysis [42]. Vector control is, at best, an adjunct strategy with limited efficacy [37]. Both strategies have the same social, logistical and economic issues with long treatment lengths, remote areas of distribution and the development of some drug resistance [37]. In livestock, the development of resistance to these drugs is widespread [34]. Consequently, a need exists for novel anti-helminthic treatments with a new mechanism of action to put an end to the unnecessary disease that is curable and help prevent the onset of parasitic drug resistance in humans.

1.2.2 The host-parasite interaction

Helminths are complex eukaryotic organisms that are characterized by their ability to maintain chronic infections in a host. The host-parasite interaction is a complicated physiological response for several reasons: (1) *Lifecycles are characteristically complex resulting in the host being exposed to unique antigen repertoires throughout an infection.* For example, in lymphatic filariasis, the host is exposed to larvae at the skin, lymph nodes and lymphatics; adult worms in lymph nodes; and microfilariae in peripheral circulation [43]. (2) *Unique tissue tropism in each area of infection (cutaneous, pulmonary, and intestinal) and migration patterns of the helminths means that the immune-stromal interaction that is activated can be highly variant.* For example, an *Ascaris* infection in the lung must mediate the inflammatory response because gaseous exchange is vital for host survival. (3) *This is further complicated by the fact that an infected host could be exposed to multiple stages of the parasite concurrently if they remain in an endemic area.* The following immune response will not only be a reaction to the new parasite but also have the imprint of the current or previous infection on immune response [42] [44].

The longevity of helminth infections may be at least partially attributable to the ability of the parasite to alter regulatory immune responses within its host. Host death before parasitic lifecycle is complete would be biologically redundant. This has led to an almost unified host-parasite interface creating carriers that are relatively asymptomatic reservoirs for transmission [45]. This alteration of the immune response can also fundamentally change the way our bodies respond to other immune stimuli [45]. Over time parasite infection down-regulates the type 2 immune response [46], towards both helminth antigen and non-related antigens [44], explaining the success of parasite invasion in the tightly regulated areas such as the mucosa. The immunoregulatory effect of these bystander responses also critically affect immune reactions to viruses and bacteria [47], causing a critical downregulation of cellular immunity that is required to prevent intracellular infection, such as HIV and TB [47].

1.2.3 Classical host immune response

Despite the wide variation, there remains a distinct type 2 skewing of the immune system [48] [49]. This is depicted by release, activation and expansion of Th2 cells

producing several key cytokines; IL-4, IL-5, IL-9, IL-10, IL-13 and the activation of plasma cells secreting immune isotypes IgE, IgG1 and IgG4 [50]. This, in turn, leads to the expansion in cell populations of eosinophils, mast cells, type 2 innate lymphoid cells and alternatively activated macrophages. Type 2 cytokine production mediated correctly triggers eosinophilia, mast cell activation, long term immunoglobulin release (IgG1, IgG4 and IgE), mucus secretion, goblet cell hyperplasia and fibrosis, which act together to expel helminths (Figure 1.4) [44]. However, mediated incorrectly, the type 2 cytokines initiate pathology associated with excessive inflammation and fibrosis characteristic in allergic responses, including asthma [44]. Understanding this interplay of cellular effectors (Figure 1.4) generated by a helminth infection becomes critical when searching for novel means by which helminth infections can be combatted [51]. Whilst the predominant response is Th2 in nature, there is increasing recognition for a regulatory component of immune response involving a repertoire of regulatory cells and cytokines.

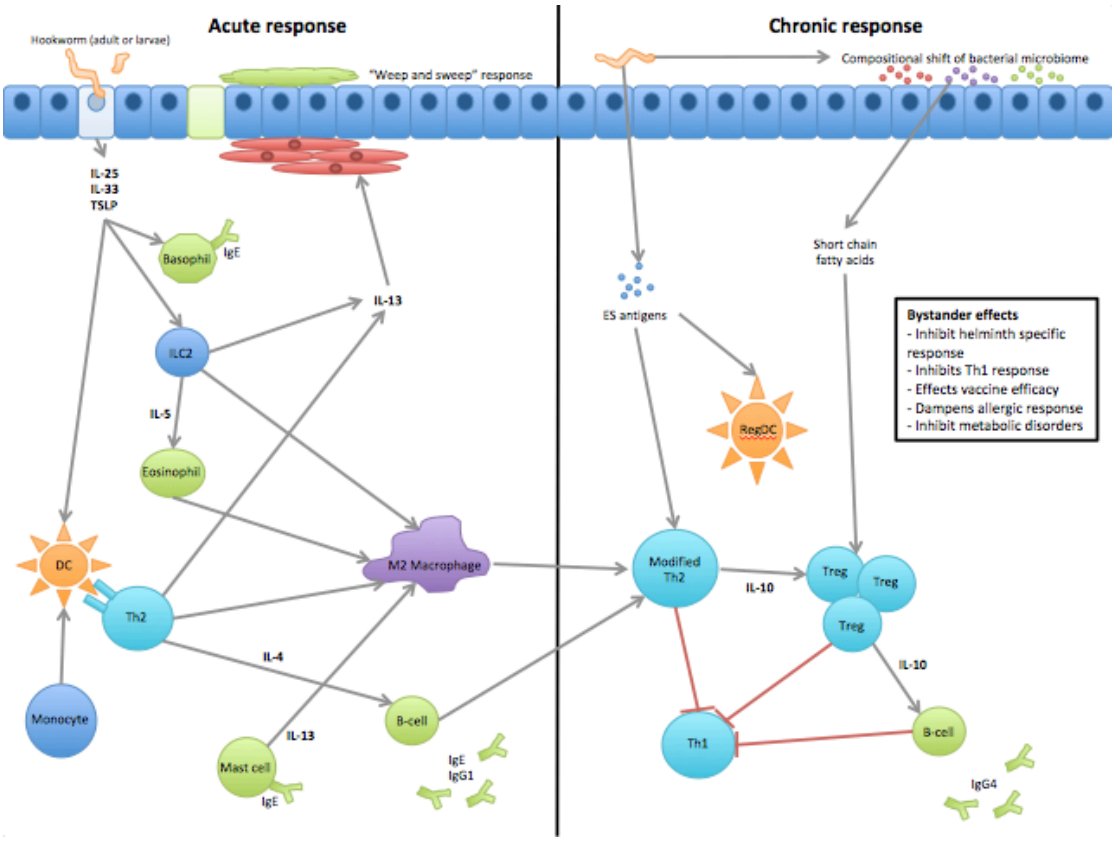

Figure 1.4: Overview of a human immune response to helminth invasion. The human immune response with (1) acute and (2) chronic reaction to helminth invasion. Acute reaction causes epithelial damage, releasing IL-25, IL-33 and TSLP triggering downstream activation of ILC2s and dendritic cells. ILC2s are fast responders, releasing a large amount of IL-13 and activating eosinophils, slower to respond are dendritic cells that prime Th2 cell activation. This activation primes B-cells to produce both helminth specific and non-specific antibodies (IgE and IgG1). Both ILC2, T cells and Eosinophils

polarise macrophages to M2 phenotype. This M2 phenotype leads to modification of Th2 cells and chronic response of the human immune system. The chronic response becomes regulatory through the activation of Tregs and the inhibition of Th1 cell. B-cells also isotype switch leading to release of IgG4. Bystander effects include inhibition of helminth specific response, Th1 inhibition, vaccine efficacy reduction, dampening of allergic response and inhibition of metabolic disorders. Diagram created by Zoe Baker.

The initiation, progression and culmination of the immune response to helminths involves an essential set of innate and adaptive cellular interactions with (1) epithelial/stromal cells, (2) innate lymphoid cells (ILCs), (3) dendritic cells (DCs), macrophages and monocytes; (4) T cells; (5) B cells; (6) eosinophils; (7) mast cells/basophils; and (8) neutrophils. Critically helminths have an effect on (9) allergy and (10) modulation of immune responses.

Non-haematopoetic stromal cells, such as keratinocytes, epithelial cells and smooth muscle cells also play vital roles in helminth interactions due to their proximity to the infection sites. Epithelial cells are the first barrier layer exposed to the invading helminth (Figure 1.4). These cells initiate an alarm response releasing TSLP, IL-33 and IL-25 as well as some other alarmins [52]. This stimulates dendritic cells to mount a type 2 immune response and inhibition of IL-12 release [53], Th2 response downregulates the Th1 response and initiates the expansion of effector cell populations of ILC2s, basophils and mast cells. Smooth muscle cells contract, mucus production increases and fluids flow in the gut lumen, which contributes to "weep and sweep" action on the live adult pathogen [54, 55]. Smooth muscle cells are highly effective antigen presenters [56] which elicit robust Th2 responses [57] with the induction of lung pathology during *N. brasiliensis* infection recently attributed to IL-4Rα signalling on smooth muscle cells [58].

Type 2 innate lymphoid cells, expand robustly in response to exogenous IL-25 or IL-33, providing the critical linkage to adaptive defences [25]. Defined by their expression of IL-33 receptor (IL-33R) under the transcriptional control of GATA3 and RORα; ILC2s respond to activation by releasing type-2 cytokines **IL-13**, **IL-5** but also IL-4, IL-9, granulocyte-macrophage–colony-stimulating factor (GM-CSF) and amphiregulin [26]. What proceeds is a potent induction of eosinophilia, mucus production (goblet cells), macrophage activation, smooth muscle contraction and tissue repair.

There is considerable overlap between Th2 cell and ILC2s. The variant speed at which

each of these cell types releases cytokines (ILC2s are innate and rapid) and ILC2s ability to directly regulate T cell activation means that there is room for coordinated strategies for functionality. IL-13 and T cells are essential for worm expulsion, but T cell secretion of IL-13 is not necessary for expulsion [59]. The initial discovery of ILC2's came from rag knockout mouse models (RAG-1 deficient mice produce no mature T and B cells) [60] which, upon stimulation with IL-33 and IL-25, produced key type 2 cytokines chief of which were IL-13, attesting to the existence of another cellular mechanism which bypassed the adaptive arm of immunity [25]. After infection with *N. brasiliensis*, and they are the major innate IL-13 expressing cells. In the same model, a transfer of wild type ILC2 cells to rag knockout mouse rescued the anti-helminthic response [61].

Interestingly reports have now linked ILC2s with metabolism. They affect homeostasis and are also more active in cases of obesity and starvation [62]. This could hint at a link through which helminths can modulate host metabolic function during colonization [63, 64].

Dendritic cells. Even though the classical mode of action for dendritic cells is the activation of T cell phenotypes Th1, Th17 and Treg, studies have shown that DCS is required for optimal Th2 responses *in vivo.* The depletion of DCs in both *S. mansoni* and *H. polygyrus* models inhibits the induction of Th2 responses [65]. The helminth products can prime DCs towards Th2 phenotype through its interaction with PRRs, TLRs and CLRs (C-type lectin receptors) and therefore suppress antigen presentation and the expression of Th1 cytokines through direct interference with the pathways. There is a general trend of modulation of DCs by helminths and this by default impairs their ability to defend against other infectious stimuli [65].

Monocytes have emerged as facilitators of divergent immune functions during infectious disease with Turner *et al.* [12] suggesting both pro-inflammatory and anti-inflammatory states for the monocyte, not unlike macrophage classification. Classification of monocytes by classical, intermediate or non-classical derives from the expression of CD16 and CD14 in monocytes circulating in peripheral blood. Interestingly, looking at the rough perimeters of the subsets, the grouping of monocytes would fluctuate based on a variety of factors and may influence STH infection

outcomes. Appleby *et al.* [66] also illustrated that CD16 (IgG receptor) expression increased with parasite-specific total IgG and IgG1 in healthy populations, and was associated with protection against schistosome infection. CD16 expression correlates with the pro-inflammatory phenotype of monocytes with cytokine secretion profile (TNFa in response to LPS) and ability to present antigen. By modelling the *in vitro* interaction between the filarial worm *W. bancrofti* and classical, non-classical and intermediate monocytes, the functional variation in response to helminth infection becomes apparent [67]. A notable association from this study was CD16 expressing monocytes with their ability to interact with a filarial antigen and simultaneous production of regulatory cytokines such as IL-10. Notably, the large proportion of human monocyte studies have not involved populations with direct exposure to tropical disease, where helminth infections are most prevalent. Instead, they have taken place in high-income countries reviewing non-communicable diseases [67]. To this effect, an African population negative for helminth infections, malaria and HIV, were compared to a Caucasian population. Changes in expression intensity of monocyte markers based on ethnic background were reported, highlighting the need to diversify monocyte studies [67].

Macrophages are an essential class of antigen-presenting cells that serve as protector cells in parasitic infections. The polarisation of macrophages is mediated by the types PAMPs (pathogen-associated molecular patterns) and cytokine profile it receives. Helminth interaction produces a population of Alternatively activated macrophages (AAMs or M2), which are defined by their expression of arginase instead of nitric oxide, are rapidly recruited to the sites of helminth infection during Th2 immune response [5-7] triggered by IL-4, IL-13 and IL-10 [4, 8] and increased mannose receptor (CD206). Their primary functions during helminth infection involve the down-regulation of Th1 response through IL-10 [9] [7] and wound healing caused by parasite migration [10]. For example, H. polygyrus is mediated by IgG-dependent induction of arginase in AAMs [68] and SP-D directly interacts with N. brasiliensis and lung macrophages that polarise macrophages to AAMs in the lung [69].

T cells. Helminth infections typically produce a robust immune response that enhances the expression of IL-4, IL-5, IL-9, IL-10 and IL-13. The central cells are the CD4+ Th2 cells expressing IL-4Rα, which is a component of both IL-4 and IL-13 receptors is at

the centre of Th2 immunity [70]. IL-4 and IL-13 are separated by expression in the body, with IL-4 being pronounced in the lymph nodes and IL-13 being pronounced in the tissues. IL-4 is a critical $CD4^+$ T-cell cytokine, which, when released, can sufficiently support Th2 cell expansion during *N. brasiliensis* infection [71]. Aside from the classical Th2 activation, there are other T-cell activation states present in helminth infection; a new subset of T cells that expressing IL-9, IL-10 and no IL-4 has been described in allergy and intestinal helminth infection [72]. Th9 cells are associated with host protection in *N. brasiliensis* and *T. muris* infections. Th17 cells express the cytokine IL-17 that in helminth infection models (i.e. *S. mansoni*) has been associated with immune-mediated pathology [73].

A strong example of host-parasite compromise comes from $CD4^+$ T-cells response in schistosome infection, whereby the stimulation of parasite development and inflammation is required for migration of the eggs [74] but granuloma formation in host gastrointestinal tract and liver allows the eggs to mature but protects the host from severe inflammation. In mouse models, this is illustrated when the balance is not achieved, resulting in severe mouse pathology stemming from unchecked activation of CD4 Th1 and Th17 cells [75].

Cytokine responses initiated by Th2 cells promote effector functions in both bone marrow-derived haematopoietic cells and non-bone marrow-derived cells and tissues. Helminths can potentially function as a type of adjuvant, enhancing or shifting bodies response to antigen, with recent studies showing their role in Th2 differentiation to non-helminthic antigens [71] [76]. Interestingly, the linkage between the innate and adaptive arms of immunity comes from the crosstalk between ILC2s and $CD4^+$ T-cells, respectively. Both arms are critical in helminth expulsion, but the very recent discovery of ILC2s means the exact mechanisms by which it communicates with adaptive cell populations is as of yet undetermined.

B-cells are activated and produce associated cytokines, namely IL-10 (an anti-inflammatory cytokine that inhibits Th1 cells, NK cells and classical macrophages [77]) and IL-2 (supports correct development of effector and memory Th2 cells [78]). Secondly, their most notable role is at the level of antibody production. Infection with protozoan parasites leads to the production of IgG and IgM, with helminths, there is additional production of large amounts of IgE [79]. Antibody acts to; neutralize

parasites by binding to, or blocking the function of various cell surface markers, recruit phagocytic cells to the site of infection and trigger neutralization through complement activation [79].

IgG is the primary antibody present in human sera functioning to activate complement and bind to the parasite, marking it for phagocytosis. It is mostly present towards the end of infection is responsible for antigen-specific neutralization of 'known' parasites. Chronic helminth infection elevates subtypes of IgG, namely IgG4 and IgG1 [80] [81]. IgE functions as a parasite and allergen-specific immune effector; it's the least abundant in human sera but elicits some of the most potent inflammatory reactions [82]. Like IgG4, IgE's production is dependent on the release of Th2 cytokines IL-4/IL-13 from $CD4^+$ T-cell and ILC2s. However, IgE is a lot faster and short-lived. IgE plays important roles not only in infection of multicellular organisms (i.e. helminth infection) but also in allergic conditions due to its high-affinity binding of Fc receptor on both mast cells and basophils, triggering their effector functions [83].

Interestingly, parasite-specific IgE in patients' sera, has been associated with natural resistance to infection [84] or re-infection [85] [86]. IgG4 is the rarest subtype of IgG and only forms when antigen persists. Therefore IgG4 relates to a long term asymptomatic or chronic infection, competing with the same epitopes as IgE it perhaps mediates a long term massive allergic/inflammatory response [87]. The co-evolution of parasite and human immune interaction could be at play here, with a long term survival strategy for helminths involving the elevation of IgG4.

Eosinophils in systemic circulation sharply increase in the early response to helminth infection [88] being rapidly recruited to the site of infection mediated by IL-5. This increase, both in blood and tissue, falls under the influence of ILC2s. Their effector functions involve the rapid release of immunomodulatory factors including cytokines, chemokines and growth factors through which they mediate IgE production and goblet cell mucus production [89]. New eosinophil functions have uncovered a more subtle immunomodulatory role in mouse models, supporting nematode larvae survival by promoting Th2 cell accumulation and preventing macrophage or neutrophil induction via iNOS [90]. Eosinophils do mediate the damage during *N. brasiliensis* challenge in the absence of $CD4^+$ T cells [59] although the primary response to the nematode is unaffected in an eosinophil-depleted mouse model [91]. They are also powerfully

linked to host resistance to helminths and allergic inflammation, including asthma [92] [93].

Basophils, like eosinophils, increase in number during helminth infection; they are essential in helminth infections secreting histamines, cytokines (an important source of IL-13 and IL-4) and lipid mediators all to promote Th2 response [94]. They are antigen-presenting cells that drive Th2 cell differentiation and generate large amounts of IL-4 in both an IgE dependent and independent manner [94]. Basophils, therefore, play a role in primary infection resistance in secondary infection protective immunity. In infection with *N. brasiliensis* basophils are found dispersed throughout the lungs, spleen and liver [95]. The release of IL-4 during early infection aids in Th2 activation and up-regulation [95].

Mast cells are a potent arm of the inflammatory response in innate immunity. They reside in peripheral tissues, making them an immediate responder, expressing an Fc receptor that is sensitized with antigen-specific IgE [96]. Basophils and mast cells both share expression of Fc receptor for IgE as well as TLR2 and TLR4 and also release IL-4 due to activation, but mast cells differ in their rapid speed of activation [97]. Hepworth et al. [98] have recently shown that mast cell degranulation is required to enhance the expression of tissue cytokines IL-25, IL-33 and TSLP during early helminth infection which primes type 2 immunity. They are also classically considered alate-stage effector cells that crosslink antigen-specific IgE and cause degranulation [42]. The type of mast cell response correlates with distinct microenvironments that nematodes colonize, *H. polygyrus* and *N. brasiliensis* colonize the intestinal lumen whereas *T. spiralis* inhabits the intestinal epithelium (arguably where mast cell action becomes more vital and effective) [45].

Neutrophil recruitment to a helminth invasion site has also been demonstrated [5] and is thought to be, along with macrophages, part of the team of first responders that contains or destroy parasites. Recently Chen *et al.* demonstrated, using a *N. brasiliensis* mouse model that neutrophils in parasite-infected mice showed a distinct transcriptional profile that promoted alternatively activated macrophage polarization through the secretion of IL-13. Therefore a distinct activation state for neutrophils, in the context of type 2 immune response, primes a macrophage phenotype that directly mediates rapid nematode damage and clearance [99].

1.2.4 C-type lectins and helminth infections

There is evidence to suggest that C-type lectins play a role in driving helminth immune responses [100]. Lectins are abundantly present on immune cells and with their structural commonality being that they share at least one or more C-type lectin domain (CTLD), which determines its carbohydrate specificity. These families of calcium-dependent receptors are abundantly present on immune cells, and in the microenvironment, collectively, they recognize a variety of glycan's (abundant on the surface of helminths) [101]. Examples range from mannose receptor (CD206), which is a surface receptor found on the surface of macrophages, all the way to a soluble receptor-like surfactant protein D. Increasing our understanding of how these molecules interact with immunity and pathogens, or beneficially modulate inflammation could help future research surrounding treatment and interventions.

1.3 Atopy

1.3.1 Introduction to atopy and atopic dermatitis

Allergies or atopy are inflammatory disorders where the immune system recognizes non-harmful environmental stimuli as pathogenic and generates an inappropriate immune response. Allergen-specific responses are initiated via the response of CD4+ Th2 cells, ultimately driving the production of allergen-specific IgE that leads to memory [79]. An individual with an unusual IgE or sometimes IgG response is deemed 'sensitized', and exposure to an allergen leads to diseases such as asthma, atopic dermatitis, allergic rhinitis and food allergy [79].

Allergy prevalence has significantly increased over the last 50 years, with more than 30% of children allergic, 10% of children having asthma and allergic rhinitis, and 5-7% of children with food allergy [102]. Queue the hygiene hypothesis, the idea that a lack of early childhood exposure to pathogens, healthy microbiota and parasites increases susceptibility to allergic disease because of suppression of the development of immunity [103, 104]. However, meta-analysis and grand assumptions made over the hygiene of third world environments is controversial and sometimes inaccurate. An extension or shift from this is the old friends' hypothesis, which states that increase in allergic disease is due to a loss of symbiosis between the human biome and parasites and bacteria, which were once evolutionarily beneficial [105].

The concept of atopic march describes the sequence and progression of atopic disorders from atopic dermatitis in infants on to food allergy, allergic rhinitis, and then eczema later as children. To understand the interplay between helminth infection immunology and allergic disease, the context of child age and progression of allergic disease must be taken into account (Figure 1.5).

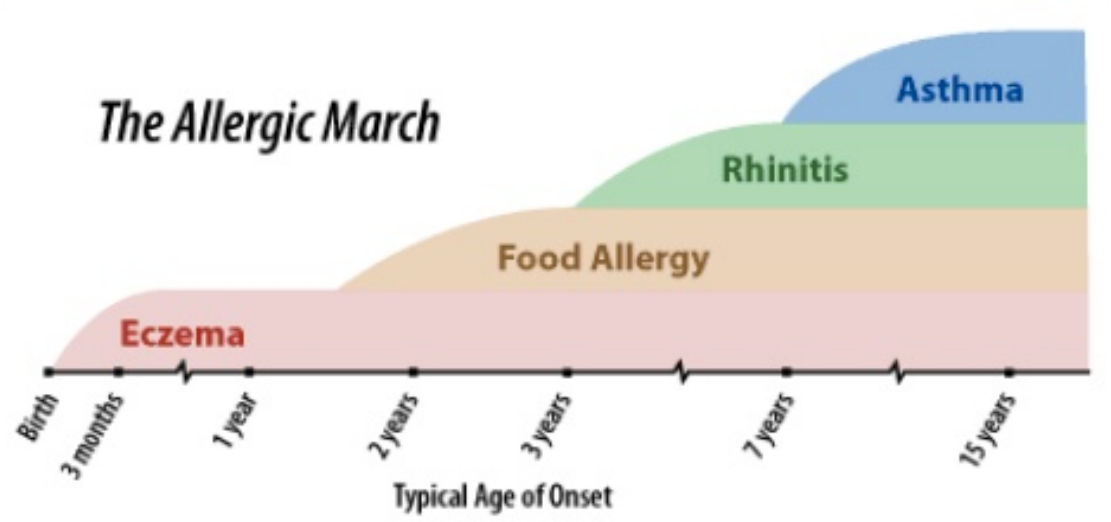

Figure 1.5: The allergic march during childhood. Progression of atopy from birth to 15 years old with different allergic disease appearing at different ages beginning with atopic dermatitis (eczema), on to food allergy, rhinitis and then asthma. This is just a representative plot, and there is much overlap in disease (adapted from LEAP study, 2011).

1.3.2 Atopic Dermatitis

Atopic dermatitis (eczema) is a particular chronic inflammatory disorder (allergy) that affects the skin. The resultant inflammation leads to itchy, red and swollen lesions on particularly sensitive areas of the body. The condition can occur at any age but is most commonly developed in children (see figure 1.5). The risk of concurrent development of other allergic conditions is also increased (see figure 1.5). SCORAD (SCORing Atopic Dermatitis) is a test to assess the severity of atopic dermatitis as objectively as possible; the scale runs from 0-100 based on various metrics surround size, inflammation and discomfort of lesions [106]. It is a useful benchmark for understanding the individual severity of atopic dermatitis and can be directly compared to SP-D to assess for any associations.

The causes root in genetics, environmental exposure and skin permeability issues that cause immune dysfunction leading to chronic inflammation. Mutations to the gene

encoding for filaggrin (FLG), which among many things ensures a well-functioning and hydrated epidermis, have been heavily associated with the development of AD [107] [108]. Interestingly, the discovery of faulty production of FLG indicates that AD, rather than just a primary immune problem, revolves around the structure and function of the barrier itself and causes resultant immune inflammation. The FLG mutation is a significant risk not only for AD but for all aspects of atopy (rhinitis, asthma, food allergy), illustrating how heavily interlinked allergic diseases are [108]. This supports the model that skin barrier dysfunction is a key driver in allergic disease and the onset of AD, or skin barrier dysfunction leads to allergen exposure and is the first step in a cascade of inflammatory disorders [109].

1.3.3 Helminth infections, allergy and the modulation of immunity

Chronic hookworm infection (*Necator americanus*) protects against asthma, but roundworm (*Ascaris lumbricoides*) infection aggravates allergic disease [110]. It is shown that repetitive anti-helminthic treatments to children in endemic areas produce an increase in allergen skin test reactivity [111] and that children in helminth endemic areas might have lowered risk of the development of allergy [111]. Study variation and conclusions could reflect a wide array of factors relating to species of helminth, the intensity of helminth infection, type of allergic disease and timing of infection concerning immune maturation. There is compelling evidence that suggests a switch in immune responses, from the initial onset of acute helminth infection with early exposure driving inflammatory response, creating an allergy, to the classic long term chronic response that attenuates host inflammation and allergy [112] [113].

The helminth-mediated modulation of allergic responses is hugely complex. Consideration must be made for the type of helminth, secreted products, site of infection and the local microbiome. Studies have observed various mechanisms at play. IL-10 induced suppression of Th2 polarisation and expansion of Treg cells (both natural and parasite-induced) [114]. IL-10 inhibition of IgE signalling in basophils, which, as previously mentioned, are important players in an allergic response. Upon helminth infection, B-cells differentiate into a regulatory B-cell type that produces IL-10, which would suppress Th2 response and expand Treg cells [115]. Parasites drive the effect of Treg cells by secretion of TGF-β clones that bind to TGF-β receptors triggering Treg cell ($FoxP3^{+}$) expansion [116]. In *H. polygyrus* infections (mouse modelled), type 2

immune response is downregulated by secretion of Hp-secreted protein, which inhibits IL-33 release [117].

The local microbiome interaction with helminths and their secretory products must also be considered. The capacity of an allergen to cross a mucosal barrier is dependent upon the condition of the epithelial barrier with mucosal layer formation and anti-microbial peptide release mediated by IL-22 [118]. These are released by healthy mucosa-associated bacterial communities, compositional changes within these communities through things like diet or antibiotic use could alter the bacterial induction of protective mucosal responses, which could lead to type 2 inflammatory response [119]. This is another example of the intertwined co-evolution between helminths and human immunity, with the modulatory response of the helminth or their derived proteins contributing indirectly to the modulation of allergic response.

There is also evidence to suggests that some parasitic helminths or systemic treatment with helminth products can lessen atopic disease and autoimmune disorders, e.g. Irritable bowel disease [120] [121], multiple sclerosis [122] [123] and arthritis [124]. The hypothesis being that if immune modulation of helminth infection strikes a particular balance, inducing no obtuse pathology, helminths function almost like commensal organisms and not pathogens that need to be removed.

