Can a gut helminth parasite influence Th2 inflammatory responses in the skin?

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Abstract

Helminth parasites are one of the most common infectious agents of humans and cause significant health and economic burdens in the countries they are endemic in, making elimination an important goal. However, epidemiological studies have suggested an inverse correlation between the incidences of infections by helminth parasites in humans and autoimmune and allergic disease prevalence worldwide; it is thought the eradication of parasites in more affluent countries through improved hygiene is an important factor for the increasing incidence of autoimmune and allergic diseases encountered in the Western world.

A Th2 immune response is central in providing immunity against helminth parasites, while suppressing T helper (Th) 1/Th17-mediated inflammation and inducing wound repair mechanisms. Helminths have developed strategies to directly regulate the immune response against them to ensure their own survival. Experimental evidence has demonstrated helminths are also able to dampen inflammatory bystander immune responses in their host, via induction of regulatory mechanisms such as regulatory T cells. These studies have focused primarily on the suppression of food and airway allergies in mouse models and there is limited data on the effect of helminth parasites on skin allergy e.g. atopic dermatitis.

Atopic dermatitis (AD) is a chronic/chronically relapsing Th2 inflammatory skin condition, characterized by skin lesions, dry itchy skin and impaired skin barrier function. This is believed to allow the entrance of other allergens into the body more easily, leading to sensitization and initiation of other allergies later in life, a process termed the 'Allergic March'. With the increased incidence of allergy in the Western world, it is desirable to find new therapies to suppress AD and the onset of the allergic march.

During my Masters, I have investigated whether the gut-dwelling mouse parasite *Heligmosomoides polygyrus* was able to suppress Th2 responses induced in skin tissue using two different allergy models: 1) intradermal injection (ID) of whole mashed-up house dust mite (HDM), which induces Th2 inflammatory responses, and 2) topical application of the chemical hapten dibutyl phthalate-fluorescein

isothiocyanate (DBP-FITC), mimicking allergic responses seen in AD. The results show that *H. polygyrus* induces interleukin (IL)-4 production in tissues distal to the gut, including the ear skin tissue, mainly from cluster of differentiation (CD) 4$^+$ T cells. Furthermore, helminth infection was able to suppress Th2-mediated inflammation in the skin in both house dust mite and DBP-FITC models, coinciding with an increase in the proportions of regulatory T cells (Tregs) in skin-associated lymph nodes (LNs). This research further demonstrates the potential use of helminth parasites, or their products, as a therapy for allergic diseases, including those of the skin.

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Abbreviations

AAM	Alternatively activated macrophage
AD	Atopic dermatitis
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
BCG	Bacille Calmette-Guérin
Breg	Regulatory B cell
CAM	Classically activated macrophage
CCL	Chemokine ligand
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CHS	Contact hypersensitivity
CLR	C-type lectin receptor
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
DBP	Dibutyl phthalate
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box protein 3
GWAS	Genome-wide association studies
HDM	House dust mite
HES	*H. polygyrus*' excretory/secretory products
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell co-stimulator
Ig	Immunoglobulin

IL	Interleukin
ILC	Innate lymphoid cell
iTreg	Induced regulatory T cell
KLRG	Killer cell lectin-like receptor subfamily G member
LN	Lymph node
LPS	Lipopolysaccharides
MFI	Median fluorescence intensity
MHCII	Major histocompatibility complex class II
miRNA	Micro ribonucleic acid
mMCP-1	Mucosal mast cell protease-1
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
NKT	Natural killer T cells
NLR	Nucleotide-binding oligomerization domain-like receptors
NOD	Nonobese diabetic
nTreg	Natural regulatory T cells
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PAR2	Protease activated receptor 2
PD-L1	Programmed death-ligand 1
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
RAG	Recombination activating gene
RBC	Red blood cell
RELM-α	Resistin-like molecule-α
SEM	Standard error of the mean