1.4 Lung immunology

1.4.1 Lung structure and function

The lungs are the major organs of the respiratory system housing the mechanics required to facilitate gaseous exchange. A process by which the oxygen is filtered into the body and carbon dioxide is filtered out. This process happens over a vast epithelial surface area (+- 70m^2) that is highly permeable to gases. The lungs transfer air from the conducting zone, where the air is funnelled through, into the respiratory zone, where gaseous exchange takes place. The exchange centres on the basic unit of ventilation known as alveoli (300-500 million in the adult lung), which are sacs that expand and contract during inhalation and exhalation [125]. These alveoli have a single layer of epithelium consisting of two different cell types; type 1 epithelial cells (single-layered and squamous) take up the most surface area and facilitate the gaseous exchange; type

2 alveolar cells are the most numerous but take up a fraction of the space. Type II alveolar cells (AT2 cells) perform a vital maintenance function through the release of pulmonary surfactant [126]. Pulmonary surfactant is a lipoprotein complex, made up of mostly phospholipids (dipalmitoylphosphatidylcholine), neutral lipids (cholesterol) and the proteins SP-A, SP-B, SP-C and SP-D. Surfactant contains both hydrophilic and hydrophobic domains, organizing in such a way that causes adsorption to the inner surface of the alveoli [125]. This adsorption fulfils its primary function, to decrease alveolar surface tension, stopping the collapse and allowing for the easy re-expansion of the alveoli on inhalation [127]. This function is performed by 2 of its subcomponents surfactant protein B (SP-B) and surfactant protein C (SP-C), which are hydrophobic, in conjunction with the phospholipids to optimize surfactants adsorption [128]. The other 2 subcomponents, known as surfactant protein A and surfactant protein D, are collectins (collagen-containing C-type lectins) that form a niche part of innate lung immunity through their interaction with pathogens [129].

1.4.2 Lung immunology

Immunity in the context of the lung is a double-edged sword. It faces the dual task of maintaining an inflammation free mucosal surface whilst still eliminating inhaled pathogens. Fine-tuned maintenance of this pulmonary homeostasis is critical to our survival, with any breaks in gaseous exchange due to inflammation or illness being life-threatening. Physical barriers are designed to filter out most of the inhaled material. However, a large proportion of small (<5um) infectious or allergenic particles still reach the distal air spaces. In a functional lung, the particles would then encounter components of the innate immune system including alveolar macrophages, dendritic cells and lung collectins [125]. The ability of the lung to remain infection and inflammation free is due to the small family of calcium-dependent C-type lectins. SP-D and SP-A are expressed on most mucosal surfaces but exist at much higher concentrations in the lung [129]. SP-A knockout mice display no lung pathology; however, SP-D knockout mice show chronic inflammation, highlighting the niche role for SP-D specifically regarding the inflammatory homeostasis of the lung and communication with innate immune cells [129].

SP-D is structured as a large hydrophilic protein with an N-terminal triple-helical collagen region and a trimeric ligand carbohydrate recognition domain, called a C-type

lectin [130]. The direct mechanism of modulation is unclear, but the C-terminal lectin end of SP-D is involved in suppression of immune cell activation, therefore suppressing inflammation itself, with the N-terminal collagen domain facilitating the clearance of pathogens and apoptotic cells through phagocytosis [131]. This is further complicated by the fact that SP-D modifies grouping structure based on oxidative changes in the lung environment as well as genotype of the protein [132].

1.4.3 Surfactant protein D and its role in helminth infections

The type 2 immune cascade in helminth infection involves damage to the epithelial layers in the body. Figure 1.4 shows that epithelial damage leads to the release of IL-25, IL-33 and TSLP, which triggers the downstream activation of effector populations in an attempt to expel the helminth. As previously mentioned, in helminth infections involving lung colonization, the primary cell type releasing IL-33/IL-25 and priming anti-helminthic responses are alveolar type II cells (ATII cells) [133]. Another major immunomodulatory protein released by ATII cells is surfactant protein D (SP-D). Classified as a hydrophilic pulmonary collectin (soluble C-type lectin), as previously stated, SP-D plays a vital role in the modulation of mucosal immunity during exposure to microbes [134].

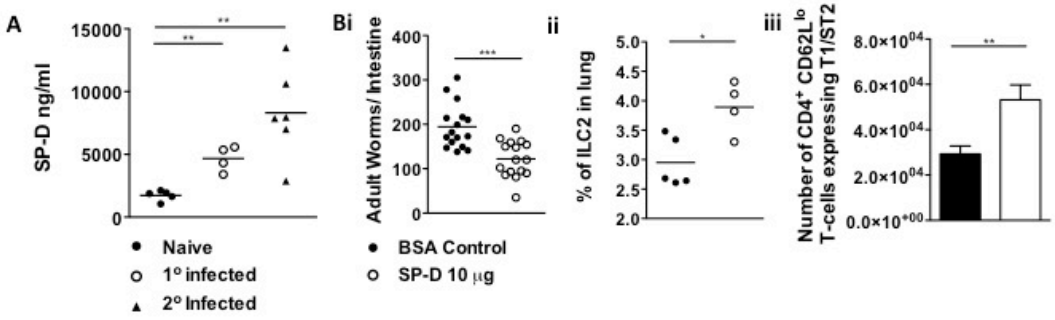

Figure 1.6: Surfactant protein D levels are increased and protective following *Nippostrongylus brasiliensis* infection. SP-D levels are significantly increased in bronchoalveolar lavage (BAL) following primary and secondary N. brasiliensis infection (A). Intranasal administration of SP-D to naïve mice reduces host-parasite burden (Bi) and associates with increased protective ILC2 (Bii) and IL-33 responsive activated $CD4^+$ T-cell populations (Biii). Figure sourced from our lab at UCT [69].

SP-D binds directly to helminths such as *Schistosoma mansoni* [135] and allergens protecting host lung from asthma [136]. A STAT6 knockout murine model shows greatly reduced SP-D titres [137] demonstrating its induction is dependent on type 2 immune activation. Recently, data emerging from our research group at UCT [69] links

the specific control of mouse-modelled nematode infections with SP-D. The research demonstrated that infection with *N. brasiliensis* correlated with increased lung SP-D levels (Figure 1.6A) and the administration of recombinant SP-D protects against *N. brasiliensis* infection by reducing worm burden (Figure 1.6Bi). This protection is associated with increased populations of ILC2s and $CD4^+$ T-cells (Figure 1.6Bii and Figure 1.6Biii))

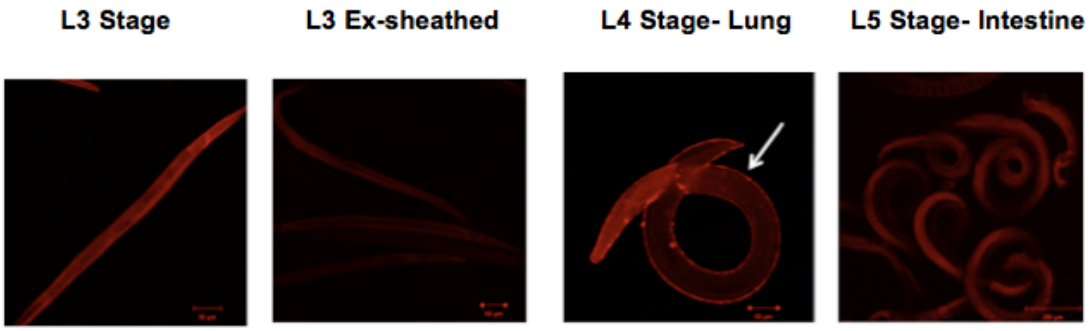

Figure 1.7: *Nippostrongylus brasiliensis* incubated with recombinant surfactant protein D. Illustrating the interaction of SP-D with the L4 lung stage of an N. brasiliensis infection Figure sourced from Thawer et al. [69].

As a C-type lectin, SP-D includes an N-terminal triple helix collagen region and a carbohydrate recognition region (CRD) [138], functioning as an immunomodulator with an affinity towards various immune cell types [139]. Both surfactant protein A (SP-A) and SP-D are essential in defence against respiratory pathogens and allergens [140] [141]. SP-D's helminth pathogen recognition abilities fall under the influence of Th2 cytokines such as IL-4, IL-13 and TNFα [142]. Unlike its counterpart, SP-A, which is at its highest levels in the alveolar epithelium, SP-D is found at its highest levels in bronchoalveolar lavage fluid (BALF) (75%) [143]. This could be due to SP-D's role in immune responses towards extracellular pathogens and SP-A's role in immune response towards intracellular pathogens. Due to the size and nature of helminth infections in the lung, the interface with effector molecules such as SP-D would be more critical extracellularly, at epithelial surfaces, explaining the heightened concentration of SP-D in secreted BALF [143].

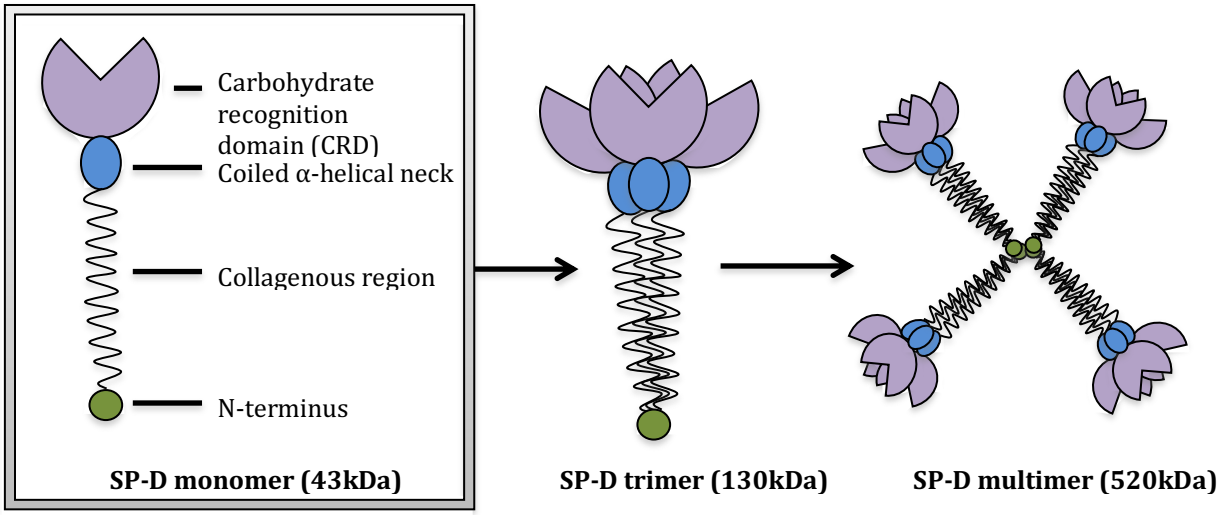

Figure 1.8: The structure and formation of surfactant protein D. The structure and formations of surfactant protein D. Surfactant protein D is a large hydrophilic protein known as a C-type lectin. Its basic subunit contains an N-terminus, a helical collagen region and a ligand carbohydrate recognition domain. Dependent on environment and genotype, SP-D can group as a trimer or multimer, which determines its recognition and interactions to pathogens, allergens and immune mediators. Diagram created by Zoe Baker.

Other C-type lectins also show roles in helminth infections with Ritter *et al.* reporting on schistosomal egg antigens that interact with the C-type lectin, dectin 2 [144]. There is also membrane surface receptor CD206 (mannose receptor) mentioned earlier. This molecule is key in the kinase signalling pathway and induces potassium efflux and the production of ROS, ultimately decreasing the immunopathology of Schistosome infections [144]. A knockout model of another C-type lectin, mannose-binding lectin-A, showed mice with irregular IgM responses that exhibit increased susceptibility to nematode infection [145]. Evidence of other C-type lectins and their immune interactions coupled with the established presence of SP-D, not only in the lung but other peripheral tissues and mucosal surfaces alludes to its powerful role in innate immunity outside of the pulmonary environment [130].

As previously stated, SP-D modifies grouping structure based on oxidative changes in the lung environment [132]. Normal lung environments suppress phagocytosis, retaining the ability to perform effective gaseous exchange, and an inflamed lung will promote apoptotic cell clearance. A key determinant is SP-D's grouping structure and the orientation by which it binds to effector cells [146]. SP-D induces a classically anti-inflammatory phenotype which is important in mucosal barriers, such as the lungs, however, when the pulmonary system is overwhelmed by pathogenic insult, a pro-

inflammatory role can be assumed to aid immune activation [147]. For various reasons (low protein concentration, biochemical modification and proteolytic breakdown) SP-D can decompose from multimeric form to low molecular weight (LMW) forms [148]. This decomposition can induce pro-inflammatory signalling, which is characterized by binding to LPS and lipoproteins [129] to a more considerable degree than HMW SP-D. SP-D shares characteristics with both under- and overexpressing macrophage models [131], with Clark et al. supporting this information showing 10 fold increase in apoptotic alveolar macrophages in SP-D knockout mice, with a reduction coming from the administration of recombinant SP-D [149]. The model for how SP-D operates in the lung remains type 2 driven and anti-inflammatory, with this inflammatory phenotype only generated under very specific circumstances.

1.4.4 Surfactant protein D and its role in atopy

As previous stated SP-D has been implicated in various respiratory diseases, including pulmonary dysplasia, allergic asthma, and chronic obstructive pulmonary disease. The abnormal modification of the pulmonary environment leads to the breakdown or modification of SP-D; this changes its homeostatic balance and can shift the lung into a different inflammatory state. Phenotypic characteristics of SP-D in asthma include increased systemic SP-D levels, which could be due to the induction of SP-D synthesis in airway epithelia and the change in barrier integrity [150]. This shift in structure leads to systemic leakage from the lung, increasing serum SP-D concentration, the systemic effects of which are unknown. This also proves to be a promising biomarker for lung injury. SP-D levels are increased in cases of severe allergic asthma [151] and are correlated with BAL eosinophils and neutrophils. These BAL eosinophils are in turn correlated with NO content and the level of oxidized SP-D species, which, as previously stated, could affect the inflammatory profile of SP-D. As stated above the variability of oxidation state changes the inflammatory effect of SP-D.

The serum concentration of SP-D rises in asthma [151] [152] but also allergic rhinitis, which has the same underlying disease process as asthma, involving the respiratory tract and its adjuncts [153]. Research demonstrates the link between SP-D and Th2 mediated inflammation associated with allergy, which is modelled not only in murine allergy models but also human data [153]. However, there is substantial variation in clinical data; it is postulated that this is due to a lack of sample stratification. Allergy

can be characterized based on its up-regulated Th-2 profile, and in murine allergy, models using *Sftpd*$^{-/-}$ mice as allergic asthma models, the administration of both SP-D and rfhSP-D appear to dampen aspects of the allergic phenotype [154]. In murine models of hypersensitivity induced by allergen challenge, administration of both SP-D and rfhSP-D suppress IgE (antigen-specific) in serum, reduces eosinophilia and causes Th-1 cell polarisation [155].

Allergen-specific responses are initiated via the response of CD4+ Th2 cells, ultimately driving the production of allergen-specific IgE that leads to memory [79]. An individual with an unusual IgE response is deemed 'sensitized', and exposure to an allergen leads to diseases such as asthma, atopic dermatitis, allergic rhinitis and food allergy [79].In humans, the loss of barrier integrity associated with respiratory disease can cause pulmonary SP-D to leak into serum; this detectably increases serum SP-D. This leakage changes the ratio of BAL SP-D to serum SP-D and is effective in determining certain disease types. One major issue is the invasive nature of BAL SP-D sampling; it limits the use of this ratio in wide-scale disease monitoring [156].

Therefore it is observed that SP-D is not only as a marker for asthma and rhinitis but also as a functional suppressant of hypersensitive allergic responses. The protective mechanisms by which SP-D suppresses allergenic challenge involves various levels; direct interaction with an allergen, crosslinking IgE and allergen with histamine release, important immune cell suppression (mast cells and eosinophils), suppression of T and B cell proliferation and modulation of DCs and macrophages [141].

1.5 IgE-IgG4 BALANCE in allergy and helminth infection

The complex cascade of immunoglobulins at various intervals has led to the proposal for a time mediated model of human IgE and IgG function [157] with the persistence of the antigen finally stimulating high-affinity IgG4 that outcompetes other isotypes and can terminate IgG1-mediated activation via the inhibitory FcγRIIB [31]. The basic effect of Th2 cytokines IL-4 and IL-13 on B-cells during clonal expansion induces the switching of immunoglobulins to IgE but also IgG4 [158]. The research suggests that during helminth infection, immune responses and hyper-reactivity is mediated by a balance of parasite-specific IgE and IgG4.

There are two mechanisms by which this would occur; **firstly** because plasma cells switch sequentially from producing IgM via IgG4 to IgE [33], a cessation of class switching would lead to a cessation in production of IgE expression at germline. **Secondly**, IgE is bound to high-affinity Fcε receptors on cells (basophil, eosinophil, or mast cell) [83], and IgG4 is free in serum [80]. IgG4 as a subclass functions as a sort of 'blocking antibody' resulting from its direct competition with IgE for antigen binding in serum, when both isotypes are close to helminth antigen. IgG4 does not fix to complement and binds weakly to Fcγ receptors [159] therefore when it outcompetes IgE (bound to functional immune cells) it causes minimal or very small consequences, therefore down-regulating the effects of antigen-specific IgE. In patients with lymphatic filariasis even though IgE is associated with natural resistance to infection [10,11] or re-infection [12–14] elevated levels of IgG4 is related to asymptomatic infection state, with IgG4 competition with IgE leading to this [15,16]. This points to a mechanism by which IgG4 enhances parasite survival by inhibiting IgE-mediated effector responses.

Blockage of specific IgE antibody by IgG4 is not just at the level of helminth but also in allergy. Allergic disease that is in a controlled phase is associated with the balance shifting towards increased allergen-specific IgG4 and decreased allergen-specific IgE [160]. The data suggests that this control is mediated through IL-10, which acts indirectly to modulate the production of IgE and directly on B-cells to drive IgG4 production [160]. IL-10 has also been implicated in the dampening of allergic responses and increased production of allergen-specific IgG4 in patients receiving allergen-specific immunotherapy [39]. The modified Th2 environment that is created by chronic helminth infection is also characterized by high levels of IL-10 secretion [40]. Therefore the elevation of IgG4 in the same environment indicates IgG4's direct involvement in the underlying regulatory response of chronic helminth infections.

It is postulated that the balance of immunoglobulins IgE and IgG4 in helminth infection and allergy could have a role in determining long term infection status and hypersensitivity reactions. Research shows this effect in endemic areas of *Ascaris* infection. ABA-1 is an *Ascaris* protein and potent allergen, but immediate hypersensitivity reactions (characterized by pulmonary *Ascaris* syndrome) are hardly seen [161]. By contrast, in seasonal areas of *Ascaris* infection, immediate

hypersensitivity responses are more common [162]. The mechanism at play here that prevents widescale hypersensitivity in endemic areas has got to do with sustained chronic exposure to *Ascaris* [161]. The research postulates that a different balance of ABA-1 IgG4 and ABA-1 IgE expression exists. A larger quantity of specific IgG4 is important in limiting pathology, in endemic areas, but also enough specific IgE still allows for control of infection and limiting worm burdens.

Interestingly, because of the tight immunomodulatory mechanism under which SP-D operates, and its link to type 2 immunity, could SP-D influence the IgE-IgG4 balance? SP-D binds to IgG, IgM, IgA, IgE, in a calcium-dependent manner [51], also specifically recognizing both Fc and Fab domains of IgG [147]. When IgG-coated beads were incubated with SP-D, the immune complexes formed large aggregates. Notably, macrophages avidly recognize these SP-D-induced IgG bead aggregates enhancing phagocytosis in murine macrophages [163]. Recent studies indicate that SP-D is present in the blood, and its concentration in the serum is significantly determined by genetics [164]. The SP-D-IgG interaction and following enhancement of immune aggregation and phagocytosis provide a means by which IgG-mediated responses are amplified [163], not only in the pulmonary environment but at various other mucosal tissues.

1.6 The old friend's hypothesis relating to regulatory DCs and the activation of TLR2 through SP-D

As stated previously, the hygiene hypothesis is based on the premise that exposure to pathogens during development and life of immunity primes the immune system and by default limits unwanted hypersensitivity responses (allergic disease). The old friend's hypothesis elaborates upon that with the essential insight still being pertinent, microbial exposure modulates our immune system. The development of allergic disorders and exposure to childhood disease is a small part of a much bigger evolutionary story; the old friend's mechanism is a much broader term not solely based around allergic response. It encompasses the idea that there is a broad spectrum of interactions between mammals and their microbial environment. Mammalian microbial interactions fall along a spectrum from endosymbiotic to various microbiotas in physiology to harmless organisms' encounters (commensals) to dangerous pathogens. This type of microbial

interaction, with dangerous pathogens, forms the original basis for the hygiene hypothesis.

The reasons for microbial exposure during youth are apparent in the sense that it provides signals for the development of the immune system. The more exposure to broad biodiversity of microbes builds up a repertoire of molecular structures that allows for more rapid detection of novel pathogens [165]. We also need background activation of innate immunity through microbial components such as LPS [166]. What is less obvious is that we also need to develop regulatory/control mechanisms to stop the immune system from becoming hypersensitive to inappropriate stimuli. If these regulatory mechanisms do not develop, we develop allergic disease [167] autoimmune disorders [168] and inflammatory gut diseases [169]. The rates of all of these conditions in high-income countries are increased. Therefore in areas that are classically defined as high income and urban, there seems to be a failure to shut off unwanted inflammation. Comparatively the low-income setting inflammation appears when needed but then is completely shut off when the immune threat (resolution of infection) has passed.

Organisms with which we co-evolved, therefore, had to be tolerated to ensure the survival of mammal and microbe. These included commensals in the microbiome, harmless organisms from the environment and a specific set of infections. These include helminth infections because they cause non-fatal or subclinical carrier states. The molecules produced by helminth infections have unique mechanisms that drive regulation and specifically expand Treg populations and cause DCs to switch to regulatory phenotypes [116]. An example of this would be when patients with multiple sclerosis are infected with helminths, their rate of relapse slows or stops altogether, and this is associated with increased Treg (myelin-specific) cells in peripheral blood [170]. This identification of regulatory mechanisms for chronic inflammation is well supported by evidence, with 'old friends' driving this regulation. It is postulated that this is interrupted by a modern urban lifestyle shift, not only in geography but diet, exercise etc. The mechanism of activation involves selective activation of TLR2 by regulatory DCs; curiously another molecule that activates TLR2 is SP-D. What could the effect be of SP-D on this system?

1.7 Aims and objectives

The first part of the work looked to identify if the **key markers of protection** identified in the mouse study are also found in human samples. It was hypothesized that there would be an overlap in the immunological effect between mouse and human protective immunity to helminth infections. It also looked at the atopic status and reviewed whether there was any influence on SP-D or helminth infection.

1) *Determine whether the exposure to a soil-transmitted helminth alters serum concentrations of surfactant protein D.* For analysis 2 cohorts of bio-banked samples recruited in helminth endemic areas of South Africa were obtained.
    i. **Cohort 1:** Women from Masiphumelele, Western Cape. Collaboration with Assoc. Prof. Jo-Ann Passmore. University of Cape Town.
    ii. **Cohort 2:** Rural and Urban Children Eastern and Western Cape: collaboration with Professor M. Levine, Red Cross Children's Hospital, Cape Town, SOSALL

2) *Determine if there are any correlates of protection or exposure that exist in human serum from the cohorts mentioned above.* In particular, the focus was on either marker of **acute** helminth exposure (i.e. participants with raised helminth specific IgE) or to markers of **chronic** helminth exposure (i.e. participants with raised helminth specific IgG4).

3) *Determine the role of **atopy** in the onset of helminth infection and functionality of SP-D in cohort 2.* This will be a comparison of the prevalence of atopy and helminth infection.

4) *Determine whether helminth infection has an influence on atopic disease, specifically atopic dermatitis.* This will be to determine whether the helminth infection influences the severity of atopy with particular focus on SCORAD (SCORing Atopic Dermatitis) values.

The second part of the work looked to identify if SP-D **influences the magnitude of anti-nematode responses** in human immune cells. IT has been hypothesized that the

association between helminth exposure and SP-D relies on immune cell interaction, the mechanism for which is undefined, but could involve type 2 innate lymphoid cells (ILC2s), monocytes and macrophages.

1) *A robust method of review was setup for cellular phenotypes of ILC2's, monocytes and macrophages isolated from human PBMCs.* Here flow cytometry was used to create a robust method of visualization due to the relative rarity of these immune cell types in human serum.

2) *This method will be used to review the influence of SP-D and helminth antigen and other cytokines on these immune cells.* The aim was to review particular cellular phenotypes and how they shift under *in vitro* stimulation that mimics the basic interactions; of human peripheral blood mononuclear cells with *Ascaris lumbricoides* antigen and SP-D. With particular focus on the effect of the following combinations; **(A)** SP-D and *Ascaris* individually or combined on monocyte CD16, **(B)** SP-D and *Ascaris* individually or combined on macrophage CD16, **(C)** SP-D and *Ascaris* individually or combined on macrophage CD206. It is worth noting that time constraints did not allow for experiments 2 (A-C) using ILC2s.

2 Materials and methods

2.1 Ethics and recruitment for human work

All experimental procedures were carried out in accordance with protocol number 066/2015 (SOSALL cohort) and 258/2006 (Masiphumelele cohort) and approved by the university of Cape Town Faculty of health science human research ethics committee.

The first cohort originates from **Masiphumelele** in Cape Town surrounds, a research project in collaboration with Associate Professor Jo-Anne Passmore (UCT) focusing on a cohort of 48 HIV- women [171].

The second cohort, **SOSALL**, aims to review helminth infection and sensitisation in rural and urban Xhosa children with or without atopic dermatitis. It is a nested case study with selected 12-36 month Xhosa children recruited from both urban (Red Cross War Memorial Children's Hospital, Cape Town) and rural environment (Nelson Mandela Academic Hospital, Eastern Cape) with a positive diagnosis of atopic dermatitis (AD), using scoring atopic dermatitis (SCORAD) method [172]. Age-matched control participants from rural and urban cohorts of SAFFA (South African Food Allergy study) were then recruited. These recruits had no evidence of atopy or food allergy. Figure 2.1 is a visual representation of recruitment, 220 participants recruited with stool available from 158 and serum available from 129.

Prior to this work, stool samples were analysed for worm ova and parasite load (Kato Katz examination kit, Vestergaard-Frandsen). *Ascaris* sensitisation determined measuring Asc-specific IgE (ImmunoCAP, ThermoFisher). Anti-*Ascaris* IgE levels of >0.35 kU/ℓ were considered positive.

Human PBMC (Peripheral blood mononuclear cell) samples obtained from four blood donors, two male and two female, in the form of buffy packs from the Western Province blood service with all samples being HIV and TB negative. This was used for cellular stimulation work.

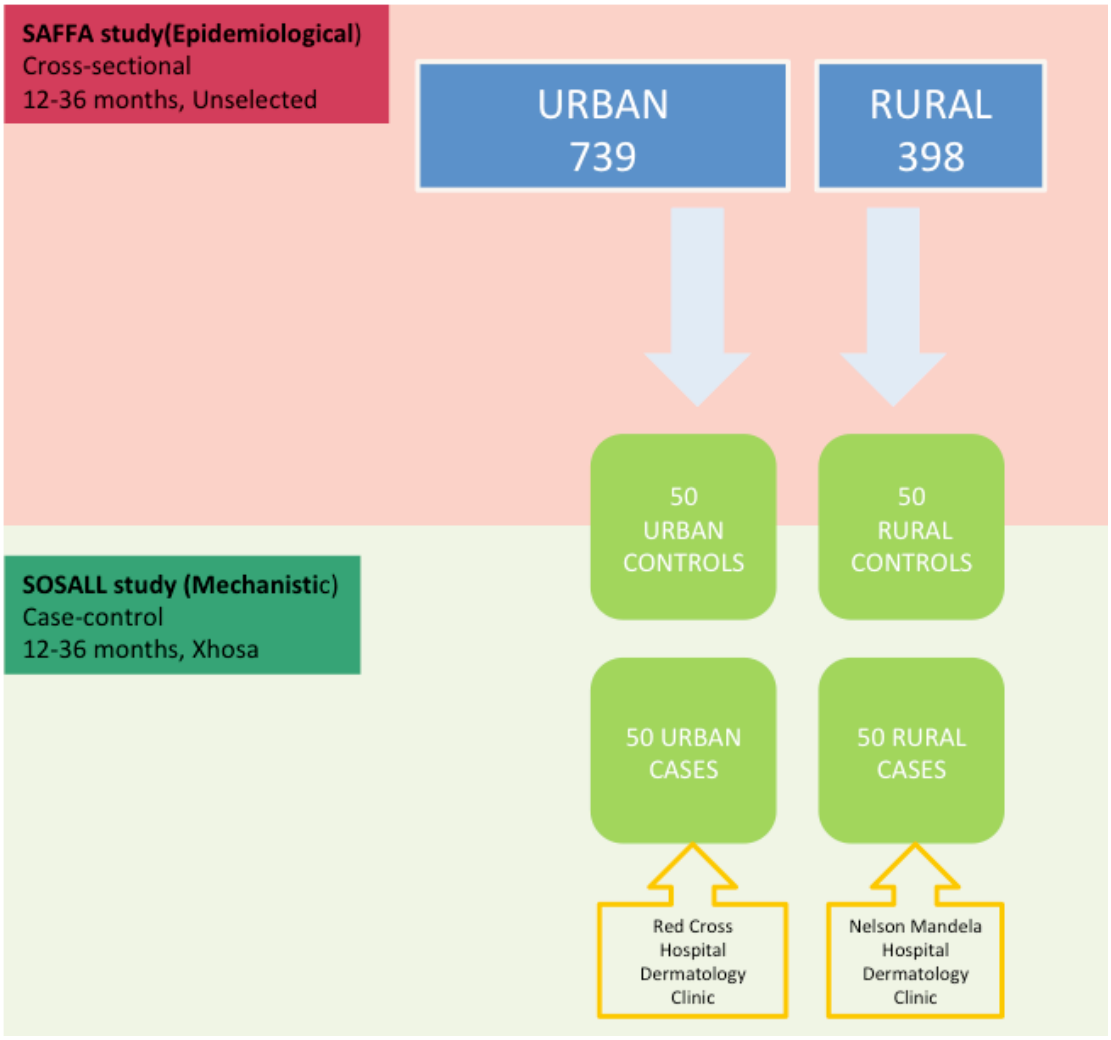

Figure 2.1: Visual representation of SOSALL cohort structure. A sample pool of 50 children was selected from rural (Nelson Mandela hospital) and urban (Red Cross hospital) locations that were atopic dermatitis positive (Skin prick test larger than 3 mm). The umbrella SAFFA study provided rural and urban control children that were atopic dermatitis negative.

2.2 Live worm and antigen preparation

To assess cellular responses to specific helminth infection *in vitro*, two different forms of antigenic stimulation were created.

Ascaris homogenization

The first type of helminth antigen used was created through the emulsification of *Ascaris* worms harvested from the small intestine of infected children at Red Cross Children's Hospital, Cape Town. These samples were then frozen at -80°C until needed. On-demand *Ascaris* worms were thawed added to an aliquot of Pen-Strep (10x) and fungizone and incubated for 1 hour. Segments from each worm were dissected and

pooled together in 50ml falcon tube with the remainder of worms frozen at -20°C for future use. Segments of the worm are mixed with PBS and homogenised to smooth "milkshake" consistency. Homogenous mix aliquoted into 2ml tubes and centrifuged at 1000g for 30min, supernatant sucked off and pooled into Eppendorf tubes. BCA was then performed to determine protein concentration. Samples then aliquoted and frozen at -80°C for use when necessary.

Ascaris larvae hatching

Larvae harvested from pig faeces on a Swiss farm and removed from sample tube using a serological pipette (add to 50ml falcon). Sample centrifuged at 500g for 5min and then resuspended in gravel media in falcon tube on the side and taped to shaker. Concentration calculated and the remainder of the sample containing eggs added to a flask of hatching beads (these are glass beads that slowly break away capsule surrounding eggs) with a magnetic stirrer for 10min. The magnetic stirrer moves very slowly, and this ensures the eggs hatch open but sensitive larvae are not destroyed. Check larvae under a microscope (+-80-90% eggs hatched is considered a good yield). Live worms are then used as a stimulant for *in vitro* cellular assays.

2.3 Western Blot

To assess variation in human Surfactant protein D levels with high specificity, a western blot analysis was performed. The kit used contained all hardware necessary (Mini-PROTEAN® Tetra Cell, Bio-Rad Laboratories, Inc.). The running and stacking gel was made as a 1mm 10% reducing gel. Human serum samples standardised by nanodrop in order to ensure that 10ug protein loaded per well. Samples diluted in PBS according to protein concentration and 10ul was removed and combined with 10ul SDS running buffer. The samples were then centrifuged and placed on a heat block at 90°C for 5min to ensure the reduction of proteins. Samples were then loaded, and then gel electrophoresis was performed at 100V for 15min and stepped up to 200V for 20min. The electrophoresis was then stopped and running buffer discarded.

The gel was then gently removed from gel cassette stand taking care not to touch or tear it. Layering the following made the sandwich for gel transfer: sponge, filter paper, gels, nitrocellulose, filter paper, sponge. This was then placed back into a mini tank and

submerging in transfer buffer. They were transferred for 1 hour at 100V. Once transferred, the nitrocellulose is removed from the sandwich and covered in ponceau stain, which indicates the presence of protein on the membrane it was then rinsed in blocking buffer (2% Milk Powder in PBS with and 0.1% tween20). The membrane was then topped up with blocking buffer and incubated for one hour. The membrane was then washed three times (10min each) with PBS 0.1% tween20 at room temperature on a shaker. Primary antibody was diluted (1:1000) in 5% milk powder (milk powder into 1x PBS and 0.1% tween20) and incubated at 4˚C overnight on a shaker. Wash step was repeated as above. The secondary antibody (1:5000) was diluted in 5% milk powder (milk powder into 1x PBS and 0.1% tween20) and incubated for 1 hour at room temperature. The chemiluminescent signal generated through the addition of LumiGLO substrate (Protein Detector™ LumiGLO® Western Blotting Kit, KPL). Detection and densitometry performed using BioSpectrum® Imaging System, UVP**.**

The SP-D western blot on human serum had not been performed in our laboratory before; therefore, the protocol had to be optimised to the sensitivity of the antibodies provided by Professor Howard Clark's lab in Southampton. Initially, the secondary antibody overdeveloped with a large amount of non-specific binding, above what should be expected in human serum western blots [173]. Some notable improvements upon this experiment include the following; addition of positive control for SP-D to quantify the samples directly; although samples were nanodropped and standardised to 10ug protein loaded per well the addition of loading control to ensure consistency in protein loaded.

2.4 Enzyme-linked immunosorbent assay (ELISA)

Human serum samples from a cohort with known IgE specific *Ascaris* and IgG4 specific *Ascaris* titers were quantified by sandwich ELISA to assess Surfactant protein D levels. The capture antibody (rabbit polyclonal anti-human SP-D antibody) was diluted at 1:1000 (stock 1.61mg/ml) in carbonate buffer (pH9.6), this was added at 50ul per well to 96well plates (Nunc Maxisorp; Thermo Fisher Scientific) and incubated overnight at 4°C. The plates were washed four times with wash buffer. Unbound sites were blocked using 200ul per well, of blocking buffer (2% Milk Powder in PBS) and incubated for 3 hours at 37°C. Wash step was repeated. The sample was then added at

50ul/well at dilutions of 1:4, 1:12, 1:48 in dilution buffer. The standard, rfhSP-D, was added with a starting concentration of 500ng/ml and titrated out (five-fold dilution series). They were incubated overnight at 4°C. The plates were washed further and 50ul per well of the biotinylated detection antibody (Hyb 246-4 biotinylated anti-Human SP-D antibody) added at 1:2000 in dilution buffer and then incubated for one hour at room temperature. Wash step was repeated, and 50ul of streptavidin-coupled Horse Radish Perioxidase at 1:5000 in dilution buffer was added. Wash step was repeated. The plates were developed with TMB microwell peroxidase substrate, and the reaction was stopped with 1M H_3PO_4. The plates were read at an absorbance of 450nm using a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.5 Cell bank

To optimise and construct the multi-colour flow cytometry panels, a stock of peripheral blood mononuclear cells (PBMCs) was created using buffy packs of blood obtained from the Western Province Blood Transfusion Service. Below is a visual representation of the workflow from collection through to experimentation (Figure 2.2). These buffy packs are ideal as they contain a high concentrate of cells from donors that are HIV negative. Two male and two female donors were utilised.

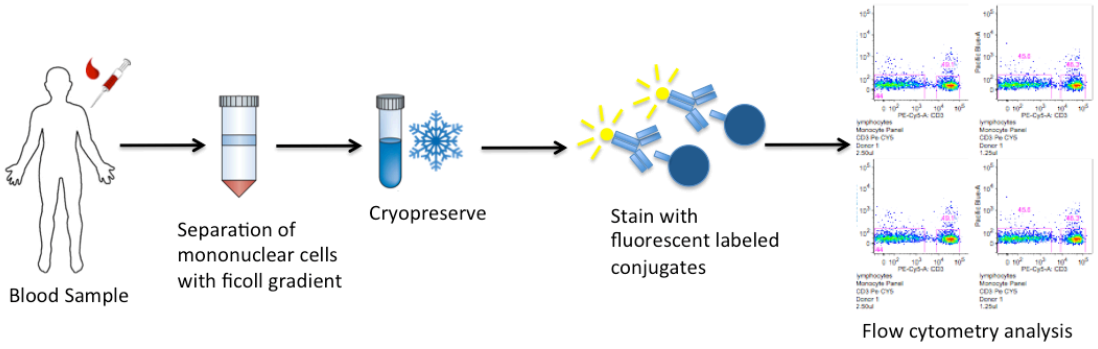

Figure 2.2: Workflow for isolation, cryopreservation and analysis of peripheral blood mononuclear cells. Starting with the extraction of blood samples and storage at the western province blood bank, separation of mononuclear cells with ficoll gradient, cryopreservation, staining with fluorescently labelled conjugates to data collection and analysis via flow cytometry.

2.5.1 Isolating PBMCs

This isolation used Ficoll-Hypaque (Sigma Aldrich) for density gradient centrifugation to separate PBMCs from blood plasma, erythrocytes and granulocytes. After centrifugation, plasma and platelets are located at the top of the density gradient since they have a lower density compared to red blood cells, ficoll and PBMCs. The next

layer formed is the PBMC layer, which consists of lymphocytes and monocytes, and below the ficoll layer are the red blood cells with the greatest density. The Ficoll, Fetal Calf Serum (FCS) (heat-inactivated for 30min @ 60°C) and wash buffer (1% FCS HI in PBS) was equated at to room temperature. A large 50ml leucosep tube (Greiner Bio-one) was used due to the quantity of blood in buffy packs. The tube consists of a filter disk, which allows for initial separation of blood and ficoll. 15ml of ficoll was added to 50ml leucosep tube and centrifuged for 1min at 2200g brakes on, forcing the ficoll below the leucosep gradient. The buffy pack or blood tube was mixed thoroughly before opening. For Buffy packs, 30ml was removed into cell culture flask, and 60ml PBS was added. Approximately 25ml of blood was then layered on top of the leucosep filter disk in each 50ml tubes and centrifuged for 18-22min at 2200g, brakes OFF to ensure the separation was not disturbed during centrifuge deceleration. The tubes were gently placed back into the rack, and opaque PBMC layer was identified. This layer was then harvested using a pipette being careful not to dip into the ficoll layer underneath which can affect cell survival. Donors were then pooled if centrifuged in separate falcon tubes. A top up with wash buffer and spin at 1500g for 10min (Wash 1 completed) was then performed. Only if a pellet had formed the supernatant was decanted. Wash buffer was topped up again, and the pellet was resuspended (Wash 2 completed) and centrifuged down again at 1500g for 10min. (*note buffy packs can house a lot of debris with 1-2 more wash steps being beneficial in removing this excess)

2.5.2 Cell count

Approximately 10ul of sample per donor was removed and aliquoted into 96well plate (Nunc Maxisorp; Thermo Fisher Scientific). Then an equal quantity of trypan blue was added. 10ul of this sample was then pipetted into a haemocytometer and placed under the microscope at 40x magnification. The PBMC's were counted in the top left and bottom right squares, being careful to dissociate them from red blood cells, with care taken not to count any dead cells identified by trypan blue.

Sample concentration = Count x ml x 10^4

The sample count was equated to an optimal freezing concentration of 20 x 10^6 cells. The cryovials containing sample did not exceed 1ml in volume.

2.5.3 Cryopreservation

This procedure involves preserving cells at very low temperatures, in liquid nitrogen, to re-analyse samples at a later time with minimal damage. To ensure PBMC survival during freezing, the cells are resuspended with 10% DMSO in FCS. The Dimethyl Sulfoxide Freezing Media (DMSO) is a polar solvent that penetrates cell membranes without damage, protecting the cells from ice crystals that form whilst maintaining the integrity of cell membranes. Fetal calf serum (FCS) contains essential growth factors and proteins required for cellular survival. The required volume of freezing solution (10% DMSO in FCS) was made up and refrigerated (4°C) before use, as it is temperature-sensitive. The tubes were centrifuged down at 1000g for 5min and pellet resuspended with 10% DMSO in FCS (added dropwise) at an optimal concentration of 20 x 10^6 cells. 500ul quantities of samples were aliquoted into freeze proof labelled cryovials. A Mr Frosty cryopreservation unit (Thermo Scientific) contains isopropanol and ensures slow step freezing of samples. Cryovials were placed in the Mr Frosty and into -80°C freezer for 24hours, ensuring slow step freezing of the PBMC's at -1°C per minute. After step-freeze, they were transferred into a liquid nitrogen storage tank (-192.2°C).

2.5.4 Thawing

Frozen PBMC samples were thawed by being placed in a 37°C water bath for 1-2 min until a tiny ice crystal remained. They were then transferred to FACS tubes and processed according to overnight incubation stage or direct staining protocols.

2.6 Macrophage culture and growth

This involved the preparation of monocytes and monocyte-derived macrophages for harvest and staining. Once PBMCs have been isolated (see 2.5.1) via density gradient centrifugation, with ficoll-hypaque, they are refrigerated and ready for the start of magnetic-activated cell sorting. To create isolated pools of monocytes and monocyte-derived macrophages, the sorting is based on the presence of CD14. Cells are centrifuged and resuspended in cold MACS buffer at 1x10^7 cells and incubated for 30min. Microbeads (specific for CD14) are added then another incubation for 30min. The sample is centrifuged at 500g for 5min and then resuspended in the buffer to prepare for magnetic isolation. The cell suspension is added to a LS column that has been rinsed with MACS buffer, unlabeled cells are collected in flow through, with

multiple washes. The column is then placed outside of the magnetic field in a tube and rinse to collect labelled cells. Cells are then counted and divided between cells to be cultured and cells that will directly go to FACS. Cells are resuspended in M-CSF at a final concentration of 20ng/ml and then plated out at $5x10^6$ cells (10ml) in Petri dishes. At day three cultured cells are replenished with 5ml of M-CSF medium. At day seven cells are counted (MDMs are strongly adherent therefore they must be scraped from petri dish with cell scraper then transferred to falcon tubes before counting) and the sample removed for FACS control, the rest is centrifuged at 1000g for 5min and dissolved in complete medium ($0.4x10^6$) and stimuli is added to cells. The remaining cells are centrifuged at 1000g for 5min and dissolved in freezing medium recipe. For the final macrophage harvest and staining; cultured macrophages (isolated CD14+ PBMCs) that have been in GM-CSF for seven days, are removed from media (via scraping technique (see above), washed and then incubated with PBS & EDTA for 10min (1ml 5uM EDTA in 50ml PBS) cells are then counted and stained for FACS. The stimulation of monocytes and macrophages in vitro was performed over 24hour period after culture and growth of the cells. GM-CSF media in addition to hatched larvae and/or rfhSP-D was added and incubated overnight.

2.7 Flow cytometry

2.7.1 Machine selection and mechanics

The critical aspect of designing a multi-colour flow panel is to understand the various machines and their configurations that are available. Each flow cytometer retains a different internal setup with variations in bandpass filters and the number of lasers. The initial design revolved around an LSR II (BD Biosciences) with three lasers. During the optimisation, the panel was moved over to a Fortessa (BD Biosciences) (Figure 2.1) with four lasers to allow for more markers to be added to the panel.

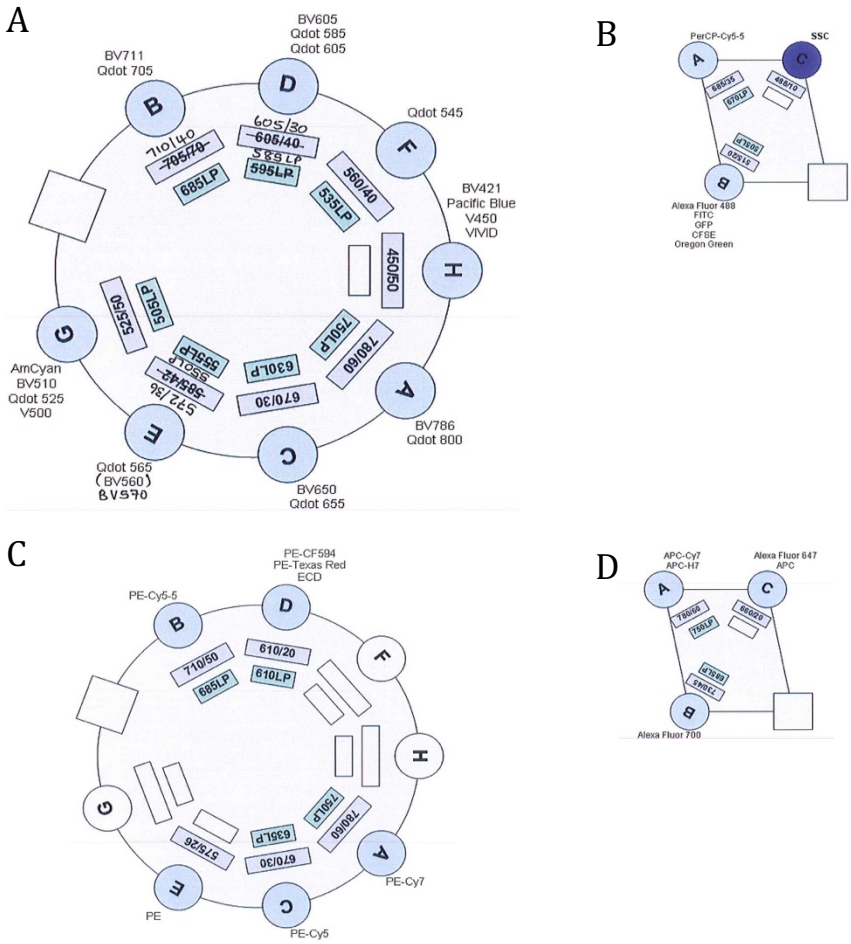

Figure 2.3: Fortessa flow cytometer configuration. Configuration of the Fortessa flow cytometer used for the largest portion of flow cytometric analysis. Another machine used was SATVI LSR II. (A) Filter configuration of Violet laser (405nm). (B) Filter configuration of Blue laser (455nm). (C) Filter configuration of Green laser (544nm). (A) Filter configuration of Red laser (633nm). Diagrams supplied by the South African Tuberculosis Vaccine Initiative (SATVI).

2.7.2 Marker selection

The markers present on specific cell types, both surface and intracellular, needs to be identified to obtain the correct anti-marker antibodies. Choosing the markers for monocyte panel was relatively straightforward with human subsets defined as classic, intermediate and non-classical subsets based on surface receptor repertoire of HLA-DR, CD14 and CD16 [67] [174]. Recently genome-wide expression studies [175] have further sub-divided the intermediate monocytes into regions of increasing CD16. Defining the phenotypes of helminth infected and uninfected children in a rural setting would then involve CD14, CD16 and HLA-DR. Additionally, a lineage cocktail to gate out lymphocytes (CD3, CD19). To date, only two studies have characterised innate

lymphoid cells in human cohorts with infectious disease. Firstly Boyd *et al.* defined all ILCs as Lin-CD127+CD45+ and the expression of cKit (CD117), showing that exposure to microfilariae can attenuate ILC2 responses [176]. Most recently, Nausch *et al.* [177] performed a study on a rural cohort of children in Zimbabwe and created a simplified gating strategy, defining ILC2s as Lin-CD45+CD127+CD161+CD294+. This formed the basis for our choice of marker and fluorochrome combinations. When our research moved to Switzerland, adding a CD206 (Mannose receptor), which is present on macrophages.

Table 2.1: Monocyte and macrophage antibody panel for flow cytometry

Monocyte panel	Fluorochrome	Cellular expression	Function	Monocyte presence
CD3, CD19 (lineage)	Pe/Cy5	T-cell lineage, B-cell lineage	Ig complex required for cell surface expression of TCR, part of BCR complex	-
CD14	Brilliant violet 605	Monocytes, Macrophages, Neutrophils, Eosinophils	LPS receptor	+
CD16	Brilliant violet 421	NK cells, Macrophages, Monocytes, Neutrophils	Component of Fc receptor for IgG	+
HLA-DR	FITC	Antigen presenting cells (Macrophages, DCs, Monocytes etc)	MHC class II cell surface receptor	+

Table 2.2: Type 2 Innate Lymphoid cell antibody panel for flow cytometry

ILC2 panel	Fluorochrome	Cellular expression	Function	ILC2 presence
CD3, CD14, CD16, CD19, CD20, CD56, CD11c, CD123	FITC	Various immune cell types	N/A	-
CD45	Alexa Fluor 700	All hematopoietic cells (highest on lymphocytes)	Antigen receptor mediated interaction	+
CD127 (IL-7Rα)	Pe/Cy7	Lymphoid progenitor marker	IL-7 receptor chain	+
CD161	Brilliant Violet 421	Th17 cells and NK cells	Inhibit NK cytotoxicity	+
CD294 (CRTH2)	PE	activated Th2 cells and Eosinophil's	Chemoattractant receptor (in allergy and inflammation)	+
CD117 (c-KIT)	PerCP/Cy5.5	Hematopoietic cells, T and B progenitor cells, mast cells	Interaction with signaling molecules	+

2.7.3 Fluorochrome selection

After markers of interest had been identified, the next step was to place them onto fluorochromes that would best suit their rarity. Generally, the brightest fluorochromes are placed onto the rarest of markers. Separation of significant populations also needs to be considered, which is best with a bright marker, although too much brightness can lead to unintended spill over into other channels. The final consideration is that not all markers are available commercially on all fluorochromes. The monocyte panel's arrangement was adapted from our lab's current human monocyte panel (Table 2.1) replaced with brighter and more accurate fluorochromes. On the ILC2 panel, there were three rare markers, which were updated with brighter options based on the Nausch *et al.* panel (Table 2.2).

2.7.4 Assessing spectral overlap and bleed through

The spectral overlap of fluorochromes was critical in determining how functional the panel became – too much spillover would lead to problematic compensation. Therefore it was in our best interest to choose markers that were situated with the largest gaps between peak emissions. This overlap was modelled on Biolegend's Fluorescence Spectra Analyser available on their website (www.biolegend.com). This allowed us to view the emission and excitation of various antibodies selections and overlay them onto the flow cytometer setup by inputting laser lines and filters (Figure 2.1).

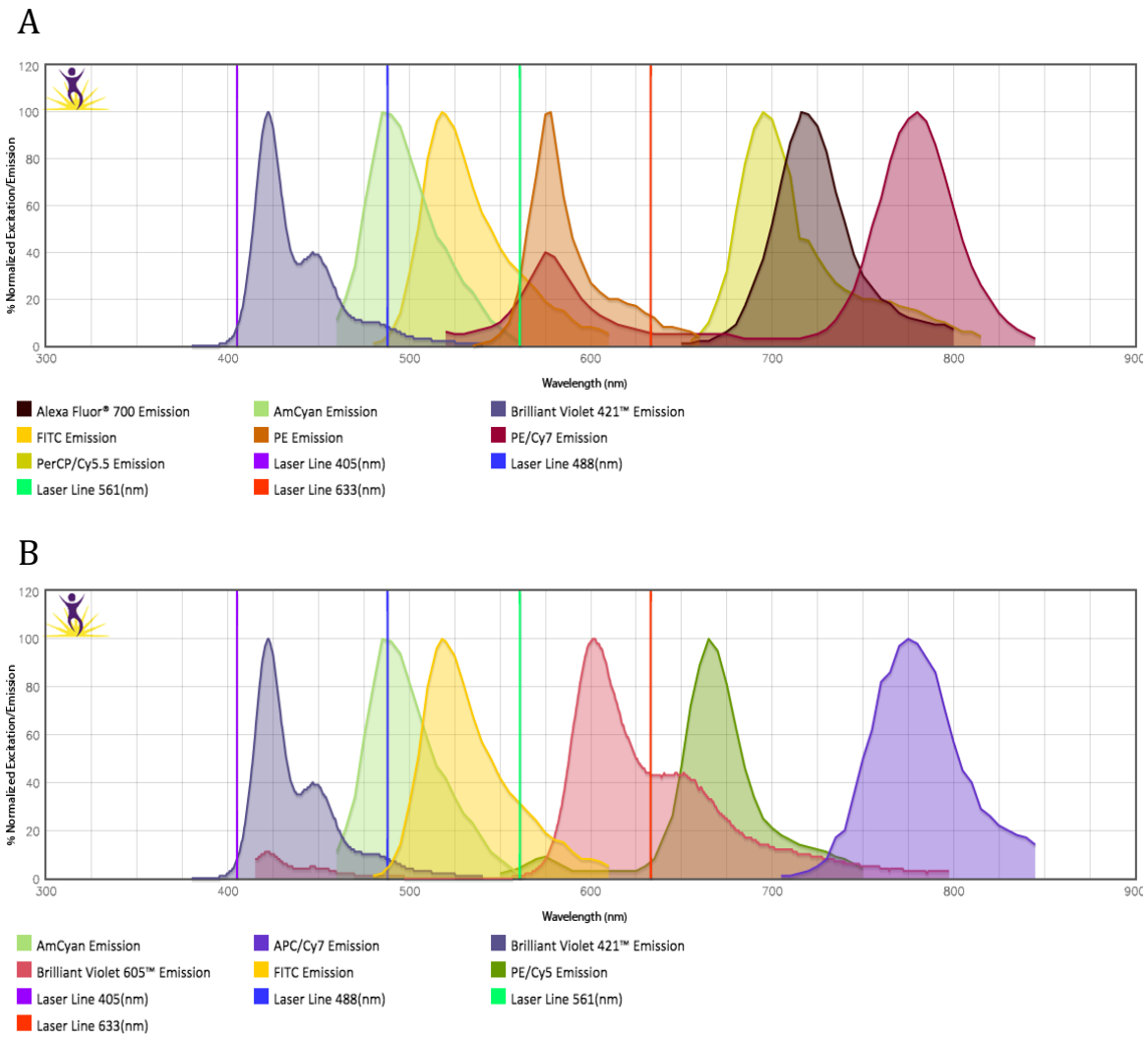

Figure 2.4: Spectral overlap of fluorochromes used to assess flow cytometry panels. Biolegend spectral viewer tool used to design the panels in best combination possible taking into account available fluorochrome-antibody combinations. Both panels exclude the viability marker, which emits as Amcyan. (A) ILC2 panel. (B) Monocyte panel.

2.7.5 Surface staining

Table 2.1 and 2.2 highlights a list of antibodies used in this protocol. Individual antibody titrations then determined the concentration at which antibodies were used—staining approximately $1x10^6$ cells per sample. The samples were stained in 96-well V-bottom plates with 50µl antibody master mix (FACS buffer 2% FCS in PBS with optimised antibody dilution) for 45min in the refrigerator before the cells were washed twice in FACS buffer to remove excess unbound antibodies. The cells were resuspended in FACS buffer and read by a three laser Becton Dickinson LSR II machine and the data was collected DIVA© BD (San Diego, CA). FMO's were used as controls. The data was analysed by FlowJo© Treestar (Ashland, OR) and graphed

with GraphPad Prism software. Appropriate FACS compensation beads (BD bioscience) were used to compensate between the signal intensities of each fluorochrome to avoid spectral overlap.

2.7.6 Viability marker

The use of a viability marker to exclude cell death from the analysis is critical when it comes to using frozen down PBMCs. The inclusion of dead cells in marker specific gates could create false positives and increase non-specific background noise.

2.7.7 Titrations

Staining performed as per surface staining protocols listed above—titrations designed as single stain samples. Antibody volumes serially diluted from manufacturers recommendations (5ul per million cells for everything except lineage cocktail, 1ul per million cells) down to 1/8 of recommended volumes. No compensation required for single stain cell tubes. Optimal antibody concentration is calculated by measuring Mean Fluorescent Intensity (MFI) on cell populations positive for the antibody and measuring the MFI of the negative populations, which do not express the antibody. The positive MFI divided by the negative MFI gives the staining index or signal to noise ratio. These are compared over a titration series. A greater staining index is associated with better separation; however, over-straining can increase background noise.

2.7.8 Fluorescence minus one (FMO)

FMO's performed as per surface staining protocols listed above. Designed to full stain cells of interest minus the antibody being tested. All antibodies required an individual FMO tube. Compensation performed using single stain compensation tubes and beads (for rare markers). Plotting the FMO channel versus all the other fluorochromes reveals any fluorescence spillover. To determine this experimentally, cells were stained with all antibodies in the panel, excluding the one of interest. This is performed for all antibodies in the panel.

2.7.9 Preparation of compensation controls (cells and beads)

For antibodies that were abundant and highly expressed on PBMC's cell compensations were performed. For cell compensations a single stain tube created with the antibody of interest. No viability stain was added. For antibodies that were rare and ranged in the expression on PBMC's bead compensations were performed. For bead compensations,

the isotype of the antibody was sourced from the manufacturer. According to isotype, either rat or mouse compensation beads were used (BD Biosciences). A single drop of beads was placed into a FACS tube, and the antibody of matching isotype was added. A single unstained tube per isotype was also made. These were then incubated in the dark for 10min. To that mix 500ul of PBS was added and the tubes were centrifuged at 2100g for 3min. The supernatant was decanted, and the dead volume was resuspended to be analysed with flow cytometry.