SPF	Specific-pathogen free
STAT6	Signal transducer and activator of transcription 6
T1D	Type 1 diabetes
TCR	T cell receptor
Tfh	T follicular helper cell
TGFβ	Transforming growth factor β
TEWL	Transepidermal water loss
Th	T helper (cell)
TLR	Toll-like receptor
TN	Triple negative
Tr1	Type 1 regulatory cell
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor

Routes of injection

IP	Intraperitoneal
IG	Intragastric
ID	Intradermal

Chapter 1.   General Introduction

1.1 The hygiene hypothesis and helminth parasites

Parasitic worms (helminths) and humans have co-evolved for many years.[1] From the 19th century, with increased industrialization, urban living, and improvement of hygiene, these helminths have been eradicated in the Western world. However, allergic disorders such as hay fever have emerged as a "post-industrial revolution epidemic" and over the last 50 years, a steady rise in prevalence has been seen in autoimmune and allergic disorders in Westernized countries.[2] In 1968, Greenwood *et al.*[3] reported a low incidence of rheumatoid arthritis in West Africa, where helminth infection is endemic, and first noted an inverse correlation between incidence of helminth infections in developing countries and immune disorders in more developed countries. In 1989, Strachan found the prevalence of hay fever was increased in people with smaller family sizes and the position they held in a household.[4] It was hypothesized that declining family size and improvement in hygiene reduced chances of cross-infection in young families, which could result in increased clinical expression of allergic diseases.[2, 4] This theory was named the "hygiene hypothesis".

1.1.1 Epidemiological studies of helminth infections and immune disorders

Many epidemiological studies over the intervening years have focused on the hygiene hypothesis. It was found that factors associated with a Western life-style could be responsible for an increased risk of atopy, as with families in higher social economic groups having a higher prevalence of atopic disorders than poorer families in the same country.[2, 5] Furthermore, keeping pets and the sharing of bedrooms is protective, most likely due to the early exposure to allergens, whereas antibiotic use and poor diet (i.e. leading to changes in microbiota) positively correlate with prevalence of atopy in children.[6-8]

In developing countries, significantly lower rates of immune disorders have been recorded, and this is often linked to the endemicity of helminth infections in equatorial and sub-tropical regions. Humans infected with helminths have a lower sensitivity to skin-prick tests and lower levels of allergen specific immunoglobulin

(Ig)E antibodies.[9, 10] This protection was reversed when infected people were de-wormed with anti-helminth drugs, as demonstrated by an increased sensitivity to allergens during skin-prick tests.[11] Furthermore, studies have shown that helminths are able to directly induce regulatory mechanisms in humans, demonstrated by an increase in the anti-inflammatory cytokine interleukin (IL)-10 and IgG4 antibody production (discussed further below).[9, 12]

Based on their ability to induce immune-regulatory mechanisms in humans, and in numerous mouse studies,[9, 12-17] helminths are being trialled as a new therapy for autoimmune and allergic diseases. So far, the pig whipworm *Trichuris suis* and hookworm *Necator americanus* have been used in several trials and proven to be safe and effective in reducing symptoms of immune disorders such as inflammatory bowel disease (IBD), Crohn's disease, asthma and multiple sclerosis (MS).[18-21]

1.1.2 Parasitic diseases: symptoms and harmful effects of infection

Helminths infect more than 1 billion people worldwide;[22] these include schistosomes, hookworms, flatworms, filarial worms, and roundworms. They infect humans via different routes, cause a variety of symptoms and differ in the morbidity they cause.