2.7.10 Flow cytometry

Flow cytometry is a powerful technique that can be used to investigate immune responses. Characteristics of an individual cell can be identified within a cell suspension by detecting cell surface and intracellular markers [178]. It requires a specialised instrument, a flow cytometer, to measure the fluorescence of each cell [178]. The measurement is performed through specialised lasers that excite the fluorochrome-conjugated to the antibody that binds to the target of interest on the cell. The fluorochromes are excited at a particular wavelength and emit light at a longer wavelength. The filter system on the flow cytometer is designed with specific fluorochromes in mind to capture as little background noise as possible. In this context, flow cytometry can give us unique insight into the phenotypes of our cells of interest.

2.7.11 Data acquisition and gating

Samples were stored in the dark at 4°C after staining and acquired within 12hours of storage. Data was acquired on the LSRII (BD Biosciences) and Fortessa (BD Biosciences) with FACS Diva software. The number of events collected for samples ranged from 500,000 to 2,500,000. The analysis was performed using FlowJo version 10.0.8 (Treestar) and Prism GraphPad (version 6).

3 Systemic SP-D levels in humans with exposure to soil-transmitted helminths

3.1 Introduction

SP-A and SP-D contribute significantly to control mouse-modelled nematode infections (Thawer *et al.*, Plos Pathogens [69]). This work has shown that infection with *N. brasiliensis* correlated with increased lung SP-D levels (Figure 1.6) and that administration of recombinant fragment human SP-D (rfhSP-D) protected against *N. brasiliensis* infection, as demonstrated by reduced worm burdens in rfhSP-D treated mice (Figure 1.7). This protection is associated with increased populations of type 2 innate lymphoid cells (ILC2) and $CD4^+$ T-cells (Figure 1.7). Subsequently, we hypothesised that SP-D could function as a novel anti-helminthic in human infections.

In this chapter we investigate whether an association exists in humans between systemic levels of SP-D and previous or current exposure to 3 species of helminths endemic to South Africa; *Ascaris lumbricoides*, *Trichuris trichuria* and *Toxocara spp*. These soil-transmitted helminths are not only crucial in the context of Southern Africa but also worldwide, causing unnecessary morbidity specifically in young children [35].

Classified as a hydrophilic pulmonary collectin, SP-D plays a vital role in the modulation of mucosal immunity during exposure to microbes [134], mediating a range of infections [138], chronic non-communicable diseases [140] and allergy [139]. With the exact mechanism of interaction still to be identified, we postulate that the homeostasis of surfactant, as well as its homeostatic abilities, has a vital role in the physiology of disease. Damage to the epithelial layers during helminth infection initiates a type 2 immune cascade leading to the release of IL-25, IL-33 and TSLP, triggering downstream activation of effector cell populations that can promote helminth expulsion (Figure 1.4). As previously stated, during helminth infections involving parasites transiting the lung, the primary cell type releasing IL-33/IL-25 and priming anti-helminthic responses are alveolar type II cells (ATII cells) [133]. Further, a major immunomodulatory protein released by ATII cells is surfactant protein D (SP-D). Previous research conducted in our group demonstrated a critical role for SP-D in

controlling helminth infection in mice, the caveat being that the data is not directly relatable to human physiology. Here we translate these findings using human samples to determine whether an association exists between helminth exposure and SP-D.

As stated in the introduction, an individual with an unusual IgE response is deemed 'sensitised', and exposure to an allergen leads to diseases such as asthma, atopic dermatitis, allergic rhinitis and food allergy [79]. The type of allergic disease presents as different pathophysiology depending on the affected area. During both allergic disease and helminth infection, the severity of the disease is mediated in part by the balance of specific IgE and IgG4. In the context of allergies, high levels of allergen-specific IgE is phenotypically associated with a heightened state of allergic disease, but when the balance shifts toward allergen-specific IgG4 it enters a more controlled form of allergic disease. Allergen-specific IgE binds to effector immune cells such as eosinophils and mast cells that activate effector mechanisms at barriers (i.e. lung) and generate allergic responses. The entry of allergic disease into a controlled stage is associated with an increase in allergen-specific IgG4; the IgG4 inhibits IgE function by competitively binding to an allergen and, due to its negligible functionality, mediates inflammatory response. In the context of helminth infection, high levels of antigen-specific IgE is related to current acute infection or natural resistance. In contrast, elevated levels of antigen-specific IgG4 is associated with an asymptomatic or chronic state of infection [158].

We postulate here that when a helminth infects a patient with atopy (in particular lung-based infection), the disease outcome will be altered in comparison to a non-allergic person based on the balance of specific IgE and IgG4 being altered. This may involve the helminth infection exploiting the mechanism of allergic disease, pushing towards the production of IgG4 (driven through IL-10 production), which creates a microenvironment for the helminth to live with negligible inflammation, or in an asymptomatic state.

This chapter uses two separate cohorts:
1) Masiphumelele, Cape Town surrounds. Samples were acquired in collaboration with Associate Professor Jo-Anne Passmore (UCT) (Protocol number 258/2006).

2) An Allergy Foundation, South Africa cohort, recruited by Prof. Mike Levin. This second cohort comes from an umbrella epidemiological study known as the South African food sensitisation and food allergy (SAFFA) study. Whereby allergy prevalence was assessed in urban and rural children by screening for food allergy, aeroallergens and companion epidemiological data. SOSALL is a case-control sub-study focusing on mechanistic, which aims to review helminth infection, and sensitisation in rural and urban Xhosa children with or without atopic dermatitis, by using 220 age-matched rural and urban children using stool and serum samples.

Referring back to aims and objectives, our aim for this section is to identify whether the **key markers of protection** identified in our mouse study are also found in human samples. We hypothesise that there would be an overlap in the immunological effect between mouse and human protective immunity to helminth infections. We also look at the atopic status and review whether there is any influence on SP-D or helminth infection. Our objectives are as follows; **(1)** Does exposure to helminth infection alter serum levels of SP-D in humans **(2)** If alteration does happen, are there any correlates of protection/exposure that exist in human serum that indicates how helminth infections navigate human immunity and how SP-D operates around this. **(3)** Do we see any correlation between levels of atopy and SP-D in atopic cases and controls **(4)** Does helminth infection prevalence influence atopy?

3.2 Results

3.2.1 The Masiphumelele cohort: The association between helminth-specific immunoglobulins and SP-D is limited to antigen-specific IgG4

To establish if any relationship existed between raised detection of *Ascaris*-specific IgE or IgG4 and systemic amounts of SP-D, we carried out western blot detection (see materials and methods) of SP-D in serum samples that were already established to be either *Ascaris*-specific IgE or IgG4 positive or negative. *Ascaris*-specific IgE (CAPRAST) or *Ascaris*-specific IgG4 (ELISA) was also established. The samples were stratified as positive or negative for immunoglobulin titre (*Ascaris*-specific IgG4 or *Ascaris*-specific IgE), with 7 IgG4 positive and 7 IgG4 negatives, 7 IgE positive and 7 IgE negative samples (Figure 3.1). There were no differences in SP-D levels between samples positive and negative for IgE (Figure 3.1 A). In contrast, in samples positive for IgG4 we found a significant difference (*p=0.026) in SP-D titer, as compared to their controls (Figure 3.1 B). This figure illustrates that, in humans, there is a positive association between *Ascaris*-specific IgG4 and levels of surfactant protein D, an association that is as of yet, undescribed.

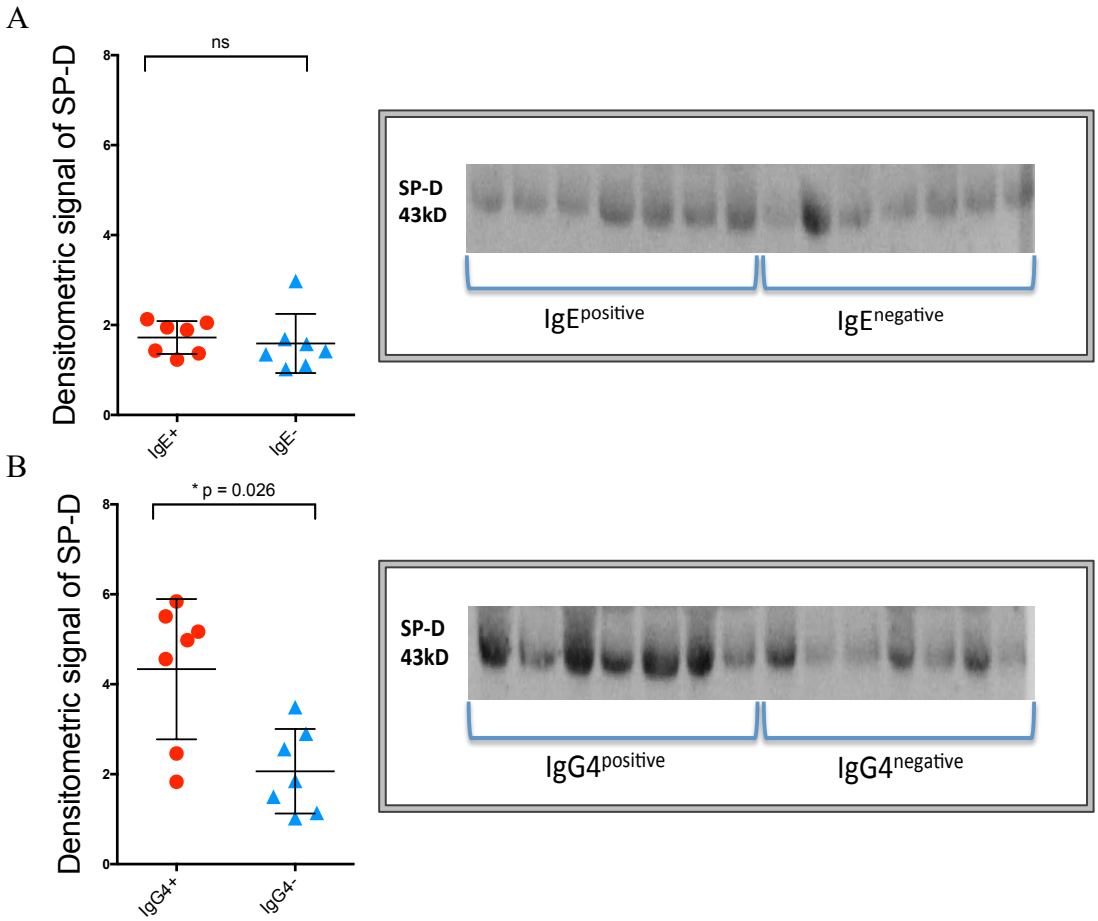

Figure 3.1: SP-D is positively associated with IgG4 levels. Serum samples with known *Ascaris*-specific IgG4 (quantified using ELISA) and IgE (quantified using CAPRAST) titres were tested for SPD by Western blot. Samples were stratified into (**A**) Women either positive (n=7) or negative (n=7) for *Ascaris*-specific IgE or (**B**) women either positive (n=7) or negative (n=7) for *Ascaris*-specific IgG4. The SP-D as the monomer is a 43kD band on the western blot. Significance was assessed using Mann-Whitney unpaired T-test.

3.2.2 Basic epidemiology of the SOSALL cohort

The SOSALL study is a follow-on of the SAFFA study (figure 2.1 in methodology). SOSALL aims to review allergic sensitisation in rural and urban Xhosa children with or without atopic dermatitis and the factors that may influence this sensitisation, including helminth infections. In total, 220 participants were recruited from the pool of SAFFA participants, with stool available from 158 and serum available from 129. The cohort epidemiological factors age, weight, height, antibiotic, asthma, allergic rhinitis (hay fever), dog owner, cat owner, combined ownership are described in Table 3.1. It is important to note that data collection was done verbally via survey and consultation with assigned medical professionals indicated some discrepancies to the verbal

responses. For example, some participants reported no allergy or eczema but tested positive (SCORAD) for eczema.

Table 3.1: Epidemiological data in the SOSALL cohort grouped based on geography and atopy.

		URBAN		RURAL	
	Total cohort	**Cases (AD+)**	**Controls (AD-)**	**Cases (AD+)**	**Controls (AD-)**
Age (median)	22 months (range 9-38)	24 months	24 months	21 months	20 months
Weight (average)	12,63 kg	13,63 kg	12,79 kg	12,11 kg	12,01 kg
Height (cm)	82,44 cm	82 cm	84,97 cm	80,21 cm	83,07 cm
Antibiotic exposure	76% (165) (n=217)	69,6% (39)(n=56)	67,3% (33)(n=49)	83,3% (50)(n=60)	82,6% (43)(n=52)
Asthma	10,1% (22) (n=217)	10,7% (6) (n=56)	16,3% (8) (n=49)	11,6% (7) (n=60)	1,9% (1) (n=52)
Hayfever	10,5% (23) (n=217)	23,2% (13)(n=56)	10,2% (5) (n=49)	8,3% (5) (n=60)	0% (0) (n=52)
Dog ownership	44,7%% (97) (n=217)	21,4% (12) (n=56)	23,7% (14)(n=49)	56,6% (34)(n=60)	71,1% (37)(n=52)
Cat ownership	19,8% (43) (n=217)	5,3% (3) (n=56)	10,2% (5) (n=49)	23,3% (14)(n=60)	40,3% (21)(n=52)
Cat or Dog ownership	50,2% (109) (n=217)	23,2% (13)(n=56)	32,6% (16)(n=49)	63,3% (38)(n=60)	80,7% (42)(n=52)

3.2.3 Urban AD negative children showed increased total IgG and IgG4 in the SOSALL cohort

In order to establish whether the positive correlation between helminth-specific antibody and SP-D is seen in a larger sample pool, we carried out a new set of ELISAs using samples from the SOSALL cohort. Baseline levels of the immunoglobulins total IgG, and total IgG4 were quantified. Samples detected above a specific value were deemed positive and quantified using arbitrary units based on endpoint titration ELISA. Results in this study were stratified into urban and rural components as well as the presence of atopic dermatitis (case)(AD+) or absence (control)(AD-). The criteria for selection of allergic cases were moderate to severe atopic dermatitis as determined by consultation with a medical professional and SCORAD analysis.

When grouped based on geographical location, total IgG was significantly (***p=0.0001) raised in urban participants (Figure 3.2 C) with that effect being a result specifically of total IgG in the urban control group being raised (**p=0.0071) (Figure 3.2 A); this is compared to urban case group as well as both rural groups. We also found the same trend in the urban population with total IgG4 significantly raised (*p=0.012)

(Figure 3.2 F), and again it was with that effect being anchored specifically by total IgG4 in the urban control group, which was raised (**p=0.007) (Figure 3.2 D). This may indicate differences in immune development or activation between rural and urban populations, which could play a significant role in the development of immune response.

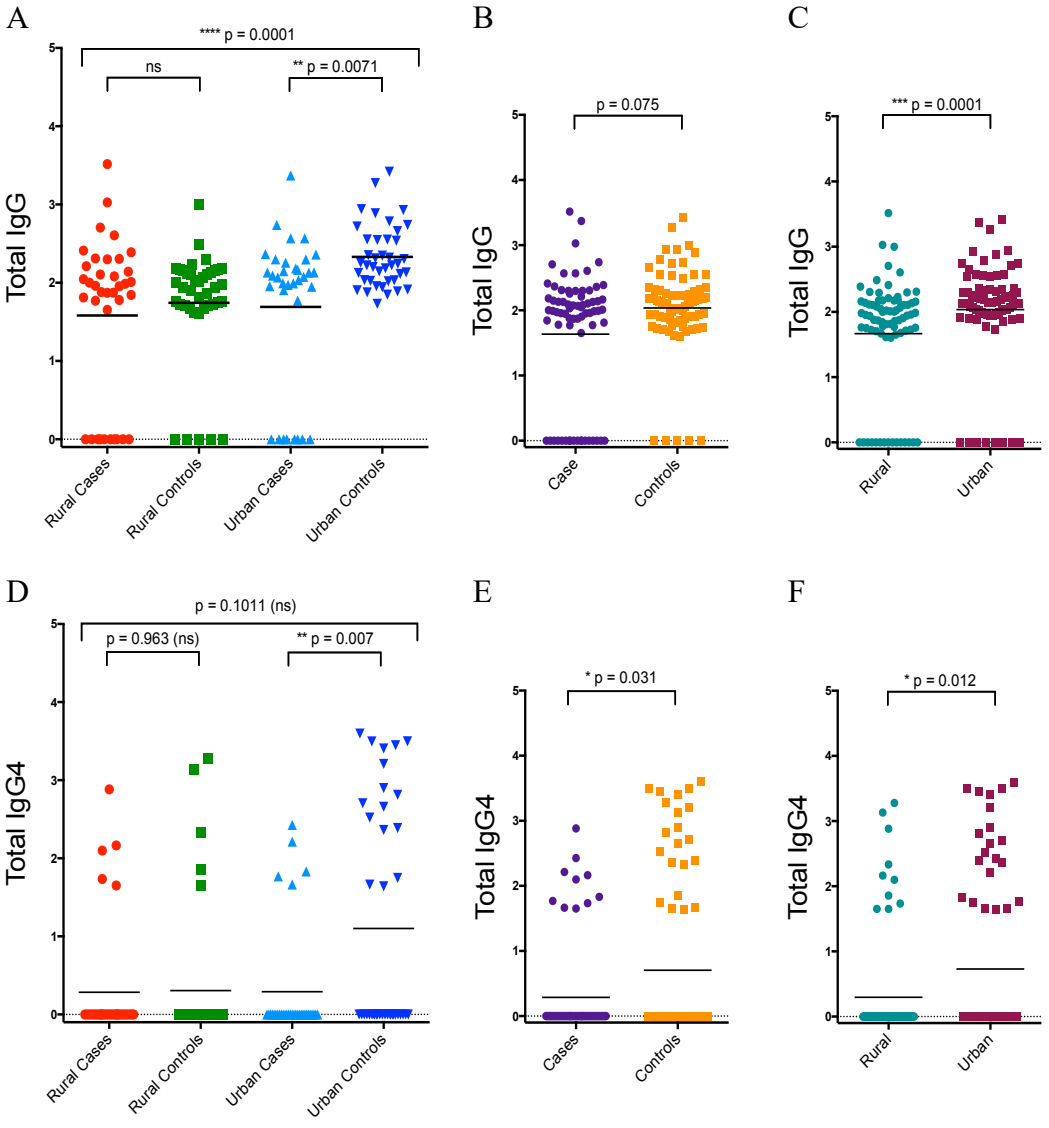

Figure 3.2: Total IgG and IgG4 titers were raised in urban AD- controls compared to rural and AD+ cases. Antibody levels from n=151 samples were measured by ELISA and stratified by the following ways: (A) Total IgG (n=131 samples above detection limit) (B) Total IgG case vs control (C) Total IgG rural vs urban (D) Total IgG4 (n=30 samples above detection limit) (E) Total IgG4 case vs control (F) Total IgG4 rural vs urban. Antibody titres are presented as log-transformed arbitrary units. Significance was assessed using the Kruskal-Wallis test or Mann-Whitney test.

3.2.4 Antigen-specific IgG4 was widespread in the SOSALL cohort. Rural AD+ participants showed increased *Ascaris*-specific IgG4

To establish whether the participants have previously been or were currently infected by specific helminth species, we quantified a known marker of helminth exposure. We tested for immunoglobulins from 3 soil-transmitted helminth species; *Ascaris lumbricoides*, *Trichuris trichuria* and *Toxocara* species (various). We used antigen-specific IgG4 as a marker of exposure [81] [157]. This is an immunoglobulin known to be produced in response to long term (chronic) or historical infection and is the rarest antibody subtype, indicating whether the individual has ever been previously exposed to the parasite, not just if there is a current infection (which would be characterised by the presence of IgE) [157] [179]. *Ascaris* (Figure 3.3 A), *Trichuris* (Figure 3.3 D) and *Toxocara* (Figure 3.3 G) show samples above cut off value stratified based on geographical location and atopy (AD+ or AD-). *Ascaris* (Figure 3.3 B), *Trichuris* (Figure 3.3 E) and *Toxocara* (Figure 3.3 H) show samples stratified based on atopy only (AD+ or AD-). *Ascaris* (Figure 3.3 C), *Trichuris* (Figure 3.3 F) and *Toxocara* (Figure 3.3 I) show samples stratified based on geographical location only.

The prevalence of helminth infection in our cohort was summarised in Table 3.2. CAPRAST detection of anti-Ascaris lumbricoides IgE detected low numbers of positive samples, indicative of low levels of acute or current exposure (data not shown). Both *Ascaris lumbricoides* and *Toxocara* spp antigen-specific IgG4 were detected in over 25% of study participants and *Trichuris trichuria* in 20% of participants, suggesting a high rate of previous long-term exposure to the parasites (Table 3.2). There is a discrepancy between the antigen-specific IgG4 and total IgG4 in our cohort. Despite having relatively large amounts of detectable antigen-specific IgG4, if we compare this to total IgG4, there are a lot fewer positive samples.

The detectable level of *Ascaris*-specific IgG4 in rural AD+ children was significantly increased (Figure 3.3). We also found significantly more *Ascaris*-specific IgG4 in rural participants overall (**p=0.0043) (Figure 3.3 C). We found significantly more *Ascaris*-specific IgG4 in AD+ participants (**p=0.003) (Figure 3.3 B). These trends are a result of significantly increased *Ascaris*-specific IgG4 in rural AD+ children seeing that detection of *Ascaris*-IgG4 in rural cases was significantly raised compared to the control group (**p=0.001) (Figure 3.3 A).

Table 3.2: Prevalence of previous helminth infection in the cohort based on the presence of parasite-specific IgG4

URBAN (Cape Town)		
	Cases (AD+)	Controls (AD-)
Ascaris lumbricoides	15% (*n*=20)	15% (*n*=40)
Trichuris trichuria	20% (*n*= 20)	25% (*n*= 40)
***Toxocara* spp**	25% (*n*=20)	25% (*n*=40)
RURAL (Eastern Cape)		
Ascaris lumbricoides	53.8% (*n*=39)	20% (*n*=40)
Trichuris trichuria	23% (*n*=39)	12.5% (*n*=40)
***Toxocara* spp**	30.7% (*n*=39)	30% (*n*=40)

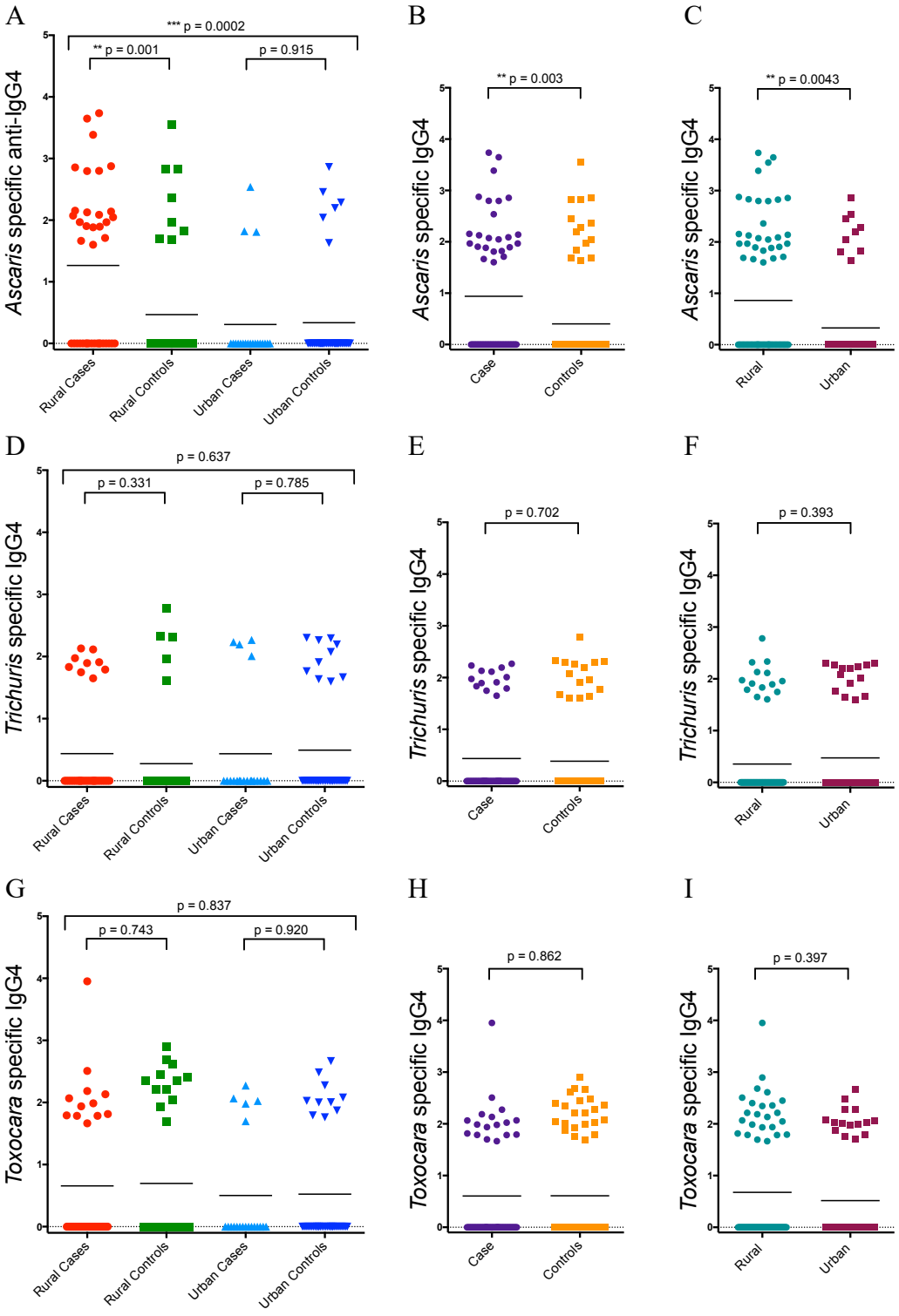

Figure 3.3: *Ascaris*-specific IgG4 titers were raised in rural cases compared to Urban cases and rural controls. Serum antigen-specific IgG4 titers from n = 151 samples were analysed by ELISA and stratified by the following ways: (A) *Ascaris*-specific IgG4 (n=38 samples above detection limit) (B) *Ascaris*-specific IgG4 case vs control (C) *Ascaris*-specific IgG4 rural vs urban (D) *Trichuris*-specific IgG4 (n=27 samples above detection limit) (E) *Trichuris*-specific IgG4 case vs control (F) *Trichuris*-specific IgG4 case vs control (G) *Toxocara*-specific IgG4 (n=38 samples above detection limit) (H) *Toxocara*-specific IgG4 case vs control (I) *Toxocara*-specific IgG4 case vs control. Antibody titres are presented as log-transformed arbitrary units. Overall significance assessed using the Kruskal-Wallis test. Intergroup significance assessed using Mann-Whitney test.

3.2.5 SP-D associated with helminth species that transit the lung stage as part of their lifecycles

To confirm the association we observed in the Masiphumelele cohort between SP-D and helminth specific IgG4 (Figure 3.1), and to assess whether this association is present in the SOSALL cohort, we compared the immunoglobulins present (Total IgG, total IgG4, helminth specific IgG4) with the samples' corresponding serum SP-D levels. SP-D was quantified using ELISA.

As was found in the first cohort, these results show that *Ascaris*-specific IgG4 was associated with raised levels of circulating SP-D (*p=0.02) (Figure 3.4 C). *Toxocara*-specific IgG4 also positively associated with raised levels of circulating SP-D (*p=0.0138) (Figure 3.4 D). In contrast, there was no association between *Trichuris spp* IgG4 and SP-D (p=0.76) (Figure 3.4 E). No association was found between show total IgG or IgG4 and SP-D, respectively (Figure 3.4 A and B). All associations were assessed using the spearmen correlation. The sample group size was n = 37 (*Ascaris*) and n = 38 (*Toxocara*), which is substantially larger than in the Masiphumelele cohort (n=7).

Ascaris lumbricoides is a common roundworm whose lifecycle involves a multi-organ migration through the host, including a vital lung stage that creates specific pathology. Larval migration, through direct mechanical force, causes substantial pulmonary damage with type 2 pathology resembling allergic airway disease (Figure 1.3) [38]. *Trichuris trichuria* is a common whipworm whose lifecycle is confined to the intestines, colonising the gut epithelium by epithelial tunnelling, but not totally exposing the mucus layer (Figure 1.3). *Toxocara* species (various) are common roundworms that when infecting humans create dead-end hosts; the lifecycle can cause serious pathology and exposure via companion animals either, domestic or livestock is common (Figure 1.3) [38]. We postulate that due to the variation of lifecycles between *Ascaris lumbricoides and Trichuris trichuria*, certain helminth infections have a greater effect on serum SP-D levels than others. In the case of both *Ascaris* and *Toxocara*, their lifecycles have stages that involve specific migration through the lung. As ATII cells in the lung are the chief source of SP-D, it stands to reason that these helminth species directly affect the production of this protein. They cause damage to the epithelial lining of the lung, just as asthma also causes epithelial damage, which leads to a release of

SP-D in the serum. We see the same effect here with helminth induced epithelial damage, with SP-D being positively associated with *Ascaris* and *Toxocara*-specific immunoglobulins.

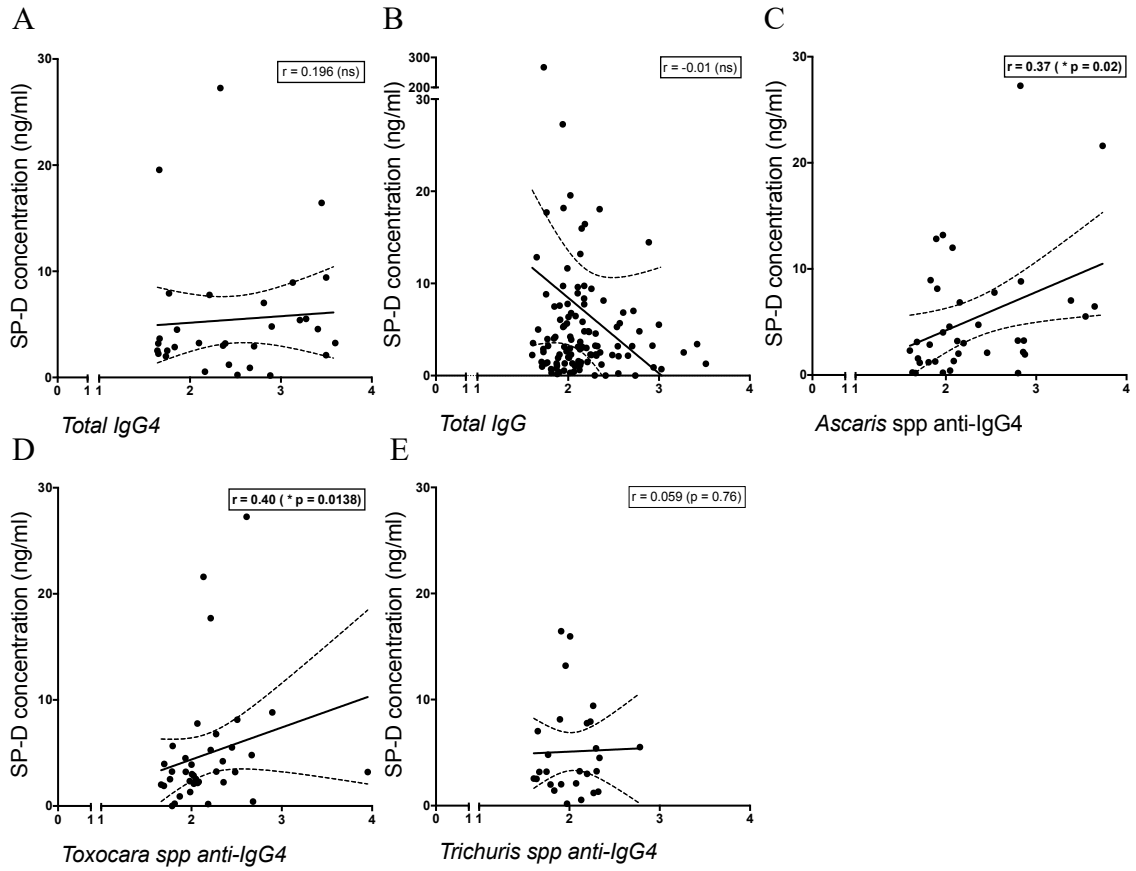

Figure 3.4: SP-D is positively associated with *Ascaris* and *Toxocara* but not *Trichuris*-specific IgG4 levels. Serum SP-D was quantified using ELISA and stratified by the following ways: (A) Total serum IgG (n=37) assessed against serum SP-D. (B) Total serum IgG4 (n=37) against serum SP-D. (C) *Ascaris*-specific IgG4 (n=37) against serum SP-D concentration. (D) *Toxocara*-specific IgG4 against serum SP-D (n=38). (E) *Trichuris*-specific IgG4 against serum SP-D (n=38). Antibody titres against helminth antigens are presented as arbitrary values—significance established by Spearman correlation.

3.2.6 Serum SP-D is not an indicator for atopic dermatitis or geographical location in SOSALL cohort

We assessed serum SP-D in the SOSALL cohort to establish whether geographical location or atopy had any effect on serum titers since SP-D is associated with protection from allergy and immunity to helminth infection [69] [149] [136]. SP-D was quantified using ELISA. We used a standardised control and to ensure as little experimental variation as possible, and this control was run on each plate at the same time using the same reagent sets for each set of samples. As with total immunoglobulin and antigen-specific data, we grouped the SP-D data according to their geographic and atopic

measures (Figure 3.5). No clear association was apparent between groups with similar medians across geography and atopy. However, rural AD+ participants showed marginally lower SP-D titers compared to rural controls, although this was not significant. This trend would perhaps this trend would become more apparent if the cohort size was increased. We also found large variations in cohort participant's serum SP-D levels. Most measured between 1ng/ml and 30ng/ml but some outliers measured as high as 1000ng/ml. SP-D can be present systemically and at mucosal layers in relatively large amounts; the reason for the outliers could be related to a number of reasons, including disease affecting the lung or other systemic conditions [141].

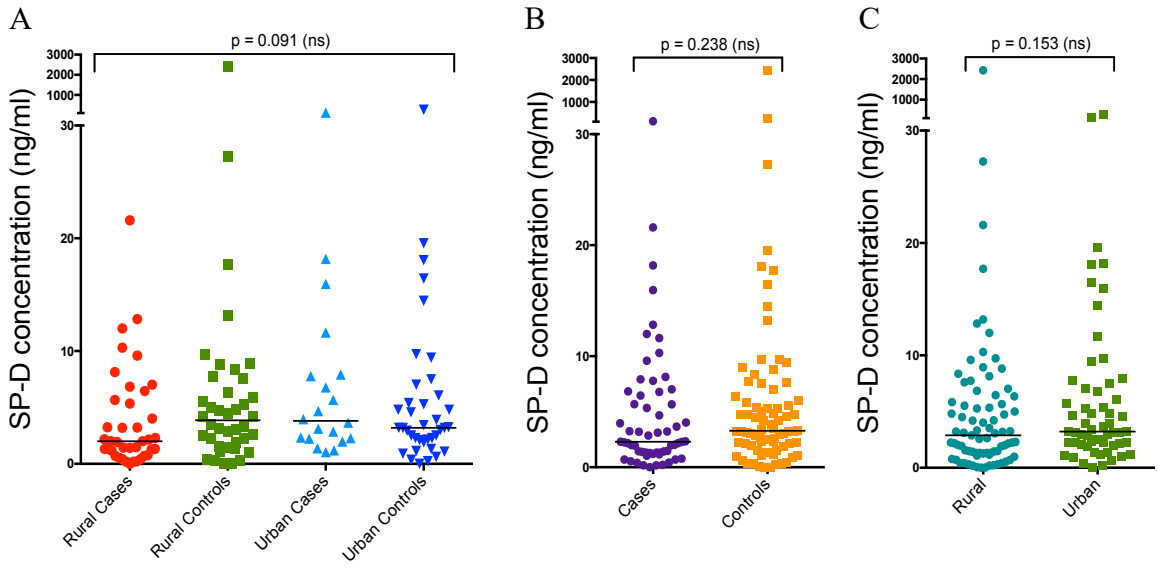

Figure 3.5: Surfactant protein D titers did not associate with atopic dermatitis or geographical location. Serum SP-D from n = 151 samples was quantified using ELISA and stratified by the following ways: (A) All cohort samples stratified based on location and allergic status (B) Samples compared based on case (AD+) or control (AD-) status. (C) Samples compared based on location. Significance assessed using the Kruskal-Wallis and Mann-Whitney test.

3.2.7 SP-D was not an indicator for the severity of atopic dermatitis

We also evaluated to what extent SP-D may play a role in allergic dermatitis. In the cohort overall, or split based on geography, we did not see any association between the severity of AD and serum SP-D levels. This means SP-D was not an indicator of the severity of atopic dermatitis. This was measured by assessing the atopic dermatitis positive children's SCORAD scores compared to serum SP-D. SCORAD (SCORing Atopic Dermatitis) is a standard test used to assess the severity of atopic dermatitis as objectively as possible; the scale runs from 0-100 based on various metrics including size, inflammation and discomfort of lesions [106]. It is a useful benchmark for

understanding the individual severity of atopic dermatitis and can be directly compared to SP-D to assess for any associations. As with total immunoglobulin and antigen-specific data, we grouped the SP-D data according to their geographic and atopic measures (Figure 3.5). SP-D concentration is reviewed against measured against all atopic dermatitis positive children in cohort regardless of geographical region (Figure 3.6 A), in the rural specific cohort (Figure 3.6 B), and against urban cohort (Figure 3.6 C). The SCORAD scores are also compared between rural and urban (Figure 3.6 D).

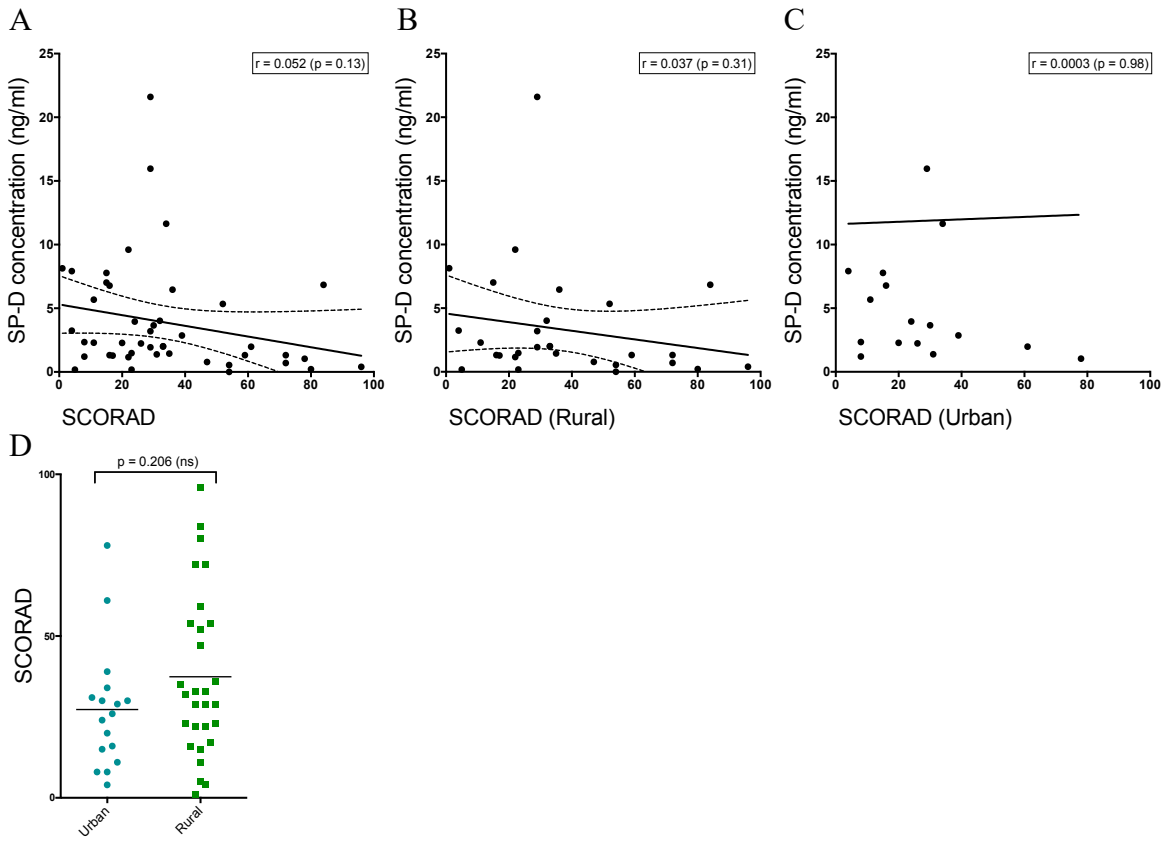

Figure 3.6: There was no association between participant's serum surfactant protein D and SCORAD scores. (A) All atopic dermatitis positive children's SCORAD scores against serum SP-D (ng/ml). (B) Rural atopic dermatitis positive children's SCORAD scores against serum SP-D (ng/ml). (C) Urban atopic dermatitis positive children's SCORAD scores against serum SP-D (ng/ml). Significance for all association curves was established by Spearman correlation. (D) Samples SCORAD scores compared based on geographical location. Significance assessed using Mann-Whitney test.

3.2.8 Participants with atopy have unusually high levels of *Ascaris* sensitisation but not active infection

These results have not been generated by this dissertation but still originate from the same study and pertain to the overall findings. Previous SOSALL Stool analysis (Kato-Katz method) was used to establish the number of actively infected participants (Data

generated by collaborators) in the SOSALL cohort; this data was compared to participants that had been sensitised to *Ascaris* (cleared or chronic infection, measured by *Ascaris*-specific IgE levels) based on whether they had atopic dermatitis or not.

Non atopic (AD-) control children had 6.7% (*n*=45) urban and 10.5% (*n*=41) rural kato-katz positive (active infection) stools; atopic (AD+) children showed no evidence of active infection (both urban and rural were 0%) (Table 3.3). The non-atopic controls also showed *Ascaris* sensitisation (Ascaris IgE >0.35kU/L) with 5.1% urban (*n*=39) and 9.7% rural (*n*=31) (Table 3.3). Despite no evidence of active infection both urban and rural case participants with atopy had high levels of *Ascaris* sensitisation, 21.4% (*n*=28) and 9.7% (*n*=31) respectively (Table 3.3).

As atopic participants had no active infection but were still sensitised to *Ascaris* (at higher rates than AD- controls for the urban group), this raises an interesting question: Are atopic children clearing active infection better than their non-atopic counterparts?

Table 3.3: Rates of active infection and *Ascaris* sensitisation in SOSALL cohort

URBAN (Cape Town)		
	Cases (AD+)	Controls (AD-)
KatoKatz Positive	0% (*n*=37)	6.7% (*n*=45)
***Ascaris* IgE >0.35kU/L**	21.4% (*n*=28)	5.1% (*n*=39)
RURAL (Eastern Cape)		
KatoKatz Positive	0% (*n*=38)	10.5% (*n*=41)
***Ascaris* IgE >0.35kU/L**	9.7% (*n*=31)	9.7% (*n*=31)

3.2.9 SAFFA cohort: Food allergy or sensitisation is lowered in the rural cohort (Data generated by collaborators)

The following results originate from the umbrella epidemiological SAFFA study of the SOSALL cohort analysed above. It has previously been established that infants with early-life food allergy are predisposed to show markers of atopy [180]. The SAFFA

study, reviewed how common food allergy is in Southern Africa, screening 1200 urban children (Cape Town, Western Cape) and 400 rural children (Mqanduli, Eastern Cape), all were healthy and between the ages of 12-36 months. All children were screened for food allergy by performing skin prick tests for the seven most common food allergens (egg white, peanut, cows' milk, fish soy, wheat and tree nuts), and parents completed a questionnaire that detailed weaning, family history, environmental conditions and dietary exposures.

Their findings demonstrated that 2.5% of 12-36 month aged children in Cape Town had a challenge confirmed food allergy, mostly to egg (1.9%), peanut (0.8%) and milk or fish (0.1%) [181]. Urban sensitization to any food was 11.4% (SPT response > 1mm) and 9.0% (an SPT response > 3 mm). Sensitization in rural cohorts was significantly lower than in the urban cohort with 4.5% (SPT response > 1mm) and 2.8% (SPT response > 3mm) [181]. The urban rates of food allergy (Cape Town) match that of middle-income countries, but the significantly lowered rate of food allergy in rural South African children suggests that rural-urban differences could be a more important factor than ethnicity and genetic determinants in the development of food allergy and potentially other allergies. The urban cohort was significantly more likely to have familial history of allergic disease (48.8% vs 7.5%, p = 0.001) and reported a higher prevalence of comorbid eczema (23.5vss. 1.8%) and allergic rhinitis (23.5% vvs3.3%) in comparison to the rural cohort [181]. The urban cohort was significantly more likely to have familial history of allergic disease (48.8% vsvs.5%, p = 0.001) and reported a higher prevalence of comorbid eczema (23.5% vs 1.8%) and allergic rhinitis (23.5% vs 3.3%) in comparison to the rural cohort [181]. In total,11.7% of the urban cohort had clinical signs of atopic dermatitis (AD), and 8.5% had signs of allergic rhinitis (AR). In the rural cohort AD and AR were significantly less frequent (0.8% and 0.0%). The urban food allergy rates did not differ between ethnicities; the urban cohort sampled black African, mixed ancestry and Caucasian.

3.3 Discussion

In this chapter, we examined three key immune components that could influence each other. Firstly, we reviewed helminth infection rates and its influence on SP-D levels. We then analysed the influence SP-D has on the atopic disease prevalence in our cohort,

and we examined the relationship between the atopic disease and helminth infection rates. We studied the system as a whole, taking into account helminth infection, SP-D influence and atopic disease together to potentially gain a broader insight into how these components work together in combination to influence human immune responses.

3.3.1 Surfactant protein D and helminths

Research performed by Thawer *et al.* in our laboratory revealed the connection between SP-D and helminth infections [69]. That study presented evidence that SP-D interacts with the helminth itself, (Figure 1.7) providing protection against helminth invasion (Figure 1.6) and leaving a long-term effect on murine immunology. Here we investigated whether this connection existed in human samples by assessing markers of helminth infection and their corresponding changes in serum concentration levels of SP-D. In agreement with the Thawer *et al.* study, our findings showed an association in 2 different cohorts between helminth specific IgG4 and surfactant protein D.

Helminth specific-IgG4 is related to SP-D. We compared children with known helminth exposure from the small **Masiphumelele cohort** to assess serum SP-D. Results indicated that SP-D levels positively associated with the development of antigen-specific IgG4, but not to the development of antigen-specific IgE. In the introduction, it was described how there are defined roles for both IgG4 and IgE in immune activation. Traditionally, in helminth infections, a short acute infection induces production of IgE, while longer more chronic or resolved infections develop an IgG4 response [158] [81]. Development of later stage immunoglobulins like IgG4 suggests a mediation of infection; our data illustrates that this mediation is associated with increased serum concentration of SP-D. This mediation not only indicates longevity of infection but potentially a shift to a less aggressive inflammatory phenotype. Over time, some parasitic infections down-regulate the hosts type 2 inflammatory immune response [46] towards both helminth antigen and non-related antigens [44]. This form of immune evasion explains the success of parasite invasion in tightly regulated immune environments such as the mucosa.

In the context of the helminth, since we have found that levels of SP-D are elevated in humans and it has been shown to be modulated by the helminth in murine studies, we can hypothesise that similar regulatory mechanisms could be employed for long term

survival in the host. The Thawer paper has functional data supporting some of these claims with SP-D-/- mice having a profound impairment of innate host immunity and the ability to resolve the infection, raising pulmonary SP-D levels prior to infection enhanced parasite expulsion and type 2 immune responses, including increased numbers of IL-13 producing type 2 innate lymphoid cells (ILC2), elevated expression of markers of alternative activation by alveolar macrophages (alvM) and increased production of the type 2 cytokines IL-4 and IL-13. It is likely that SP-D could interact not only with the parasite directly but also potentially with immune cells that may lead to a highly specific immune activation state in sensitive tissues such as the human mucosa.

SP-D and helminth association occurs specifically in helminth species that transit the lung in their lifecycles. Next, we analysed the case-control **SOSALL** cohort that originated out of the larger SAFFA umbrella study. Our analysis reviewed helminth specific markers of infection (IgG, IgG4 and IgE) and surfactant protein D. As we observed in cohort 1, *Ascaris*-specific IgG4 is associated with SP-D. Out of three helminth species tested, only two tested positive for this association (*Ascaris* and *Toxocara*); we hypothesise that apparent differences between helminths and their lifecycles underlies this finding. *Ascaris* and *Toxocara* [39] species lifecycles are systemic – specifically, their lifecycle involves a lung stage, and with the lung being the largest producer of SP-D [130], it would be logical to assume they come into contact with a large amount of SP-D protein and its producer, ATII cells. On the contrary, the *Trichuris* lifecycle, which did not correlate with increased SP-D, is localised to the gut, specifically limited to epithelial burrowing. Based on these findings, this may suggest that only helminth species (such as *Ascaris* spp and *Toxocara* spp), which transit the lung and other mucosal barriers as part of their life cycle, induce an associated increase in serum SP-D.

We might be seeing a mechanism by which low-grade helminth infection makes use of the body's own regulatory mechanism to defend against inflammation. The evidence in this dissertation (SP-D is associated with increased specific-IgG4) for the upregulation or increased concentration of serum SP-D during low-grade chronic inflammatory state could point towards it being part of the unique mechanism of helminth survival. Research shows helminth survival strategies are driven by their co-evolution alongside

humans, and helminths can turn hosts into carriers or sub-clinical patients [112] [113]. This happens through the specific regulation of chronic low-grade inflammation with the mechanism involving activation of TLR2 by regulatory DCs [65]. Interestingly, another molecule that activates TLR2 is SP-D [182]. SP-D also binds to IgG, IgM, IgA, IgE, in a calcium-dependent manner [51], specifically recognising both Fc and Fab domains of IgG [147]. The results of this study could be demonstrating an SP-D mediated amplification of IgG responses through enhancement mechanisms of immune aggregation and phagocytosis. The evidence we see for the upregulation or increased concentration of serum SP-D during low-grade chronic inflammatory state could point towards it being part of the unique mechanism of helminth survival. The interplay between a systemic helminth infection such as *Ascaris* and the long-term immune response could be reliant on homeostatic molecules like SP-D, which has both suppressive and activated states in human mucosa. *This section answers objective one showing that exposure to an STH changes serum concentration and objective 2 showing a potential correlate of exposure that exists in human serum.*

3.3.2 Surfactant protein D and allergic disease

Surfactant protein D has been implicated in various respiratory diseases, including allergic asthma [150] [183] [156], being both a biomarker of disease severity as well as being involved in pathophysiology. The abnormal modification of the pulmonary environment leads to the breakdown and modification of SP-D and its homeostatic capabilities in the lung [132] [146]. The detectable level of serum SP-D in allergic asthma has been shown to be significantly higher than non-allergic patients, and we know SP-D directly binds to and opsonises allergens. Our cohort investigated atopic dermatitis, a different allergic disease, that is not present in the lung but presents itself on the skin [184]. Therefore we investigated whether SP-D could have any associations with another allergic disease that is in a remote location from the source of the protein, concluding that SP-D titer does not indicate the severity of atopic dermatitis.

We quantified SP-D titers in the SOSALL cohort and stratified them by geographical location and AD status. There were large variations in participant's serum SP-D levels, which is expected as the serum SP-D levels from person to person has been shown to be highly variable. By comparing SP-D and SCORAD scores, we assessed the association between serum SP-D and the severity of AD. The rural AD+ SCORAD

scores trended towards being raised compared to urban AD+ children, but not significantly. We determined that neither SP-D serum titers nor SCORAD scores show any association to atopy or geographical location, and we, therefore, concluded that SP-D does not indicate the severity of atopic dermatitis. Other research shows that allergic disease may be a factor in how much serum SP-D is present in a patient and that it is a reliable indicator for the severity of the disease, but these studies are specific to allergic diseases affecting the lungs, such as asthma and rhinitis [185]. Our research points to SP-D not being an indicator of the severity of atopic dermatitis. However, in rural cases, in particular, SP-D concentration is slightly lowered compared to the control group, and SCORAD scores are slightly increased. There may be subtle immunological mechanisms at play here that the relatively crude measure of serum SP-D cannot pick up. For protein targets abundant in biological sample alternative measures of SP-D will be useful alongside the ELISA in order to fortify the findings surrounding SP-D concentration. *This section answers objective three showing that atopy, specifically atopic dermatitis, has no influence on serum SP-D. Even though SP-D is a biomarker for severity of allergic asthma, it is not a biomarker for atopic dermatitis and the trends surrounding atopy, and geographical location is too loose to pin down.*

3.3.3 Helminths and allergic disease

This section assessed whether participants with allergic disease have altered response to helminth infection and whether helminth infection alters the phenotype of allergic disease. Helminths are widespread [186], and the SOSALL cohort was drawn from rural and urban settings in South Africa; therefore, we tested for parasites endemic to the area [186]. As previously stated, helminth infections are loosely associated with a reduction in risk of allergy or allergic disease [113]. The hygiene hypothesis or old friends hypothesis dictates that organisms with which we co-evolved have developed unique survival strategies for preservation that as a byproduct downregulates or desensitises human immunity [103, 104] [105]. If through the development of immunity early on in life, we do not receive specific stimuli, our likelihood of developing a hypersensitivity response (allergy) is increased. In developing nations, in particular, an increase in affluence and environmental conditions come with decreased risk of parasitic infection. This developmental phenomenon could be a reason for increased immune-mediated diseases like allergies [187, 188]. In developing nations, considerations need to be made over socio-economic factors, such as the likelihood of

a more affluent home to respond to an ailment of their child, the access to healthcare systems both geographically and financially, cultural implications of belief systems and trust in 'western medicine'.

We assessed the trends between the rates of allergy in our cohort and the levels of helminth infection. During helminth infection, immune responses and hyper-reactivity are mediated by a balance of parasite-specific IgE and IgG4. After the initial response of IgE, which is acute and powerfully inflammatory, the development of IgG4 occurs. There is a balance between these specific antibodies, with IgG4, through various mechanisms, down-regulating inflammation and balancing tightly regulated immune areas. We postulate that the balance of immunoglobulins IgE and IgG4 during helminth infection and allergy could have a role in determining long term infection status and hypersensitivity reactions.

Upon review of the epidemiological data, we saw lowered rates of asthma and allergic rhinitis in rural AD- group. This is to be expected, due to the similarity of underlying mechanisms of allergy and if you already have an allergic disease, the likelihood of developing other allergic disease is increased [189] [190] [191]. Dog or cat ownership was also significantly higher in the rural cohort for both allergic and control children. This is important in the context of helminth infections being soil-transmitted through contaminated faeces, ownership of an animal that lives in close proximity to a child would mean exposure to a larger array of both pathogens and allergens. In accordance with research, both the "hygiene hypothesis" and "old friends hypothesis", microbial exposure across the board could influence the development of immunity in young children [103, 104] [105].

Urban AD- children have increased proportions of total IgG and IgG4 in serum. The increased proportions of total IgG and IgG4 in serum could indicate that the rural-urban divide plays a significant role in the development of immune response, with urban children producing more IgG, in response to exposure to allergens and microbes.

Rural AD+ participants have increased proportions of Ascaris-specific IgG4. Atopy can lead to a defective skin barrier as the first line of defence to pathogens, which could explain the higher rate and magnitude of infection [192]. In children, due to the nature

of the allergic disease, with consistent itching and scratching, atopic dermatitis increases the hand to skin/mouth contact [184], increasing likelihood of transmission of disease. In rural areas particularly, the chance of children being exposed to helminths at a young age is high, even though there are active anti-helminthic programs in place to combat this. Interestingly, the trends were only apparent with *Ascaris*, perhaps due to being the most widespread of the three species we tested for.

Atopic participants have high levels of Ascaris sensitisation. Data collected before this dissertation showed differences in the current helminth infection rates of AD+ children and AD- children across the rural/urban divide [181]. AD+ participants have no evidence of active infection with *Ascaris* but have all been sensitised to helminth antigen (seen in specific IgE titer). The ability of the AD+ cases to quickly clear active infection is undefined, but it is known that allergy up-regulates Th2 responses, which in turn has knock-on immune effects, i.e. B-cell upregulation and increased antibody production [193]. As atopic participants had no active infection but were still sensitised to *Ascaris* (at higher rates than AD- controls for the urban group), this raises an interesting question: Are atopic children clearing active infection better than their non-atopic counterparts? A child with allergic disease would have up-regulated Th2 and therefore, upon infection with helminth, clear it more efficiently due to increased immune activation. In the non-atopic, low-grade infestation continues asymptomatically with no large Th2 response due to the modulatory abilities of helminth itself [114] [14] [177].

Immune responses are dependent upon received stimulation, whether that is in the form of pathogen or allergen. Another critical consideration is the timing in which the stimulus is received. Whether a helminth infects during a stage of heightened allergic disease (high IgE) or controlled allergy (high IgG4) [158] [157], the level of Th2, the age of the patient and their ability to form a response; all influence variation regarding the level of Th2 activation inflammatory profiles. Our data showed rural AD+ children had increased titers of serum *Ascaris*-specific IgG4. We argue that the susceptibility of a child in an area with a high prevalence of *Ascaris* is increased, as is the weakened barrier integrity and subsequent helminth entry point. We review children that have an allergic disease and helminth infection, perhaps the increased IgG4 titers in these rural children could be piggybacking off the same mechanism, that allergic response and

helminth infection make use of the inhibitory effect of IgG4. We do not have data to the time of year and corresponding season that data was gathered, which could influence what allergic state they are currently in.