Hookworms are soil-transmitted helminths and infect over 500 million people in the world. The most common human hookworms are *N. americanus* and *Ancylostoma duodenale*.[23] They chronically infect humans, often asymptomatically, indicating adaptation of the parasite to the host's immune system.[24] They penetrate the skin and migrate through the lungs, ending up in the gut where they burrow through the wall to access the host's blood for feeding.[23, 25] The parasites mature in the gut and secrete eggs into the faeces, which hatch outside the host. The migration and blood feeding of the parasite causes tissue damage, haemorrhaging and anaemia, which can aggravate malnutrition in countries where this is already problematic, leading to growth stunting, impaired cognitive development and diminished physical fitness.[25, 26]

Schistosomes infect over 240 million people through fresh water and are also able to live within the same host for decades. The main species to infect humans are *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*.[27] Infective larvae (cercariae) penetrate through the skin and migrate through the lungs to either the perivascular or mesenteric venules. Mature parasites mate and produce eggs, which can be secreted through faeces or urine, or are trapped in tissues such as the liver and bladder leading to granuloma formation, to wall off the eggs, and fibrosis.[27] Health complications caused by schistosome infection include: anaemia, growth deficiencies, urinary tract disease, reduced reproductive health, and bowel ulceration.[28-30]

As immunity to most microbes depends on a T helper (Th) 1/Th17 mediated immune response, the skewing towards a Th2 response by helminths often has an adverse effect during co-infection with other pathogens. Helminth infections are most common in countries where malaria, tuberculosis and human immunodeficiency virus (HIV) are also prevalent. Studies have shown that helminth parasites could increase susceptibility to tuberculosis and lower efficacy of tuberculosis therapy.[31] Furthermore, urogenital schistosomiasis, caused by *S. haematobium*, is associated with increased susceptibility to HIV infection.[32, 33] Finally, there is some conflict on the effect of intestinal helminths on the development of malaria as some studies show an increased risk from *Plasmodium* species in helminth infected individuals,[34, 35] whilst others show helminths protect against *Plasmodium* infection.[36]

Some vaccines have shown a lowered efficacy in countries where helminths are prevalent, such as the Bacille Calmette-Guérin (BCG) vaccine, likely due to increased IL-10 production in helminth infected people.[37] Furthermore, the skewing to alternative-activation of macrophages rather than classical activation, could lead to impaired anti-viral and anti-bacterial immunity.[38] Latent viruses, such as γ-herpes virus, can also be reactivated by high levels of IL-4, a cytokine released during the Th2 response elicited by helminths.[39]

While helminth parasites can be protective against some infectious and non-communicable diseases, it is clear helminth parasites can cause major health issues

as well. This includes increasing susceptibility to pathogens also prevalent in those countries and altering vaccine efficacy, making the eradication of helminths in developing countries a desirable option.

1.2 The Th2 immune response

The immune system has evolved to deal with many different kinds of pathogens such as viruses, bacteria, parasites and fungi, but also bites, stings and toxins from animals and plants. These pathogens and toxins are neutralized and removed through the initiation of different immune responses in which each component has a different role to play.

Cluster of differentiation (CD) 4$^+$ T cells are central to these different responses and can be split into several different subsets based on their cytokine production and transcription factor expression.[40] While Th1 and Th17 response are important for immunity against micro-organisms such as viruses, bacteria and fungi, they can also cause immune-mediated tissue damage to the host and are involved in autoimmune disorders such as MS and rheumatoid arthritis, via the release of pro-inflammatory cytokines such as IL-12.[41-43] The Th2 response, on the other hand, is specialized in mediating wound healing after tissue damage and anti-parasitic immunity, but is also involved in mediating allergic diseases (discussed below).[44] Th2 cells secrete the canonical cytokines IL-4 and IL-13 to activate innate and adaptive cells involved in the anti-helminth and allergic response. Th9 cells, the main producers of IL-9, were found to be a separate subset of T cells important for mucus production and activation of mast cells and eosinophils in anti-parasitic immunity and allergic inflammation.[45-47] Regulatory T cells (Tregs) are known for their secretion of anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor β, TGFβ) which prevent chronic inflammation and autoimmune disorders.[48, 49] These different T helper subsets are able to balance one another to prevent tissue pathology e.g. the Th2 response can suppress Th1 and Th17 mediated inflammatory responses.[42, 50]