Relating the up-regulation of Th2 to balance of IgG4 and IgE in helminth infections and allergy. Our data indicated that AD+ participants had up-regulated Th2 in response to helminth infection and AD- participants are more likely to develop a low-grade asymptomatic infection. We postulate that already atopic children exposed to helminths will clear the infection faster, and the helminth will not have time to exploit unique modulation of immunity through antigen-specific IgG4. In contrast, a non-atopic child would develop low-grade infection allowing time for the helminth to modulate its immune environment. Here we see how helminth infection and allergy could clash or coincide when it comes to immune regulatory balance in the lung.

What can be gathered from this information is that the polarisation of an immune response, specifically in a tightly regulated area such as the lung, is not as binary as previously assumed. The IgE-IgG4 balance still remains on a scale; it's the magnitude of infection that varies. In the case of AD+ infection, clearance happens because Th2 activation is already high and in the case of the AD- the Th2 activation is lower, interestingly we do not know how the variation or effect of SP-D fits into that model.

The influences of the rural-urban divide on allergic disease. In South Africa, like other developing countries, the prevalence of the non-communicable disease in children is a growing public health concern (WHO, 2010). There has been a notable increase in the prevalence of allergic disease, in urban areas with the rate of allergy reflecting 'developed' countries [181] and rural areas have a lower prevalence of respiratory allergies [194].

The SAFFA study showed that the urban cohort was significantly more likely to have signs of AD or AR, have familial history of allergic disease and increased prevalence of comorbid allergies. The urban rate of food allergy matches middle-income countries with a significantly lowered rate of food allergy in rural South African children. In our research, we noticed a notable rural-urban variation. **First**, total IgG and total IgG4 were significantly raised in urban cohort, particularly AD- group. **Second**, *Ascaris*-

specific IgG4 titers were significantly raised in the rural cohort. **Third**, rural AD-cohort was more likely to be positive for active infection than an urban cohort. **Finally**, in the SAFFA study, we saw that urban food allergy rates were significantly raised compared to the rural cohort.

Other research also suggests variation in sensitisation and allergy. This is based on socioeconomics between children of different ethnicities, as well as children of recently urbanised immigrants, compared to local population [195]. South Africa, like many other developing countries, has juxtaposition between urban and rural populations and vulnerability to disease; when normalising for ethnic demographics, rural populations have different susceptibilities to disease entirely [181]. There are distinct parallels in the epidemiology of atopic dermatitis, asthma and allergic rhinitis. According to the model of allergic march, eczema (AD) precedes the development of asthma and allergic rhinitis (AR) [184].

Furthermore, there is a large body of evidence showing the likelihood of comorbidity of asthma and allergic rhinitis [196] [197]. In adult studies, 30% to 90% of participants with asthma also have allergic rhinitis, and these share similar pathophysiology [196]. The evidence from the SAFFA study, our data, and other research suggests that rural-urban differences could be as important a factor as ethnicity and genetic determinants, in the development of the immune response and potentially as a knock-on for food allergy and other allergic diseases.

This raises the question of how a rural-urban divide that influences quantitative outcomes must be considered whilst conducting research. How should it be considered and what exactly should be considered? Should it be used to normalise data using the urban/rural geography?

This section answers objective four, illustrating that participants with allergic disease have altered response to helminth infection and helminth infection alters the phenotype of allergic disease.

3.3.4 Chapter conclusion

There is an interplay between helminth & SP-D, helminth & allergy and SP-D & allergy, but what about the system as a whole? We know that SP-D correlates to increased *Ascaris* IgG4 and that SP-D binds to allergens and to helminths directly [69]. What is unknown is how SP-D elicits an effect. SP-D increases in some allergic disease (according to lung proximity) and our data shows an increase during specific helminth infections. Our lab also showed that addition of SP-D associates with up-regulation of Th2 responses [69]. These results point to the ability of SP-D to alter the mechanism by which Th2 immunity functions in the lung and other mucosal barriers. Under normal conditions, SP-D's CRD binds to helminth and allergens driving a Th2 phenotype, and subsequent clearance through phagocytosis or opsonisation [198]. In an allergic condition, SP-D competes with allergic immune responders and decreases the amount of available binding sites on an allergen, lowering the immune stimuli and mediating the allergic response. Upon binding through its CRD, SP-D is classically anti-inflammatory but when barrier integrity is lessened and the oxidation state of the microenvironment changes, SP-D could have an inflammatory effect due to its breakdown from multimer to monomers [148]. In helminth infection, the same mechanism of binding to available epitopes on the helminth itself could limit immune stimuli, changing of course with a shift in barrier integrity.

We know in murine models of allergic disease, administration of large quantities of SP-D and rfhSP-D suppresses IgE (antigen-specific), reduces eosinophilia and causes Th-1 cell polarisation [92]. It is larger but still comparable to SP-D leakage into serum from the lung of allergic asthma patients. We did not see the same leakage and subsequent raised level of serum SP-D that we attribute to the different pathophysiology of atopic dermatitis compared to lung allergy. With *Ascaris* and *Toxocara* infection transitioning through the lung as part of the lifecycle, where allergic asthma causes damage to the alveolar epithelium, the physical size of the helminth can cause similar damage resulting in the leakage of SP-D. This leaked SP-D would have an effect on serum immunoglobulins, throwing the IgG4-IgE balance into disarray.

This dissertation, supported by other studies, points to there being a novel association between SP-D and helminth-specific IgG4, related to the proximity of the helminths to the lung. We are also seeing an upregulation of Th2 immunity in the lung in allergic

participants which increases their ability to clear the helminth infection quickly. SP-D has an effect on the allergic disease but is not a marker of severity for atopic dermatitis. Finally, the rural-urban divide in South Africa plays a role in the prevalence of allergic disease and other undetermined health outcomes and should be considered thoroughly when performing research.

There are limitations to our analysis of SP-D. The significance of a 14-sample comparison (Masiphumelele cohort) and 30-sample comparison (SOSALL cohort) is limiting; however, in this experiment, the number of IgG4 positive samples was limited. Future work should focus on longitudinal time points per patient and the degree of exposure to *Ascaris* infections more thoroughly assessed. This would allow specific conclusions to be made regarding the upregulation of serum SP-D. Finally, owing to the limited number of samples available and the large variability typically observed in patient-derived data, the statistical analysis of this study was limited to provide definitive conclusions. Nevertheless, the results presented provide interesting avenues for further research and should be explored in a larger cohort. Age must be factored in too, with this cohort being adult (Masiphumelele) and adolescent (SOSALL) their exposure to helminths and subsequent shift in SP-D is notably different.

The SOSALL cohort showed low numbers of acute or actively infected children (previously determined IgE titer). This was expected as the tested participants are young and active anti-helminthic programs are being run in both rural and urban areas. However, previous or non-active exposure to helminths in the cohort was found to be very common with *Ascaris lumbricoides,* and *Toxocara* spp antigen-specific IgG4 detected (above cut off) in over 25% of study participants and *Trichuris trichuria* in 20% of participants. Despite having relatively large amounts of detectable antigen-specific IgG4, if we compare this to total IgG4, there are fewer positive samples. The number of samples positive for antigen-specific IgG4 should not be more than total IgG4; the only explanation that we could see was an experimental error. Another consideration is antigenic cross-reactivity, cross-reactivity happens when an antibody created against one specific antigen recognises another antigen with a similar structural region. The helminth species that we work with are distinct in many ways but do share similar surface molecules that might cause cross-reactivity in experimentation. The antibody used to identify SP-D and the immunoglobulins are polyclonal in nature,

which means there is an increased chance of antigenic cross-reactivity due to the recognition of multiple epitopes.

The use of SP-D as a biomarker is gaining traction, with other studies looking into similar potentials [199] [200], however, standardisation needs to be established. Reviewing the use of various ELISA and western blots, reported SP-D values for healthy control populations varied more than a hundredfold between studies [201]. On the one hand, the experimental methodology can have an influence on outcomes like the use of EDTA during blood collection results in inconsistent SP-D readouts [201], we should also consider that SP-D in serum in a variable between individuals due to genetics and environment. It is important to consider the use of SP-D as a biomarker but also potentially as a novel treatment for helminth infections. We know it is associated with helminth infections occupying the lung and has a role in binding to and dampening immune responses. More work should be directed towards its use as a treatment or host-directed therapy, moving away from anti-helminthics. *Could SP-D be used as a novel anti-helminthic treatment for lung-based parasites?*

SP-D research focuses on immunomodulation, specifically its role in phagocytosis, optimisation and oxidative signalling. However, SP-D also interacts with various other cell types, including immune cells, epithelial cells, fibrocytes and smooth muscle cells. Another consideration is that in the majority of research the pulmonary effect of SP-D reviews it's dampening of inflammation, there are also potential inflammatory effects of SP-D variants when SP-D does not form HMW structures. When LMW SP-D has generated research shows [14] that it can become inflammatory, these LMW bi-products develop when SP-D is broken down due to a change in oxidative state in the pulmonary environment. LMW SP-D must be reviewed in more detail, in particular, focusing on what inflammatory effect LMW SP-D has on the human immune response.

Helminth exposure and modulation of physiology are complex, and more investigation needs to be directed towards other measurable biomarkers in serum, such as antibody titers and cytokine responses to validate the concept of Th2 up-regulation. With our body of work only generated from the analysis of the serum samples, focusing in particular on IgG4 levels in the cohort.

4 Influence of SP-D and helminth antigen on cellular phenotypes relevant to helminth infection

4.1 Introduction

The relevance of surfactants in disease is multi-faceted with roles in parasitic infection [69], chronic non-communicable disease [199] [141] and allergy [202] [198]. Although the exact mechanism of interaction is still unknown, the homeostasis of surfactant, as well as its homeostatic abilities, appears to be critical indicators for disease and disease prevention. As stated previously, murine experiments within this research group have illustrated that SP-D enhances the innate immune defence in the protection against helminth infection. These results need to be translated into human studies, which was pursued in two directions.

In the first set of experiments (Chapter 3), trends were identified surrounding SP-D and parasitic infection. It was showing that antigen-specific IgG4 titers associate with increased SP-D levels (Figure 3.4). This association could be linked to the role of SP-D in the body but gives us a superficial indication of the cellular mechanistic. Flow cytometry allows phenotyping of cells based on their extracellular and intracellular marker repertoire. This section aimed to review the phenotypes of particular immune cell subtypes related to SP-D and its interaction with human helminth infections. Two cell types were chosen, **type 2 innate lymphoid cells** and myeloid lineage **monocytes/macrophages**.

Studies have linked innate cell types from the myeloid lineage with resistance to helminth infection [9]. New research classifies monocytes into distinct human subtypes and functionality during infections including valuable, but undefined, roles during helminth infections, these cells also serve as precursors to the macrophage [67]. We focused on circulating monocytes due to being readily accessible through blood samples. Human monocytes can also be derived, ex-vivo, to form macrophages, which are crucial during helminth infections. Murine macrophages are known to limit type 2 pathology by regulating cytokine production [203] [204] and are involved in tissue repair and fibrosis [205] [206]. Human monocyte subsets have functional variation in

response to helminth infection. By modelling the *in vitro* interaction between the filarial worm *W. bancrofti,* we see distinct categorisation into classical, non-classical and intermediate monocytes [207]. Macrophages and monocytes display phagocytic capabilities through an array of receptors and have been associated with particular helminth infections, both human and murine. Our cell work focused on human monocyte expression of CD16 and human monocyte-derived alternatively activated macrophage expression of CD16 and CD206.

A brief overview of CD16: Monocytes and macrophages display CD16 on their surface, otherwise known as FcγRIII, which binds to the Fc portion of IgG. The Fcγ receptor group critically links humoral and innate arms of the immune response [208]. In humans, high-affinity binding is illustrated with IgG1 and IgG3 leading to phagocytosis, the release of inflammatory mediators and clearance of immune complexes through ADCC [208]. ADCC is a dominant mechanism *in vivo* that is involved in pathogen clearance, the destruction of cancerous cells and autoimmune disorders. Monocyte CD16 expression is linked to protection against schistosome infection, with increased CD16 associated with increased total IgG and IgG1 in healthy participants, but not in Schistosoma infected patients [66]. Similar to parasite-specific IgG and IgG1, CD16 expression in healthy individuals is associated with protection against schistosome infection. Notably, monocytes categorised as 'non-classically activated' with higher expression levels of CD16 (CD14+CD16++) have a greater ability to bind to Schistosome cercarial and egg E/S products [12]. These relationships indicate a mechanistic link between the innate and adaptive immune responses to helminth infection in protection against infection, whereby CD16 plays a critical role.

A brief overview of CD206: CD206 is a pattern recognition receptor otherwise known as mannose receptor C type 1 (MRC1 or MR) present on antigen-presenting cells, including alternatively activated macrophages [209] [210] [211]. Functionally, CD206 is involved in innate immune response and active during antigen processing and mediating phagocytosis through the recognition of microbial carbohydrates [209] [210]. Macrophage CD206 recognises a variety of microorganisms, including bacteria, fungi, virus, and parasites (helminths specifically release substantial quantities of glycosylated proteins that bind to immune cells expressing MR) [211]. Many pathogens are coated with structures that contain mannose, and the macrophage MR acts as a

homeostatic receptor, by binding and clearing areas of high mannose glycoproteins [210]. We refer to MR as homeostatic because it is hypothesised that MR plays a crucial role the maintaining inflammatory stability in sensitive tissues, i.e. lung [209]. CD206 has been used widely in the characterisation of AAMs during parasite infection [212] [211]; however, the exact role for CD206 is undefined in human helminth infections. There is a broad scope to understand how CD206 mediates and is mediated by parasitic infections and the presence of SP-D.

As previously stated, after infection with *N. brasiliensis*; type 2 innate lymphoid cells (ILC2) are the major innate IL-13 expressing cells. In the same model, a transfer of wild type ILC2 cells to rag knockout mouse rescued the anti-helminthic response [61]. They have a role to play in type 2 immune cascades during parasitic infection. Mjosberg et al. were the first to describe them in humans as lineage negative CD127+ CD161+ expressing CRTH2 (CD294) and producing IL-13. The CRTH2 expression and IL-13 production separate them from other innate lymphoid cells, which are classified as lineage negative CD127+ CD161+ [213]. To date, only a few studies have characterised innate lymphoid cells in humans with a parasitic disease. Boyd et al. also defined all ILCs as Lin-CD127+CD45+ and the expression of cKit (CD117), showing that exposure to microfilariae can attenuate ILC2 responses [176]. The flow cytometry panel and gating strategy were designed for viewing total ILC populations concurrently with ILC2s, which together makes up a large part of innate immune effectors to various diseases.

Referring back to aims and objectives, here it was identified whether SP-D **influences the magnitude of anti-nematode responses** in human immune cells. The hypothesis is that the association between helminth exposure and SP-D relies on immune cell interaction, potentially involving type 2 innate lymphoid cells (ILC2s), monocytes and macrophages. Our objectives were as follows; **(1)** We set up a robust method of review for cellular phenotypes of ILC2's, monocytes and macrophages isolated from human PBMCs. **(2)** We then used this method to review whether SP-D and *Ascaris* antigen have an influence on these immune cells with particular focus on the effect of the following combinations; ***(A)*** SP-D and *Ascaris* individually or combined on monocyte CD16, ***(B)*** SP-D and *Ascaris* individually or combined on macrophage CD16, ***(C)*** SP-

D and *Ascaris* individually or combined on macrophage CD206. It is worth noting that time constraints did not allow for experiments 2 (A-C) using ILC2s.

4.2 Results

4.2.1 Establishment of flow cytometry panel to assess phenotypes of monocytes/macrophages and ILC2s

The following was the process of structuring and optimising a flow cytometry panel, which is a powerful tool to review cellular phenotypes at high concentration. The optimisation was not straightforward, particularly for cell types that are present in low abundance. The precision of marker overlap and antibody concentration must be high to robustly review these shifting cellular phenotypes. We designed our panels based upon existing literature and consensus on cell population size and shapes, using markers identified as necessary for either cell function or identification.

4.2.1.1 Antibody titrations

To determine the optimal volume of the antibody for detecting cell population, we stained cells with antibodies at a range of concentrations. This ensures that antibodies are used at a volume that allows for the optimal separation minimising background signal [178]. We surface stained cells from two individuals, as described in methods (see surface staining protocol), and optimal antibody concentrations were chosen based on the staining index. Below the workflow is illustrated, and titration calculation with the remaining antibodies and their calculated titration values left out for length's sake (Figure 4.1 and 4.2).

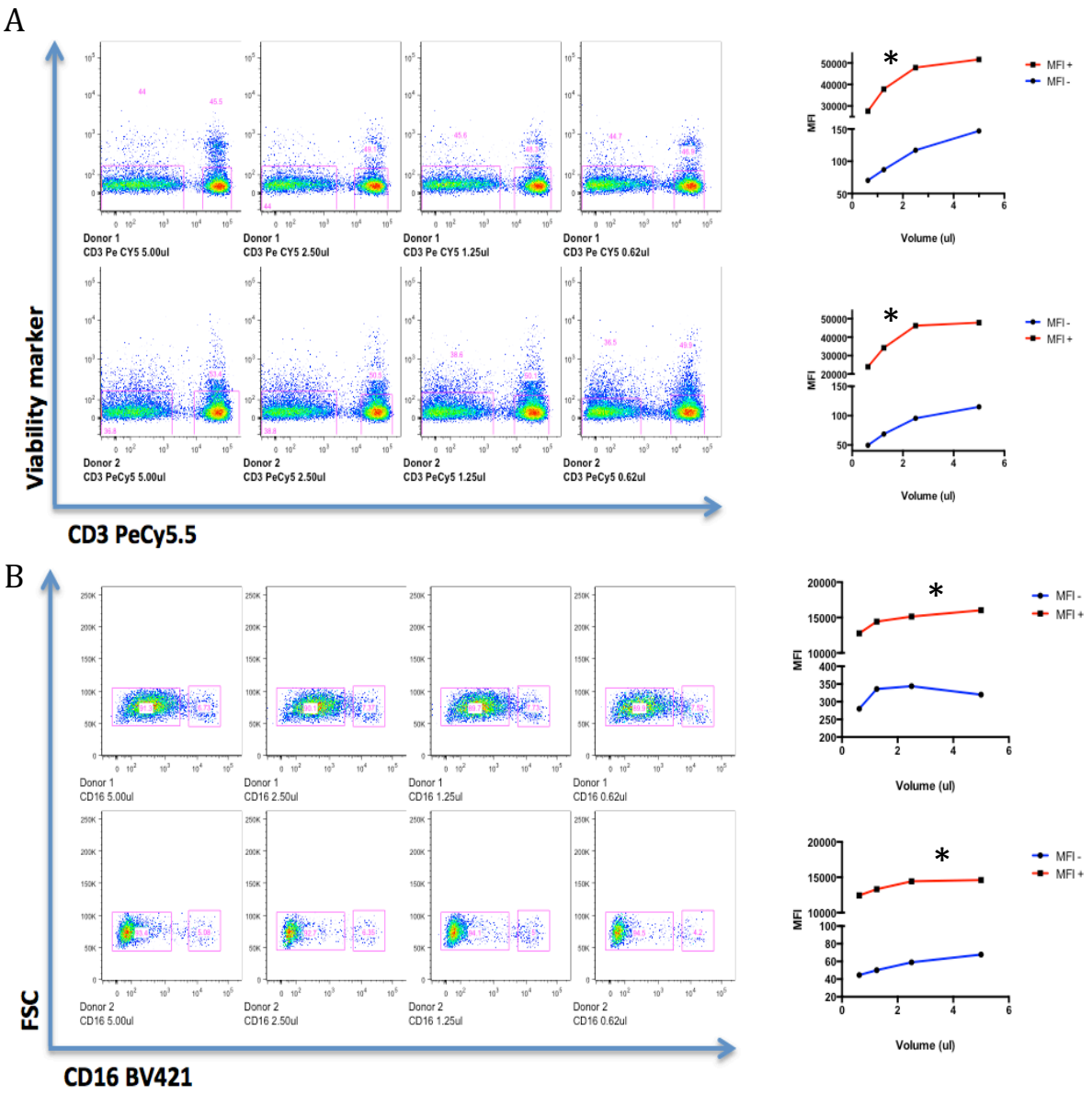

Figure 4.1: Representative monocyte panel titration of anti-CD3 PeCy5.5 and anti-CD16 BV421. (A) Represents the flow plots, where the volume of antibody added is shown on the top right-hand corner of each plot. A two-fold dilution series was performed, and PBMC were surface-stained and analysed unfixed on a flow cytometer. Cells were gated on singlet and lymphocytes. (B) The titration curve used to calculate MFI for the negative (-) and positive (+) population for all volumes tested. Asterisk (*) on MFI graph indicates optimal staining index.

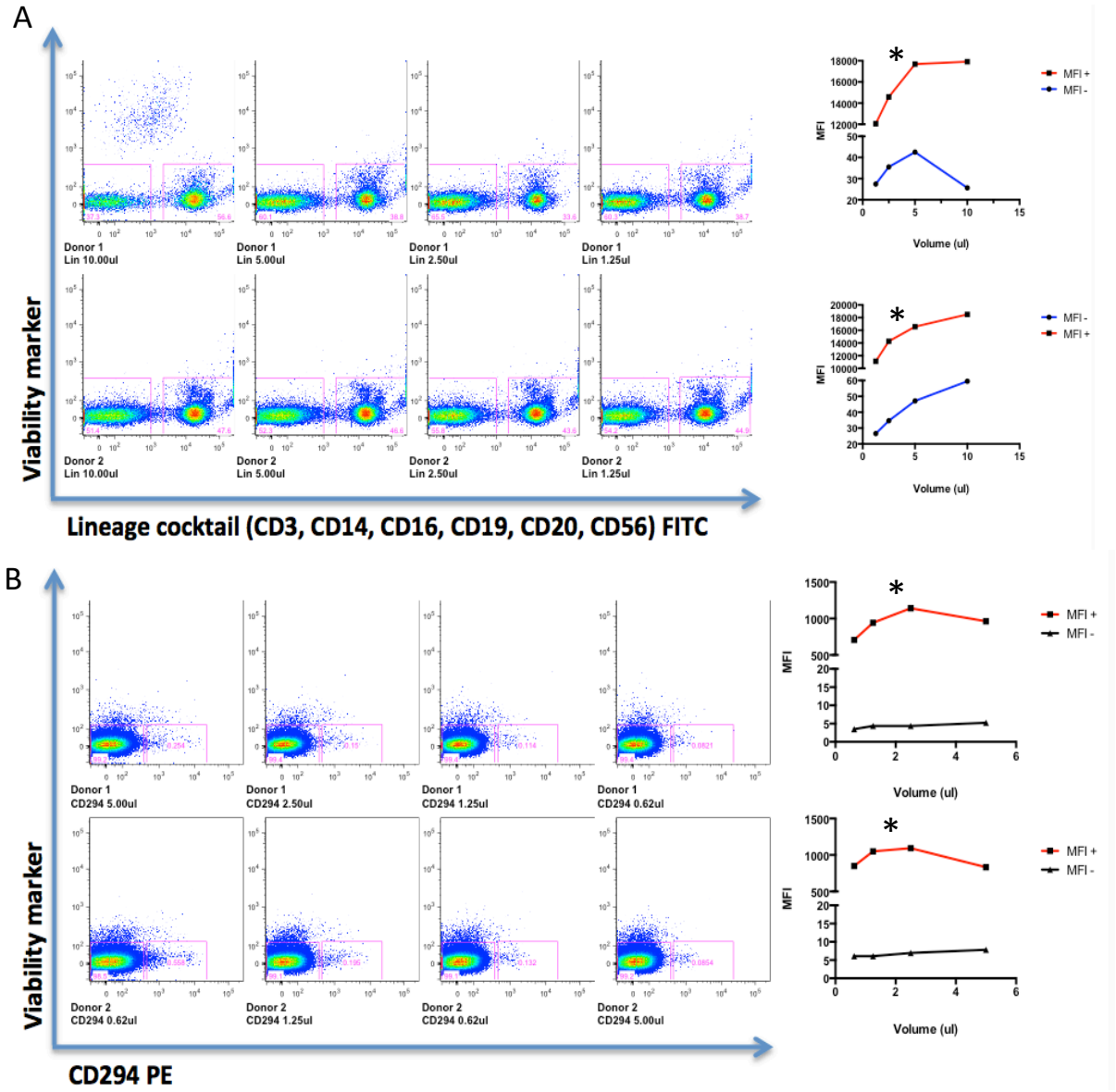

Figure 4.2: Representative ILC2 panel titration of anti-lineage cocktail FITC and anti-CD294 PE. (A) Represents the flow plots, where the volume of antibody added is shown on the top right-hand corner of each plot. A two-fold dilution series was performed, and PBMC were surface-stained and analysed unfixed on a flow cytometer. Cells were gated on singlet and lymphocytes. (B) The titration curve used to calculate MFI for the negative (-) and positive (+) population for all volumes tested. Asterisk (*) on MFI graph indicates optimal staining index.

4.2.1.2 Building the multi-colour flow panels

To build our monocyte and ILC2 panels we consecutively added titrated antibodies to develop a multi-antibody cocktail that would identify our cell populations of interest, this allowed us to compensate for optimal antibody separation and to troubleshoot any problems with interference (see methodology).

4.2.1.3 Dump channel

The inclusion of a dump channel allowed for the exclusion of unwanted cell populations, which aids in improving the quality of the data when viewing rare cell types. In this case, the monocyte panel has CD3 and CD19, both on Pe/Cy5 as a lineage cocktail (Table 2.1 methods displays cell types targeted). The ILC2 panel used a more complex lineage cocktail that contained CD3, CD16, CD19, CD56 and additionally CD11c and CD123 all on FITC (Table 2.2 in methods displays cell types and markers targeted). Lineage markers are used to gate out unwanted cell populations (Figure 4.3).

4.2.1.4 Fluorescence minus one (FMO) controls and the effects of compensation

FMOs were used to determine whether the panel of fluorochromes chosen can be combined. To develop a flow cytometry panel that included multiple antibodies, it is vital to make sure that the bleeding of one fluorochrome into another channel was minimised. This is achieved by performing FMOs. With regards to compensation, the spillover of fluorochromes into other colour channels causes false signal and irregular population shapes on FACS plots. We corrected for this using a mathematical matrix, which was applied to the fully stained samples based on single stained controls run in the same experiment. This matrix corrected for the majority of spillover but had limitations.

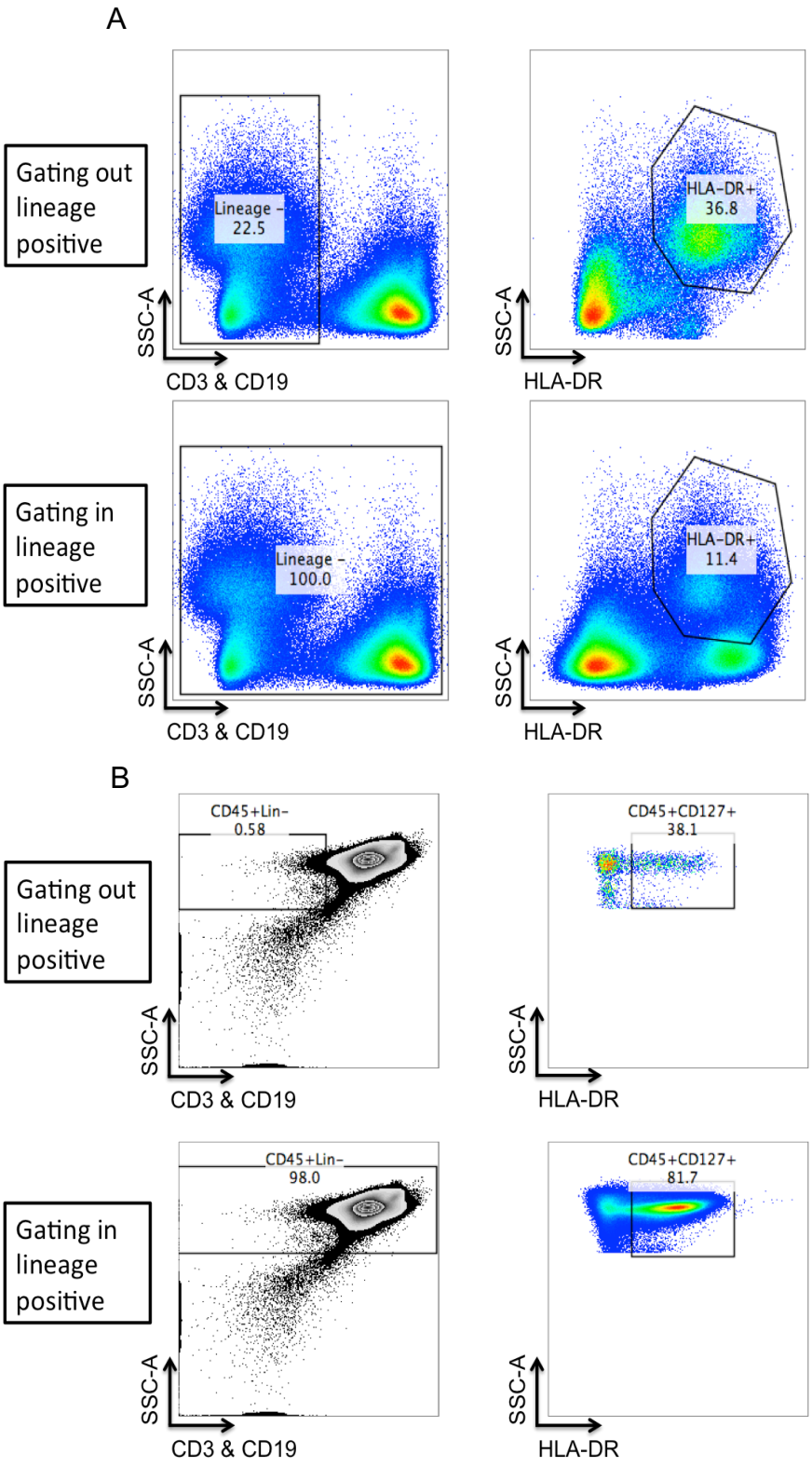

Figure 4.3: Flow cytometry gating strategies for lineage markers. This displays a comparison between inclusion and exclusion of lineage channel gate. (A) Monocyte panel: gating out the lineage cells (CD3 (T-cells) and CD19 (B-cells)) displays a large reduction in the spread of cell populations, increasing the accuracy of subsequent HLA-DR gate. (B) ILC2 panel: Gating out the lineage cells (CD3, CD14, CD16, CD19, CD56, CD11c, CD123) displays a large reduction in the spread of cell populations increasing the accuracy of subsequent CD45+CD127+ gate.

4.2.1.5 Gating strategies

To maintain consistent results, a single gating strategy was designed based on where the populations appear on the flow plots. The gates were replicated per donor to ensure precision in the inclusion and exclusion of certain cell populations. Below are gating strategies decided upon and then used for ILC2s and monocytes, respectively (Figure 4.4 and 4.5).

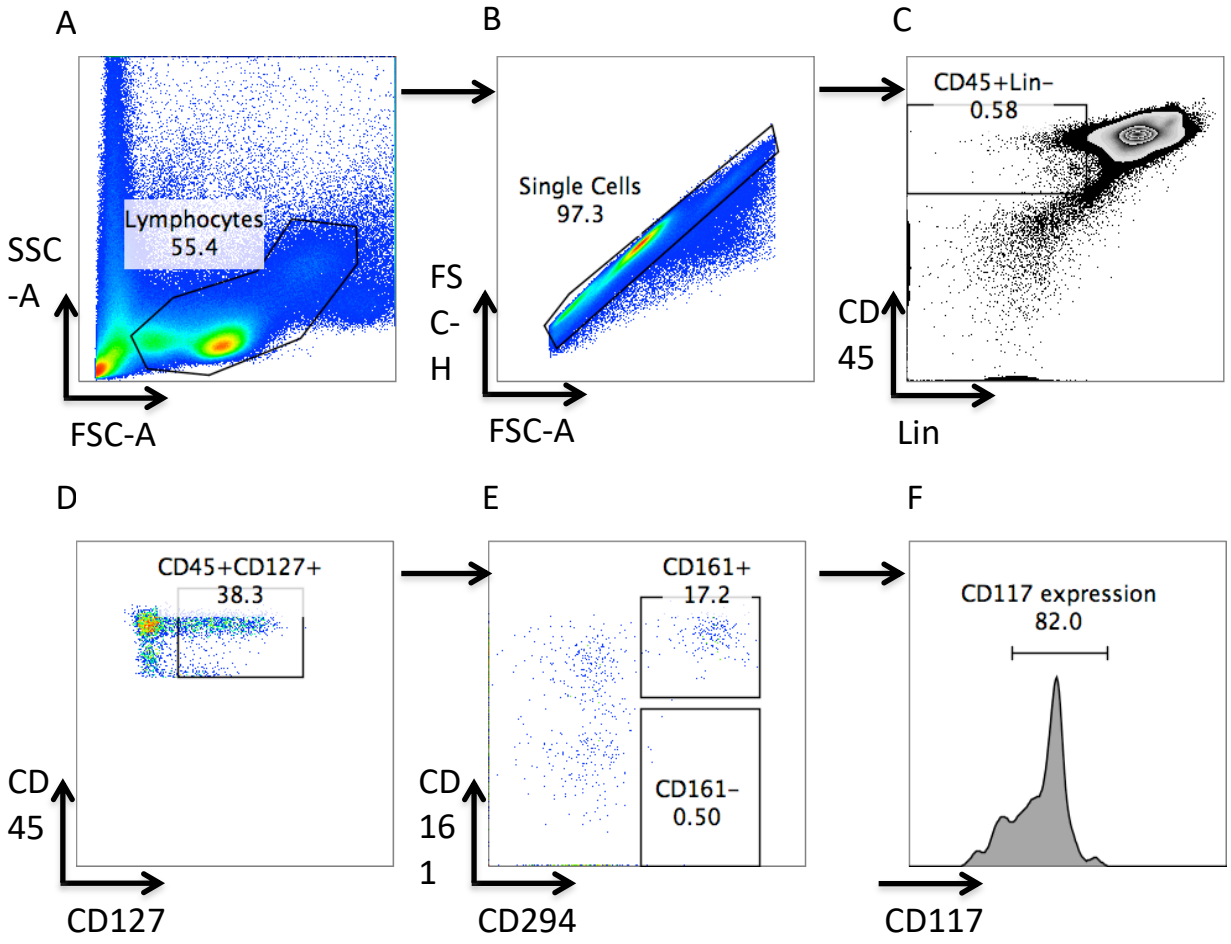

Figure 4.4: Flow cytometry staining strategies for type 2 innate lymphoid cells. Flow cytometry gating strategies for type 2 innate lymphoid cells. This displays the full gating strategy for type 2 innate lymphoid cells in samples of peripheral blood mononuclear cells collected from the western province blood bank. (A) Size based gate (B) Single-cell gate (C) Lineage – CD45+ gate (D) CD127+ gate (E) CD161+ CD294+ gate (F) Expression of CD117.

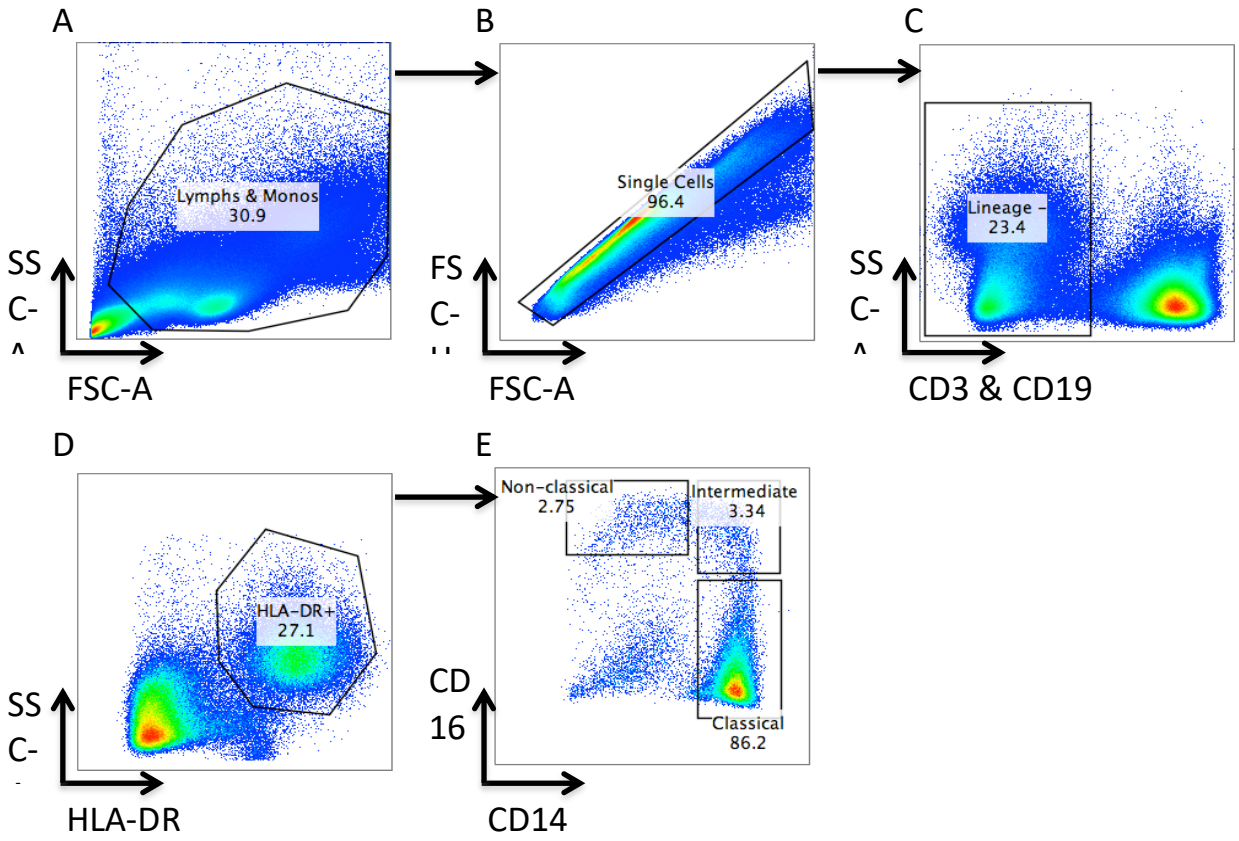

Figure 4.5: Flow cytometry gating strategies for monocytes. This displays the full gating strategy for monocytes in samples of peripheral blood mononuclear cells collected from the western province blood bank. (A) Size based gate (B) Single-cell gate (C) Lineage (CD3 & CD19) – gate (D) HLA-DR + gate (E) Expression of CD16 and CD14.

4.2.1.6 Panel validation

To establish whether we were identifying accurate cell populations, we assessed the literature and roughly compared published population size, shape and number to our own results. The spread and size of cell populations were the most important. Monocyte panel was validated against current human monocyte panel courtesy of Dr Heather Jaspan (Figure 4.7). Based on CD14 and CD16 expression monocytes are divided into three subsets; classical, non-classical and intermediate [66]. The ILC2 panel was validated against human ILC2 panel designed by Nausch *et al.*, 2014 [177] for a cohort recruitment study in Zimbabwe. This new and rare cell type has proven difficult to identify in blood without the use of magnetic isolation kits. ILC2 populations are notoriously difficult to work with due to their low circulating percentage in human serum [214], but with the validation of the panel (Figure 4.6) and the heterogeneity between donors, we can be confident that this panel was a reasonably robust identification of a small cell type. Inevitably, flow cytometry requires troubleshooting;

in this case, a change in machinery to 4-laser Fortessa was required for the inclusion of a viability dye. The PBMC viability from a cell bank was consistently high and with a diminutive need for a viability marker. However, future cohort recruitment and variation in sample processing would require more accurate cell viability control.

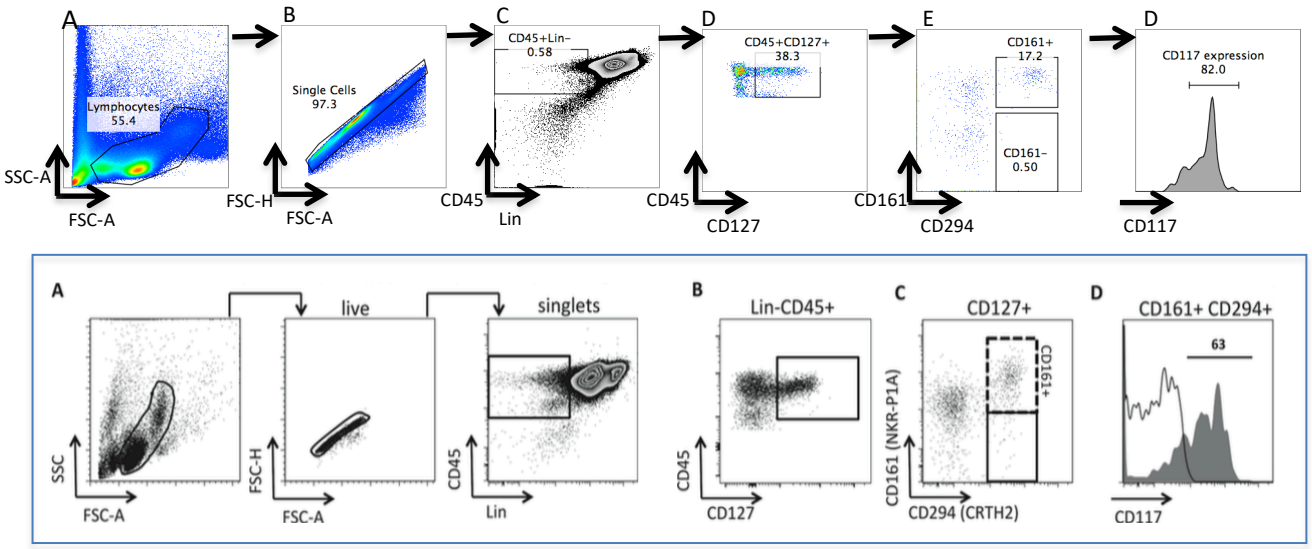

Figure 4.6: Comparison of type 2 innate lymphoid panel to existing literature. In order to gauge the accuracy of our panel, we compared our panel to existing literature from Nausch et al. 2015. The same markers were gated for, and population size and spread were compared. The upper row is the panel generated from this work, and the lower panel is Nausch et al. panel.

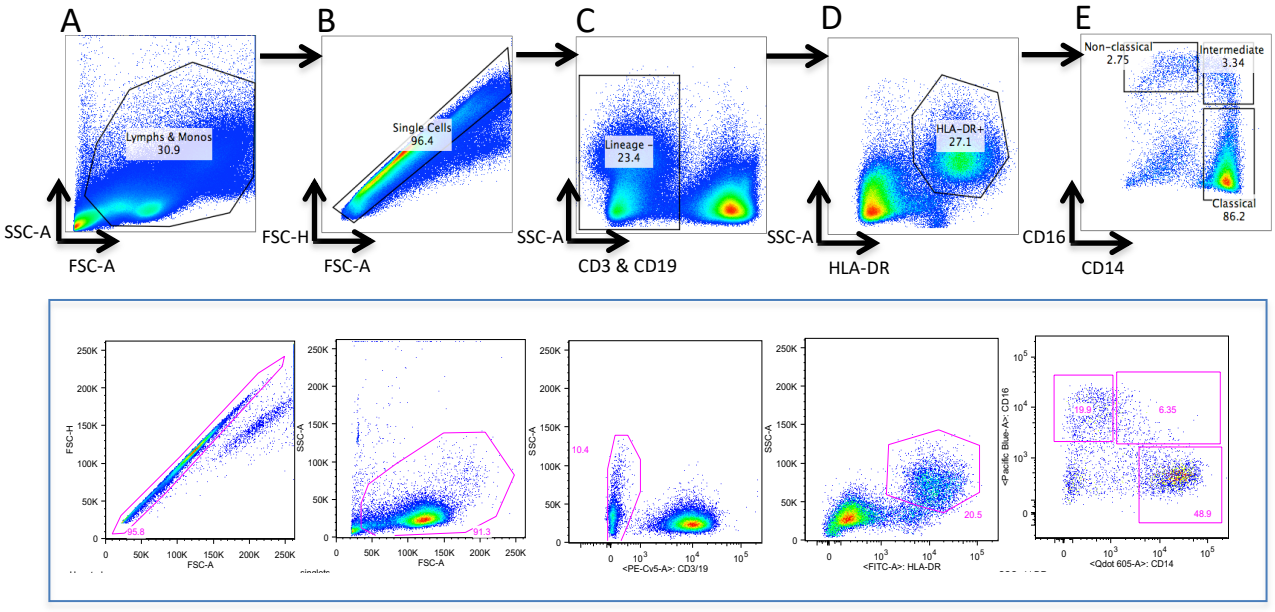

Figure 4.7: Comparison of monocyte panel to existing literature. In order to gauge the accuracy of our panel, we compared our panel to existing literature from Dr Heather Jaspan, UCT. The same markers were gated for, and population size and spread were compared. The upper row is the panel generated from this work, and the lower panel is Dr Heather Jaspan research groups panel.

4.2.1.7 Using flow cytometry panels to determine the phenotypic shift in cell types

A preliminary phenotype comparison between the four PBMC donors (Figure 4.8) was created to establish whether the flow panels were working as expected, this illustrated the populations that will be analysed in future work with ILC2s and monocytes. ILC2 populations are notoriously difficult to work with due to their low circulating percentage in human serum [214] but with the validation of the panel (Figure 4.6) and the heterogeneity but consistent proportionality between donors (Figure 4.8 A) this panel was a robust identification of small cell type. Monocyte proportionality was expectedly more variable (Figure 4.8 B), as monocyte proportionality between classical, intermediate and non-classical subsets is highly variable.

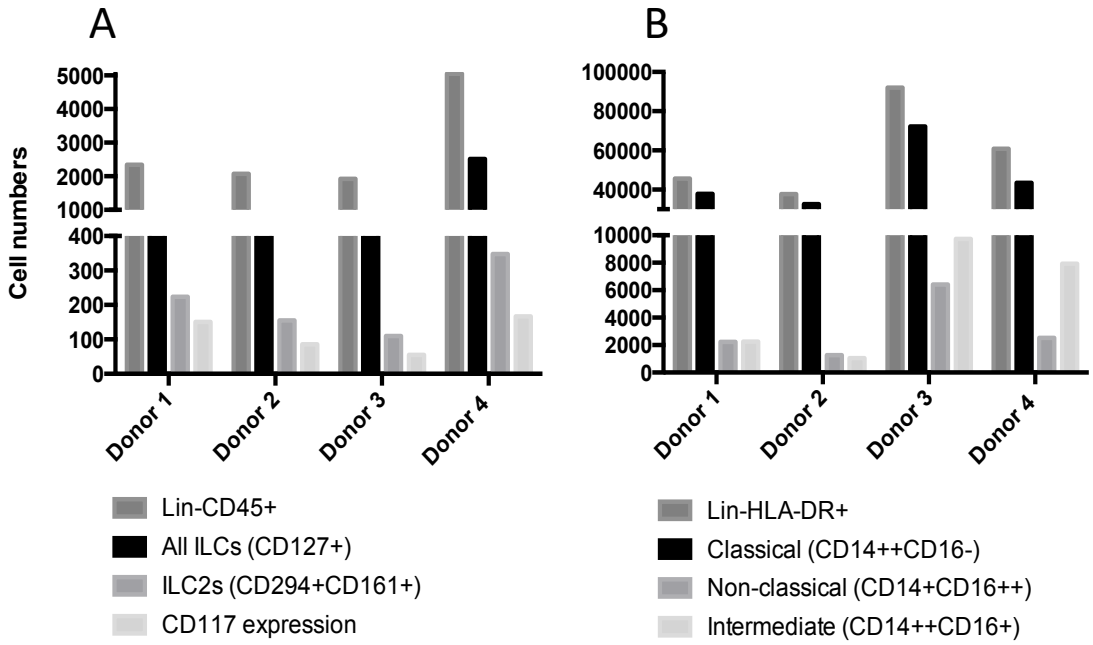

Figure 4.8: Donor comparison plots. Monocyte and type 2 Innate Lymphoid Cells absolute cell counts compared between donors. (A) Adapted Type 2 innate lymphoid cell panel utilising different fluorochrome labelled markers and identical gating strategy. (B) Adapted Monocyte panel utilising different fluorochrome labelled markers and identical gating strategy.

4.2.2 Cellular stimulation experiments

The cell work was conducted at the École Polytechnique Fédérale de Lausanne (EPFL) as part of a research internship. We focused on expanding the research previously done, looking at unexposed human PBMCs and their response to human SP-D, helminth antigen and various cytokines. The project focused on monocytes and macrophages, adapting our flow cytometry panel for outputs surrounding CD16 expression, CD206

expression and IL-13 release (no data). Human monocytes were separated using magnetic isolation kits based on their CD14 expression. Macrophages were also cultured from isolated CD14+ PBMCs (monocytes) in GM-CSF, which causes alternate activation. Our focus was on whether SP-D and helminth antigen cause a shift in expression of cellular markers on monocytes and macrophages. Stimulations were performed using PMA/Ionomycin. Limitation on time and reagents meant we were only able to perform a small number of experiments. The gating strategy used for monocyte and macrophage identification, respectively, is depicted below (Figure 4.9 and 4.10).

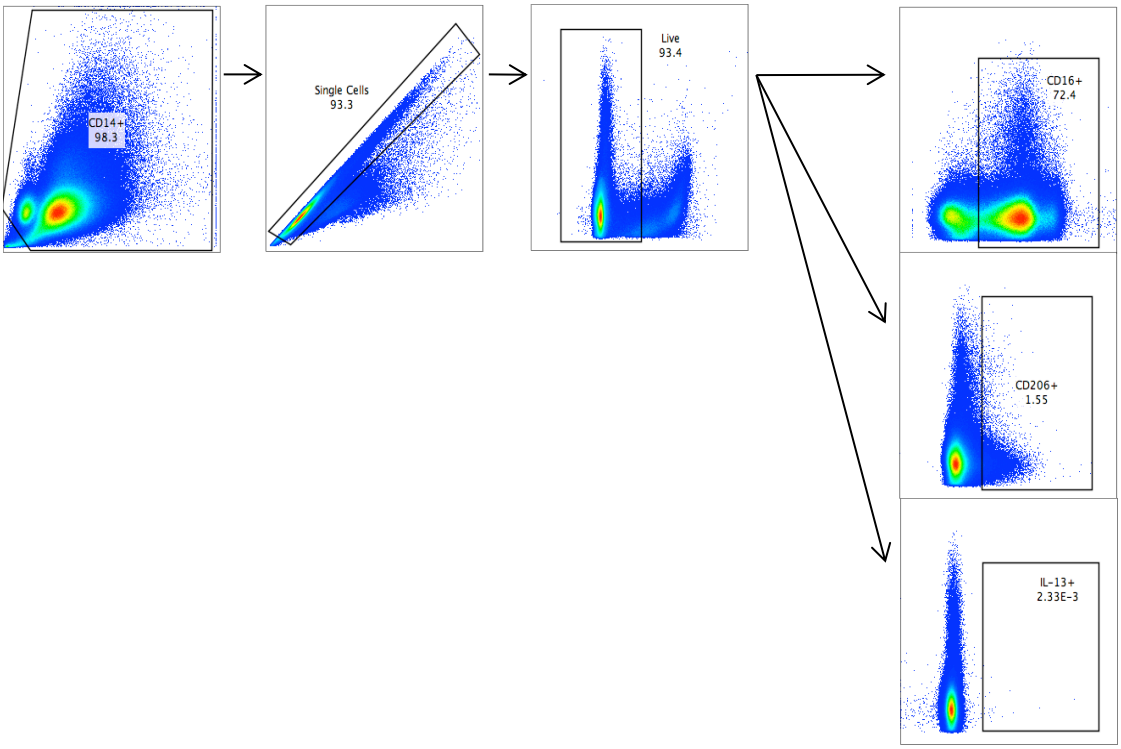

Figure 4.9: Monocyte gating strategy and stimulation with recombinant surfactant protein D. (A) Monocyte gating strategy generated in Switzerland. Gating based on CD14+, single cells, and viability marker, CD16+ or CD206+ or IL-13.

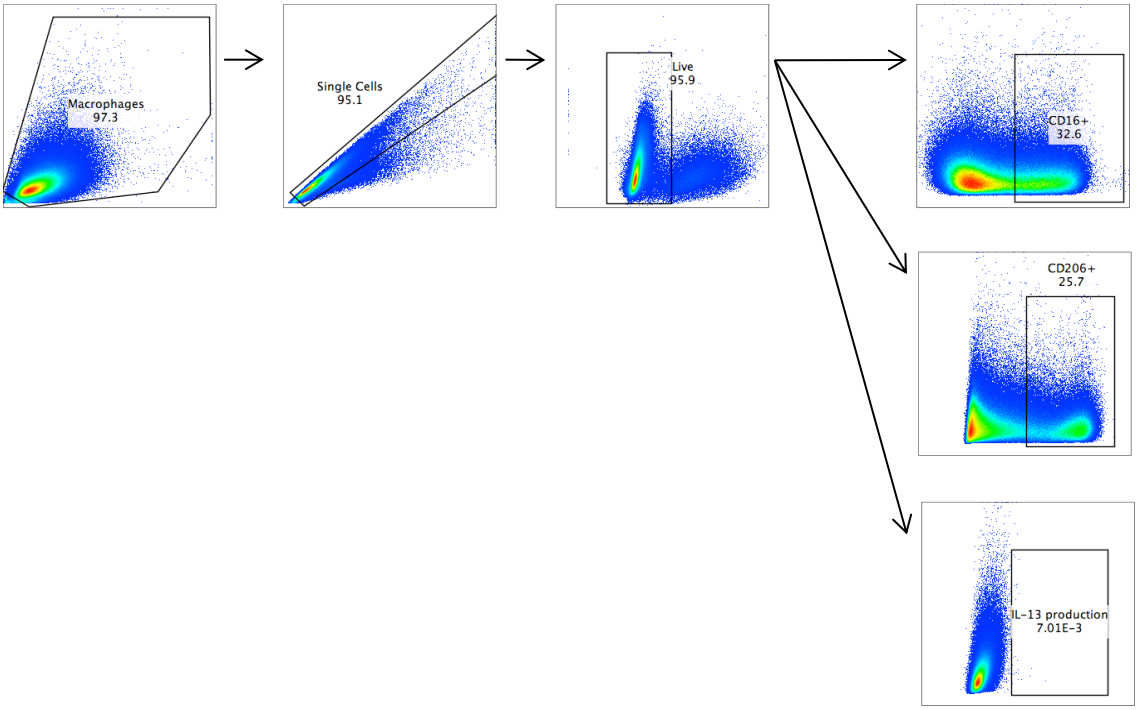

Figure 4.10: Macrophage gating strategy and stimulation with recombinant surfactant protein D. (A) Macrophage gating strategy generated in Switzerland. Gating based on size, single cells, and viability marker, CD16+ or CD206+ or IL-13.

4.2.3 Both SP-D and helminth antigen influence monocyte and macrophage phenotypes

The cell work was re-located to the École Polytechnique Fédérale de Lausanne (EPFL) for a research internship. We focused on expanding the research done at UCT; looking at human PBMCs, specifically monocytes and macrophages, and their responses to human SP-D, helminth antigen. Outputs were surrounding CD16 expression, CD206 expression and IL-13 release (no data). Limitation on time and reagents meant we were only able to perform a few experiments. Focussing on the data that was generated, we see trends surrounding both CD16 and CD206 expression. From each donor, we extracted the PBMC's then using magnetic isolation we extracted human monocytes (CD14+ cells), to either stimulate directly (PMA/ionomycin) or culture (GM-CSF) into monocyte-derived alternately activated macrophages.

4.2.3.1 SP-D has a small influence on human monocyte CD16 expression

To establish whether SP-D had an effect on human monocytes, we assessed our chosen cell population's expression of CD16. We reviewed the monocyte expression of CD16, showing four donors under three different concentrations of SP-D (no rfhSP-D, 20ug/ml rfhSP-D or 40ug/ml rfhSP-D) (Figure 4.11). We show all four donors in unstimulated conditions (Figure 4.11 A) and all four donors in stimulated conditions (Figure 4.11 B). Both donor AV and AZ under-stimulated or unstimulated conditions showed a definite decline in CD16 expression with the addition of SP-D and increased concentration of SP-D. The other two donors showed no conclusive trend. Inconclusive data from the first sets of stimulation experiments showed increased CD16 expression in 2 donors monocytes with SP-D (stimulated and unstimulated) and a negligible change in CD16 expression in 2 donors monocytes with SP-D (stimulated and unstimulated). No shift in negligible expression of CD206 monocytes with SP-D. Here we observe that SP-D has a small influence on CD16 expression of monocytes both in stimulated and unstimulated conditions.