1.2.1 Evolution of the immune system with helminth parasites

Helminth parasites are multicellular macro-organisms that do not replicate inside the host, but mature and secrete eggs, which hatch outside the host to continue the lifecycle. Some parasites such as hookworms migrate through different tissues which can cause extensive tissue damage and haemorrhaging. Many also feed on

blood causing further blood-loss and pathology.[23, 24] The Th2 response has most likely evolved to expel or contain these helminth parasites while limiting tissue damage by the immune system (discussed below).[44, 51]

Helminths have been shown to induce regulatory mechanisms that suppress inflammation and anti-worm immune responses and also limit tissue damage ensuring not only prolonged worm survival and reproduction, but also the survival of the host (discussed in chapter 1.4).[15, 24]

1.2.2 Induction of the Th2 response

The first step in the induction of a Th2 response is physical trauma of epithelial barriers and the sensing of danger by epithelial cells such as those found in the gut, lung or skin. Epithelial cells secrete alarmins such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), and chemokines, to activate innate cells such as dendritic cells (DCs).[52-54] TSLP suppresses inflammatory IL-12 production by DCs and activates DCs to adopt a Th2-activating phenotype, which then migrate to the lymph nodes to activate the adaptive arm of the Th2 response.[55] IL-25 and IL-33 have similar roles in activating innate cells such as macrophages, mast cells, basophils, eosinophils and group 2 innate lymphoid cells (ILC2s).[52, 54, 56-58]

IL-4 secretion is crucial for the Th2 response and guides the differentiation of naive T cells into Th2 cells. IL-4 signalling, through IL-4Rα (a component of the receptors for both IL-4 and IL-13), activates signal transducer and activator of transcription 6 (STAT6) phosphorylation, which upregulates the transcription factor GATA-3 to drive Th2 differentiation.[59, 60] Th2 cells in the lymph nodes secrete IL-4 to induce antibody class switching of B cells to IgE. IgE antibodies are known to activate mast cells and eosinophils in the tissue, causing degranulation and release of pro-inflammatory molecules, proteins and cytokines.[61] Th2 cells migrating to the tissue secrete the main cytokines involved in the Th2 response IL-4, IL-5, IL-9 and IL-13. Once in the tissue, positive feedback loops between IL-4/IL-13 on epithelial cells and TSLP/IL-25/IL-33 on Th2 cells may occur to amplify the Th2 response, while IL-

5 is important for the recruitment of eosinophils and IL-9 can mediate mast cell proliferation.[62-64]

1.2.3 Anti-parasitic mechanisms of the Th2 response

After infection with helminths, epithelial barriers are disrupted due to tissue migration or breaching of the gut wall for blood feeding, leading to initiation of a Th2 response (figure 1.1). This section will focus on anti-parasitic mechanisms specific for the gut-dwelling murine parasite *Heligmosomoides polygyrus*, the helminth utilised in the experiments contained in this thesis, and described in more detail in the subsequent section.

CD4$^+$ T cells are central to immunity against parasites in the gut; treatment with α-CD4 antibodies, blocking IL-4 production, or blocking IL-4Rα signalling leads to impaired worm expulsion.[65-67] Furthermore, the depletion of DCs or blocking of CD80/CD86 resulted in impaired worm expulsion, demonstrating the priming of Th2 cells by DCs is crucial for anti-parasitic immunity.[68, 69]

Basophils are activated through receptors including FcεRI and ST2 (IL-33R), upon which they secrete cytokines such as IL-4 and TSLP.[70] They have been shown to migrate to the lymph nodes (LNs) as antigen presenting cells (APCs) and are an important source of IL-4 for the priming of Th2 cells during *Nippostronglyus brasiliensis* infection.[71, 72] However, depletion of basophils did not have an effect on the priming of Th2 inflammation during *H. polygyrus* infection.[73]

ILC2s are activated by IL-25 and IL-33 and are an important source of Th2 cytokines IL-4, IL-5, IL-9 and IL-13.[74] IL-4 secretion by ILC2s was recently shown to be important for the priming of Th2 cells *in vivo*.[75] IL-13 is believed to induce goblet cell hyperplasia and stimulate mucus production and smooth muscle contraction in the gut, mechanisms crucial to worm expulsion.[57]

IL-5 is important for the maturation and recruitment of eosinophils. Eosinophils release preformed granules through degranulation, containing pro-inflammatory molecules and secrete Th2 cytokines such as IL-4.[76] Eosinophilia is a hallmark of

parasitic and allergic disease, however their exact role in anti-helminth immunity is unclear at present.

Alternatively-activated macrophages (AAMs; or M2 macrophages) are important in protection against many helminth parasites. AAMs are characterized by the expression of gene products such as Yml (an eosinophil chemo-attractant), resistin-like molecule α (RELM-α), arginase-1, IL-4Rα and mannose receptor CD206.[24, 77] AAMs are involved in trapping *H. polygyrus* larvae in the gut wall, which compromises worm vitality and slowly kills them.[24] AAMs could also harm worms directly through production of arginase-1 resulting in localized deprivation of amino acids.[78] When AAMs are depleted or arginase blocked, worm expulsion is impaired.[78]

Mast cells proliferate rapidly in parasitic infection and could damage parasites by mucosal mast cell protease-1 (mMCP-1)-mediated breakdown of epithelial tight junction proteins in the host gut, increasing luminal flow and disrupting the niche of the parasites.[79, 80]

B cells have varying roles in the protection against different primary helminth infections, and are found to be particularly important for secondary immunity to *H. polygyrus* infection – both IgE and IgG1 antibodies, through IL-4 mediated class switching, are induced during infection;[81] B cell deficient mice are unable to clear secondary infections.[81]

1.2.4   Wound repair functions of the Th2 response

Some parasites, such as *N. americanus*, migrate through several tissues and cause extensive tissue damage and haemorrhaging in the process. It is believed that the Th2 response has evolved to repair damage done to the tissue at the same time as providing immunity against parasites, as many immune cells and mechanisms are involved in both processes, e.g. mucus production is initiated after disruption of the epithelial barrier, which also functions to expel worms from the gut.[44, 51, 82]

Innate cells are important mediators of wound healing mechanisms (figure 1.1). Eosinophils communicate with other immune cells such as mast cells through the secretion of cytokines such as IL-4 and IL-13.[76] They also degranulate to release preformed granules containing cytokines, growth factors and matrix metalloproteinases (MMPs) that mediate tissue remodelling, myofibroblast differentiation and wound healing.[76, 83] Mast cells also selectively release cytokines or degranulate after activation, releasing molecules such as vascular endothelial growth factor (VEGF), which is involved in angiogenesis, an important process in wound healing.[84] AAMs secrete molecules such as VEGF, RELMα, argninase-1, Yml, MMPs, TGFβ and growth factors that are all involved with wound healing mechanisms.[85, 86] Amphiregulin, an epidermal growth factor receptor ligand, secreted by ILC2s is also involved in mediating wound repair, and was seen to help maintain lung homeostasis and repair lung epithelial damage after infection with influenza virus.[87] Basophils have a less clear role, although they might assist wound repair through secretion of Th2 cytokines such as IL-4.[86]

Innate cells also initiate fibrosis, or scarring, which is defined as over-accumulation of fibrous connective tissue such as collagen in and around damaged or inflamed tissue.[86] Fibrosis can lead to permanent scarring, malfunctioning of organs and even death. IL-13 was found to induce skin fibrosis in atopic dermatitis (AD).[88] Arginase-1 secretion by AAMs, however, was seen to suppress fibrosis through the induction of regulatory T cells.[85]

Taken together, it is clear the Th2 response utilises multiple components to maintain homeostasis by suppressing a tissue-damaging Th1 response, clearing parasites, and inducing wound healing mechanisms.[44]

1.3 Heligmosomoides polygyrus

1.3.1 H. polygyrus lifecycle and experimental model

Due to millennia of parasite:host co-evolution, helminths are highly adapted to their specific host, making those adapted to the human immune system difficult to study in the laboratory.[89, 90] Model helminths that mirror similar lifecycles and migratory pathways are therefore widely used as tools to investigate immune responses against them.