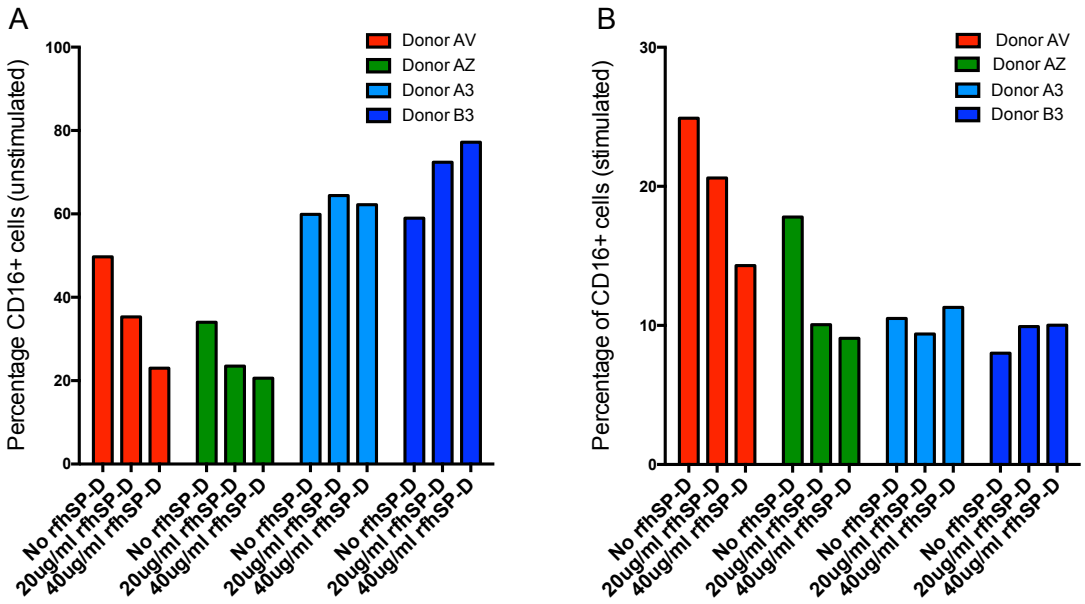

Figure 4.11: Proportion of CD16+ monocytes. Peripheral blood mononuclear cells from 4 donors treated with various concentrations of surfactant protein D (SP-D) either unstimulated or stimulated with PMA/ionomycin. Cells then analysed via flow cytometry and gated on proportions of CD14 and CD16, which is shown here as a percentage. (A) Shows the percentage of CD16+ monocytes left unstimulated and treated with variable concentrations of SP-D (B) Shows the percentage of CD16+ monocytes stimulated with PMA/Ionomycin and treated with variable concentrations of SP-D.

4.2.3.2 SP-D had negligible influence on human macrophage CD16 and CD206 expression

To establish whether SP-D had an effect on the human macrophage, we assessed our cell population's expression of CD16 and CD206. Macrophage expression of CD16 and CD206 was reviewed, with four donors in stimulated conditions under three different concentrations of SP-D (no rfhSP-D, 20ug/ml rfhSP-D or 40ug/ml rfhSP-D) (Figure 4.12). Inconclusive data with variable CD16 expression, mostly negligible in 3 donors with massive expression seen in the 4th donor (stimulated conditions) (Figure 4.12 A). A variable but an increased amount of CD206 expression in donors macrophages (Figure 4.12 B) with similar expression levels of CD206 but upon the addition of *Ascaris* alone, *Ascaris* + SP-D or *Ascaris* + SP-D + CaCl2 the CD206 expression levels dropped substantially, particularly in the case of *Ascaris* + SP-D + CaCl2 (Figure 4.12 B). Here we observe that SP-D has a negligible influence on human macrophage expression of CD16 and CD206 in both stimulated and unstimulated conditions.

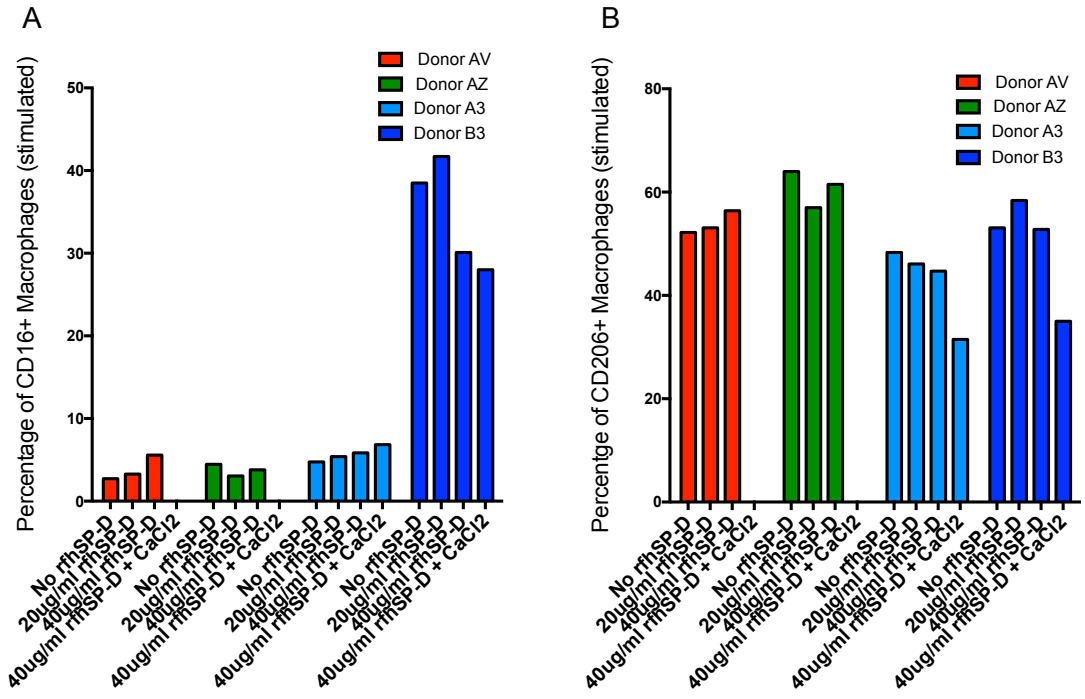

Figure 4.12: Proportion of CD16+ or CD206+ macrophages. Peripheral blood mononuclear cells from 4 donors underwent magnetic isolation selecting for CD14+, which identifies circulating monocytes. These monocytes were then cultured with GM-CSF, which generates monocyte-derived human macrophages. Cells were then treated with various concentrations of surfactant protein D and calcium chloride and stimulated with PMA/ionomycin. Cells then run via flow cytometry and gated on proportions of CD16 or CD206, which is shown here as a percentage. (A) Shows the percentage of CD16+ macrophages stimulated treated with variable concentrations of SP-D and calcium chloride (B) Shows the percentage of CD206+ macrophages stimulated and treated with variable concentrations of SP-D calcium chloride.

4.2.3.3 Ascaris antigen and SP-D upregulate expression of CD16 on monocytes separately but combined lowered it back towards baseline

We then went on to review the influence of live *Ascaris* antigen on monocyte phenotype. We looked at the monocyte expression of CD16 and CD206 in the presence of *Ascaris* antigen (Figure 4.13). The proportion of CD16+ monocytes and the influence of SP-D alone, *Ascaris* alone or SP-D and *Ascaris* in combination (Figure 4.13 A). We see an inconclusive signal from CD206+ monocytes and the influence of SP-D alone, Ascaris alone or SP-D and *Ascaris* in combination (Figure 4.13 B). We see that in both donors, the addition of SP-D or antigen alone increased the expression of CD16. Finally, the addition of *Ascaris* and SP-D combined in both donors drops the proportion of CD16 monocytes (Figure 4.13 A). Here we observe that both *Ascaris* antigen and SP-D upregulate the expression of monocyte CD16 separately but combined lower its expression back towards baseline. The nature of this effect should be further investigated to elucidate whether the effect is through direct interaction or stimulation of other receptors.

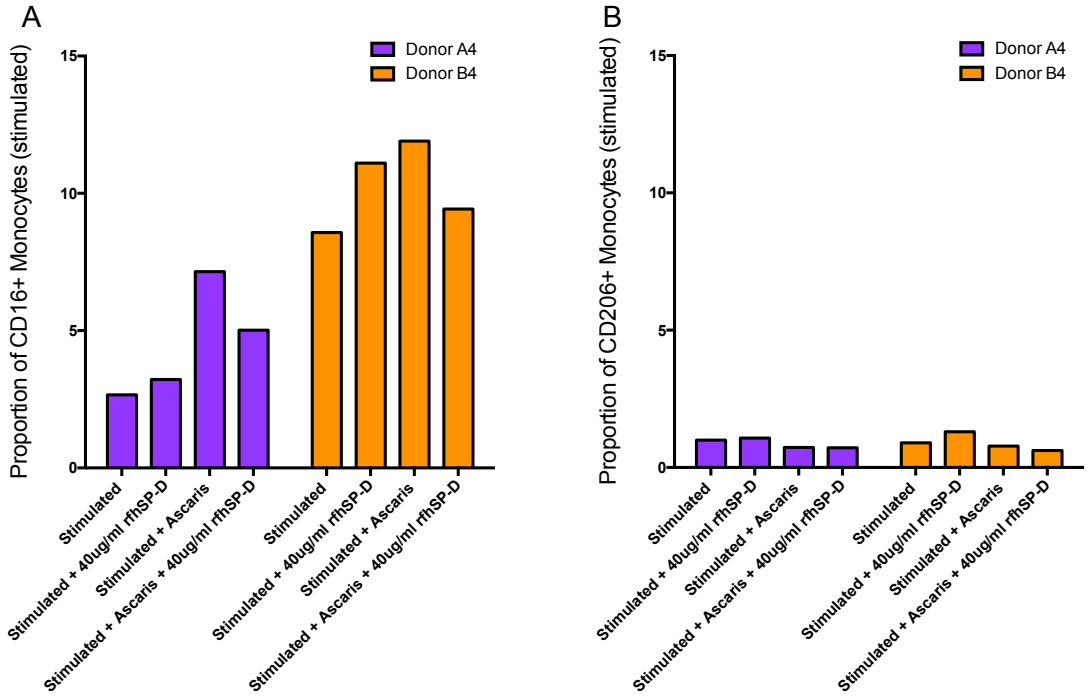

Figure 4.13: Proportion of CD16+ or CD206+ monocytes during exposure to *Ascaris*. Peripheral blood mononuclear cells from 2 donors stimulated with PMA/ionomycin and treated with various concentrations of surfactant protein D (SP-D) in the presence of live *Ascaris* (species) worm. Cells then analysed via flow cytometry and gated on proportions of CD16 or CD206, which is shown here as a percentage. (A) Shows the percentage of stimulated CD16+ monocytes under various conditions *in vitro*. (B) Shows the percentage of stimulated CD206+ monocytes under various conditions *in vitro*.

4.2.3.4 Macrophage CD206 and CD16 decreased with the addition of antigen alone, antigen combined with SP-D, decreased further with antigen & SP-D & CaCl2.

Here we show macrophage expression of CD16 and CD206 with antigen (Figure 4.14), reviewing proportion of CD16+ macrophages with SP-D alone, *Ascaris* alone or SP-D and *Ascaris* (combined) (Figure 4.14 A), the proportion of CD206+ macrophages with SP-D alone, Ascaris alone or SP-D and *Ascaris* (combined) (Figure 4.14 B). Both donors (stimulated) with a high baseline CD16 expression, the addition of *Ascaris* antigen dropped donors CD16 to negligible levels (Figure 4.14). Addition of SP-D or SP-D+CaCl2 increased the CD16 expression back towards baseline (Figure 4.14). The macrophages from both donors had similar expression levels of CD206. Still, upon the addition of *Ascaris* alone, *Ascaris* + SP-D or *Ascaris* + SP-D + CaCl2 the CD206 expression levels dropped substantially (Figure 4.14 B), particularly in the case of *Ascaris* + SP-D + CaCl2. Here we observe the effect of antigen, SP-D and calcium, which lowers the expression of macrophage CD206 and CD16.

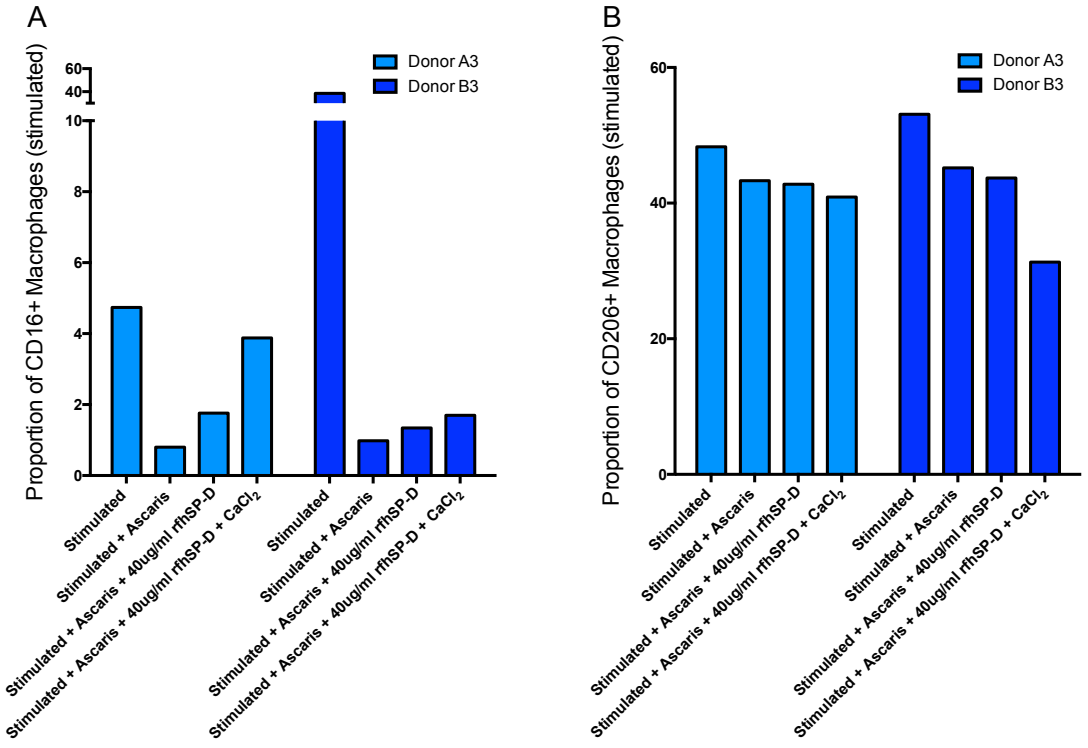

Figure 4.14: Proportion of CD16+ or CD206+ macrophages during exposure to *Ascaris*. Peripheral blood mononuclear cells from 2 donors stimulated with PMA/ionomycin and treated with various concentrations of surfactant protein D (SP-D) in the presence of live *Ascaris* (species) worm. Cells then run via flow cytometry and gated on proportions of CD16 or CD206, which is shown here as a percentage. (A) Shows the percentage of stimulated CD16+ macrophages under various conditions *in vitro*. (B) Shows the percentage of stimulated CD206+ macrophages under various conditions *in vitro*.

4.3 Discussion

4.3.1 The effect of SP-D and *Ascaris* on human monocytes and macrophage (CD16 expression) phenotypes

SP-D is known to directly bind to other helminth antigens [69] through its carbohydrate recognition domain, with no research looking into whether *Ascaris* binds directly with SP-D, we postulated that because of surface carbohydrates present on *Ascaris* there would be direct binding. Other research confirms the helminth-monocyte trend that we observed in our data, with monocyte CD16 expression increased with parasite-specific IgG and IgG1 (schistosome infection). This is associated with protection against parasites in healthy adults [66]. The study mentioned above used a cohort of patients that have already been exposed and developed resistance to schistosome infection *in vivo*. The development of protective antibodies (Fc chain of IgG) would potentially act directly with monocyte CD16. Our trend showed similar increased expression of CD16 upon exposure to live worm antigen *in vitro; however* since no protective antibodies were present, the interaction might be more complicated than simply the effect of protective antibodies reacting with CD16.

CD16 expression on both monocytes and macrophages was influenced by the addition of SP-D or antigen alone and in combination. SP-D and *Ascaris* influenced host immunity through a change in human monocyte CD16 function. The individual addition of *Ascaris* or SP-D increased expression of CD16. *Ascaris* has a larger effect in upregulation than that of SP-D. Interestingly, when *Ascaris* and SP-D are added, the combined effect was downregulated close to baseline CD16 expression. We postulate that SP-D bound directly to *Ascaris* and that SP-D indirectly interacted with monocyte due to upregulation of CD16. In an *in vivo* model, CD16 upregulation would bind to a specific antibody that activates the process of ADCC. *Ascaris* alone also upregulates CD16 through direct interaction with the immune cell. We hypothesise that SP-D could indirectly influence monocyte expression of CD16 on the cell specifically, by a mechanism as of yet, undescribed. SP-D could also compete for *Ascaris* antigen-binding sites mitigating effect on monocyte. However, the combined effect of *Ascaris* and SP-D on monocyte CD16 dampens expression back towards baseline.

SP-D and *Ascaris* influenced host immunity through a change in human monocyte-derived macrophage CD16 function. The addition of *Ascaris* antigen downregulated both donors macrophage CD16 expression to almost negligible levels, interestingly the addition of SP-D or SP-D+$CaCl_2$ upregulated the CD16 expression back towards the baseline, rescuing the response. Again, we postulated that SP-D binds directly to *Ascaris*. We know in an *in vivo* model, CD16 upregulation would bind to the specific antibody that activates the process of ADCC. However, unlike monocyte CD16, the addition of *Ascaris* alone downregulated CD16 in macrophages. The addition of SP-D or SP-D+$CaCl_2$ seems to have begun to upregulate CD16 again; we hypothesise that this is either through an interaction with the macrophage itself or because SP-D binds directly to helminth antigen competing for binding sites. As this is a time-dependent process, we would need to conduct more experiments in a time-dependent manner to confirm this.

Interestingly, we saw opposing effects of *Ascaris* on monocyte and macrophage expression of CD16. Monocyte CD16 increased in the presence of *Ascaris,* and macrophage CD16 expression decreased. In both cases, the addition of SP-D dampened the effect of *Ascaris* antigen on CD16 back towards the original baseline level of expression. *This section illustrated that SP-D and helminth antigen had an influence on human CD16 expression on both monocytes and macrophages.*

4.3.2 The effect of SP-D and *Ascaris* on human macrophages (CD206 expression) phenotypes

The three prevailing cell types in the lung alveoli are alveolar type I cells, alveolar type II cells and alveolar macrophages. MR's abundance in the lung comes from the surface expression on alveolar macrophages [215], which is how they interact with and detect helminth antigen. Alveolar macrophages are the first line of defence in the protection against not only inhaled pathogens but resident pathogens as well (i.e. lung based helminth infection). To avoid compromising gaseous exchange, they are unique in their function as a macrophage, and tightly regulated to prevent unnecessary inflammatory responses [216] [133]. Part of their unique phenotype includes greater phagocytic abilities and increased expression of receptors of innate immunity, like MR [217]. Circulating monocytes do not supply resident alveolar macrophages; instead, alveolar macrophages develop shortly after birth [218]. Although with macrophages that are not

AM's, rather human monocyte-derived macrophages, they still give us an idea of specific responses to stimuli.

Similarly, SP-D and SP-A are released by another common cell in the lung; type 2 alveolar cells, this is in response to signals from the epithelium via cytokines such as IL-33, IL-25 and TSLP. We know SP-A upregulates the surface expression of MR on human macrophages, this upregulation enhances phagocytosis of invading pathogens. It would, therefore be logical to assume that SP-D has a similar effect on the expression of MR in human alveolar macrophages [219]. In our research with mouse modelled nematode infection, SP-D has been shown to interact directly both with the antigen (via CRD) and alveolar macrophages following *N. brasiliensis* infection and to increase expression of AAM markers after IL-4/IL-13 treatment *ex vivo* [37]. We know pulmonary surfactants actively suppress alveolar macrophage activation during resting conditions [220]. Alveolar macrophages also phagocytose excessive surfactant proteins [218]. With SP-D functioning as a soluble PRR and MR functioning as a macrophage bound PRR, we see an interesting mechanism by which the balance of type 2 inflammation may be kept in check. This is an example of SP-D mediated downregulation of macrophages during helminth exposure and shows the critical linkage between innate and adaptive arms of immunity.

SP-D and *Ascaris*, alone or in combination cause a shift in macrophage expressing CD206 and potentially influences macrophage phenotype as a whole. In our findings, we saw human monocyte-derived macrophages expression of CD206 decreased, or is downregulated upon the addition of *Ascaris*, *Ascaris*+SP-D and decreased further with *Ascaris*+SP-D+$CaCl_2$. As CD206 (MR) is a pattern recognition receptor on macrophages, likely, it binds directly to surface carbohydrate molecules on *Ascaris*. SP-D is known to directly bind to other helminth antigens [69] through its carbohydrate recognition domain, with no SP-D specific *Ascaris* binding research we postulate that because of surface carbohydrates present on *Ascaris* there will be direct binding. The amount of antigen available for binding to macrophages is decreased, which could be the mechanism by which CD206 decreases. *This section illustrated that SP-D and Ascaris individually or combined have an effect on macrophage CD206.*

An observation that came out of our data was that adding $CaCl_2$ decreases CD206 on macrophages. SP-D is mediated by calcium-binding *in vivo* [129] and here we see the dampening of CD206 expression on macrophages which is enhanced by calcium. We have observed that the addition of calcium further drives the SP-D mediated downregulation of CD206 on macrophages, which is, as of yet, an undescribed mechanism that warrants further investigation.

4.3.3 Chapter conclusion

This chapter was an exploratory analysis of certain human immune cell types and the specific effect of surfactant protein D and *Ascaris*. We see that SP-D and *Ascaris* had an effect on both CD16 and CD206. Our small amount of data may point to there being both direct and indirect methods by which SP-D and *Ascaris* have an influence on CD16 and CD206 expression. We postulate that in the case of CD16 and CD206 on either cell types, there are different mechanisms at play here; direct binding, competition for binding sites or indirect activation through another receptor or cell.

There are a few considerations that must be made surrounding SP-D use *in vitro*; in these experiments, we used recombinant fragment SP-D which contains only the carbohydrate recognition domain (Figure 1.8) and whilst this is the principal means by which SP-D interacts, as discussed earlier, different oxidative states in the pulmonary environment can generate opposing effects with the N-terminal domain leading to alternate macrophage polarisation [129]. Not only does the direction by which SP-D binds have an influence on its effect but also the structures that SP-D forms before interacting with the surrounding environment, SP-D's monomeric subunits can arrange into trimers, hexamers, dodecamers and then multiunit dodecamers that resemble a 'fuzzy ball' (Figure 1.8). These more complex arrangements centre around the N-terminus, hiding it and leaving multiple CRD binding points exposed for binding. Although trends are visible, due to time and budgetary constraints, we have limited data generated from these stimulations.

The conclusion is also limited by the size of the sample pool that was used, which limits our ability to run reliable statistical analysis. We are also limited by flow cytometry and the background noise generated by this experimental technique, with smaller, more niche cells populations, it becomes difficult to discern what are truly positive results.

Future work should consider the addition of more samples, controls, different oxidative states and different calcium levels. Not only do we need to be aware of the particular molecular binding positions but also look at expanding the participants. Using 1-2 donors to understand an immune mechanism that can be primed by so many external factors is not viable, performing the experiments on a larger cohort should be considered for future work.

Circulating monocytes do not supply resident alveolar macrophages; instead, alveolar macrophages develop shortly after birth [218]. With human research, it is critical to turn focus to the cell types in question within their microenvironment and not just perform experiments on circulating cell types that are not primed in the same way as tissue-resident cell types. The context of interactions is vital; it is always wise to question these findings relate to the specific mechanisms going on in stimulation experiment and how this finding relates to the infection *in vivo*.

Type 2 innate lymphoid cells also have a large role to play in response to helminth infection, we have already created the means by which to analyse them, so it would be interesting to perform similar SP-D & *Ascaris* exposure experiments on this rare and very powerful cell type that is known to interact with helminth infections.

Research points to the assumption that SP-D binds to *Ascaris,* and it would be ideal to confirm that through binding assay experiments surrounding particular *Ascaris* antigens and SP-D in monomeric to multimeric forms.

Helminth infection induces a regulatory phenotype in DC's which in turn through the activation of TLR2, SP-D also induces this phenotype [182]. Perhaps this mechanism is a means by which SP-D indirectly causes regulation in the lung downregulating immune response. The investigation of DCs and its markers could be a vital step forward in unravelling the network of interaction.

For human monocyte experiments, expansion of the flow cytometry panel to include more of the Fcγ receptor family would be optimal to fully assess the influence of SP-D on host immunity through alteration of human monocyte function. Monocytes express Fcγ (CD16) as well as the inhibitory receptor, FcγRIIb (CD32) [221] [222], and high

affinity receptor for IgG, FcγR1a (CD64) [221] [222]. Expression levels of these receptors may further indicate how human monocytes alter in response to infection, particularly with their role in immunoglobulin interaction.

5 Overall conclusion

The type 2 immune cascade in helminth infection involves damage to the epithelial layers in the body leading to the release of IL-25, IL-33 and TSLP, which triggers the downstream activation of effector populations in an attempt to expel the helminth. In helminth infections involving lung colonisation, the primary cell type releasing IL-33 and priming anti-helminthic responses are alveolar type II cells (ATII cells). Another major immunomodulatory protein released by ATII cells is surfactant protein D (SP-D). Classified as a hydrophilic pulmonary collectin, SP-D plays a vital role in the modulation of mucosal immunity during exposure to microbes.

Recently, data emerging from our research group at UCT (Thawer *et al.*, under review PLOS Pathogens) links the specific control of mouse-modelled nematode infections with SP-D. The research demonstrated that infection with *N. brasiliensis* correlated with increased lung SP-D levels (Figure 1.6A) and the administration of recombinant SP-D protects against *N. brasiliensis* infection by reducing worm burden (Figure 1.6Bi) and drove the expansion of ILC2s and $CD4^+$ T-cells (Figure 1.6Bii and Figure 1.6Biii). Therefore we hypothesised that SP-D could function as a novel anti-helminthic in human infections.

We determined that, in human sera, and *Ascaris lumbricoides* infection alters systemic levels of surfactant protein D. Western blot analysis highlighted the unique association between SP-D and *Ascaris*-specific IgG4. We then went on to determine that association exists in a larger cohort, with it being specific to helminth species that transit the lung as part of their lifecycle. The cohort also contained data pertaining to allergic disease, in specific atopic dermatitis, we see SP-D is not an indicator for the severity of AD, unlike other diseases. The atopic participants also had increased levels of atopic sensitisation, with very low levels of active infection, we attribute this phenomenon to upregulated Th2 response in allergic disease, leading to faster parasite clearance. Our antibody data also gives insight into the role of the IgE – IgG4 balance in both allergic disease and helminth infection, a mechanism worth investigating further.

Interestingly, we also saw evidence of the rural-urban divide prevalent in exposure to helminth infections, risk of allergy, levels of SP-D, all suggestive of geography and genetic background being highly determinate in health and disease outcomes. There seems to be a clear mechanism at play here, but an expansion on cohort size as well as looking at other markers in serum should be considered. Helminth exposure and modulation of physiology are complex; more investigation needs to be directed towards other measurable biomarkers in serum such as antibody titers and cytokine responses to validate the concept of Th2 up-regulation and the homeostatic abilities of SP-D.

With the limiting nature of human sample work, this investigation progressed on to cell phenotyping and manipulating controlled environments to gather more information on surfactant protein D's immunological impact on type 2 immunity. Therefore we designed and optimised two antibody panels for flow cytometry to review monocyte/macrophage and ILC2 phenotypes in response to both helminth antigen and SP-D. When our research shifted to a lab in Switzerland, the second panel was in major use with monocytes and monocyte-derived macrophages. Human work can be fickle, and the purpose of this investigation was to move away from recruitment of samples and limited investigation through serum protein levels and on to understanding the mechanics of how SP-D effects the immunology of helminth infection in humans.

We observed the *in vitro* effect of helminth function on immune cells by first seeing that, with CD16 expression, *Ascaris* upregulates it on monocytes and downregulates it on macrophages. The addition of SP-D rescues that effect, on both cell types, towards the baseline. We then reviewed the CD206 expression on macrophages exposed to *Ascaris* and saw that CD206 is downregulated, and the addition of SP-D downregulates it further. These findings point to the modulatory role that SP-D plays during infection with a parasite, through both direct and indirect binding and competition for epitopes.

The cell types in use must be considered; they are not a true reflection of alveolar macrophages with human research it is critical to turn focus to the cell types in question within their microenvironment and not just run experiments on circulating cell types that are not primed in the same way as tissue-resident cell types. Considerations must also be made for the structural complexity with which SP-D binds.

All of this data points to expansive effects of SP-D on the immune system, with indirectly mediated downregulation of inflammatory responses the interplay between SP-D and antigen directly, specifically when that antigen is immunomodulatory. There are multiple avenues by which SP-D and helminths affect the immune system they occupy both through direct binding, competition for binding sites or indirect activation of another cell type. Future work should be directed towards other measurable biomarkers in serum, such as antibody titers and cytokine responses and expansion of the flow cytometry panels and including more cell types in the analysis.

These findings point to the need for further investigation into the novel role of SP-D in the control of human helminth infections in the context of immune mechanisms, biomarker and eventually, treatment.

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