H. polygyrus is a natural mouse parasite, and is thus highly adapted for the murine immune system. It is classified as a nematode parasite and is phylogenetically placed in the same order as human hookworm parasites *A. duodenale* and *N. americanus*.[91]

In the wild, mice get infected through ingesting the infective larvae, after which the larvae migrate to the small intestine and penetrate through the wall and undergo two developmental moults. At 8 days post-infection, the majority of worms have matured and emerge into the lumen where they coil around the villi of the small intestine, mate and produce eggs, which are released into the host faeces. The eggs hatch outside the host, undergoing two developmental moults to become infective larvae (figure 1.2).[79]

It has been demonstrated that most mouse strains, including C57BL/6 mice, are unable to expel a primary infection with *H. polygyrus*, although a full spectrum of Th2 responses amongst laboratory mice provides fast and slow responders for use in varied experiments.[79, 92] Also, *H. polygyrus* has an entirely enteric lifecycle in the host, making it an excellent model not only to study mechanisms of immunity against and immune modulation by chronic helminth infections, but also to study mucosal immunity and the systemic effects of helminth infections (i.e. changes in immune cell and cytokine profile in tissues distal to the gut).

1.3.2 *H. polygyrus* excretory/secretory products

As with human helminth infections, *H. polygyrus* has also been found to modify the Th2 response in mice and suppress allergic responses in several experimental settings, mainly through the induction of Tregs (discussed in chapter 1.4).[13, 16] Most likely this modified Th2 response is due to the release of *H. polygyrus'* excretory/secretory products (HES), as it has been demonstrated that HES is able to induce the same protection as a parasitic infection in a murine allergy model.[93] HES consists of a mixture of proteins, lipids and carbohydrates, which have been extensively analysed and many have been identified through proteomic studies.[94, 95]

More research is needed to find the full array of molecular mechanisms used by helminth parasites to induce immune tolerance, which could lead to novel therapies for common allergic and inflammatory conditions.

1.4 Immune regulation by helminths

1.4.1 Immune cells induced by *H. polygyrus*

Regulatory T cells

Tregs have an important role in controlling homeostasis and balancing immune responses during inflammation. These cells are important for the prevention of autoimmune diseases and regulating chronic inflammation, making them an interesting target for drug development.[48]

They are able to suppress T cell proliferation through cytokine deprivation (such as taking up IL-2 from the environment), and cytolysis.[96, 97] The activation and function of innate cells such as mast cells, basophils, and eosinophils can be inhibited via suppression of IgE antibody production by B cells, thereby preventing activation through Fcε-receptors.[98, 99] Antigen presentation by DCs can be inhibited by binding of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to CD80/CD86,[100] and the secretion of suppressive cytokines IL-10 and TGFβ.[101-104]

Tregs can either be induced naturally (CD4$^+$CD25$^+$FoxP3$^+$ nTregs) in the thymus and are characterised by their specific T cell receptor (TCR) for self-antigens and express the marker Helios, or induced from effector T cells (iTregs) during the inflammatory response in peripheral tissues (IL-10 producing type 1 regulatory cell (Tr1) or TGFβ producing Th3 cells).[99] During *H. polygyrus* infection Helios$^+$ nTregs proliferated early on, but iTregs start to expand one week after infection.[105, 106] This expansion of Tregs was paired with an increase in inhibitory cytokines IL-10 and TGFβ in the mesenteric lymph nodes (MLNs) from day 14 after infection.[16, 107] Early activation of Tregs was shown to dampen innate and adaptive Th2 responses and increased worm burden, while early depletion of FoxP3$^+$ Tregs was seen to increase pathology, T cell activation and Th2 cytokines secretion (IL-4 and IL-13) in the gut.[105, 107]

When *H. polygyrus* infection was combined with an airway inflammation model in mice, infected mice had diminished numbers of eosinophils and other immune cells in the bronchoalveolar lavage fluid (BALF) and IL-5 and eotaxin expression was reduced, while IL-10 expression and Tregs were increased.[13] Using anti-CD25

antibodies, depletion of Tregs was seen to restore infiltration of immune cells and eosinophils in the BALF. When Tregs from infected mice were transferred to non-infected mice, protection was also transferred. However, protection was also demonstrated when using Tregs from an infected IL-10 deficient mice, suggesting IL-10 does not play a key role in this model of suppressing allergic inflammation in the lung.[13]

Regulatory B cells

Regulatory B cells (Bregs) are a subset of IL-10-producing B cells thought to be involved in regulating inflammation in autoimmune diseases, and it has been suggested patients with autoimmune diseases have Bregs with an impaired function.[108] Moreover, Bregs are induced by helminth infections.[14, 109] *H. polygyrus*-infected mice were found to have a population of CD23^{hi} B cells present in the MLN, that secreted IL-10. Adoptive transfer of these cells from infected mice was sufficient to suppress Der p 1-induced allergic airway inflammation, reducing IL-5 secretion in the BALF, and eosinophil recruitment and immune cell infiltration in the lung. However, IL-10 was not required to provide this protection, suggesting another mechanism or molecules (such as through CD23 and/or TGFβ) were involved.[14]

Alternatively-activated macrophages

AAMs play an important role in the anti-helminth immune response in the gut by trapping helminths in the gut wall and slowly depriving them of nutrients. They are also important for the repair of tissue damage by secretion of many different products such as VEGF, arginase-1, Ym-1 and RELMα.[85, 86] AAMs have also been shown to have an anti-inflammatory role via secretion of anti-inflammatory cytokines IL-10 and TGFβ, suppression of immune cells via expression of programmed death-ligand 1 (PD-L1), and induction of Tregs via retinoic acid dependent mechanisms.[110-112]

H. polygyrus infection in mice resulted in the induction of AAMs in various tissues, including the spleen through STAT6-dependent mechanisms.[113] While these AAMs enhanced bacterial colitis due to suppression of the Th1 response needed to kill the bacteria, they were found protective in non-obese diabetic (NOD) mice which develop type 1 diabetes (T1D).[113, 114] In this model, AAMs and Tregs were induced in

the pancreatic LNs, but this protection was not dependent on the presence of Tregs or IL-10, suggesting a key role for AAMs.[114]

Anti-inflammatory cytokines

IL-10 is produced by a number of cells including Tregs (Tr1 cells) and AAMs, and drives differentiation of Tr1 cells.[115, 116] IL-10 is known for its suppressive effects on immune cells such as APCs and T cells.[117, 118] IL-10-deficient mice treated with piroxicam develop colitis, and *H. polygyrus* infection inhibited the development of this colitic inflammation.[119] Furthermore, *H. polygyrus* was protective in a murine model of food allergy, which was dependent on IL-10-mediated blocking of IgE antibody production.[120]

TGFβ has many roles in the immune response, such as suppressing inflammatory responses, inducing wound healing mechanisms, and restoring tissue homeostasis.[44, 121] *H. polygyrus* infection induces TGFβ, and it plays an important part in prolonging survival and reproduction of the helminth in the gut as neutralization of TGFβ in *H. polygyrus*-infected mice results in reduction of worm burden and faecal egg count.[122] Furthermore, TGFβ neutralization restored eosinophilic infiltration into the guts and lower levels of IgA antibodies were present in the lamina propria.[122]

One of the proteins found in HES is a TGFβ mimic, which is highly expressed by adult worms and has been found to induce Tregs *in vivo* and *in vitro* through the TGFβ signalling pathway.[15] However, the administration of mammalian TGFβ could not induce the same protection as HES in a mouse allergy model, indicating the TGFβ mimic is most likely not solely responsible for the suppressive effect of HES.[123]

1.4.2 Suppression of the Th2 response by *H. polygyrus*

The wide array of cellular and humoral immune mechanisms induced by helminth parasites has been extensively investigated.[77, 106, 124-126] Below is an overview of key immune cells affected by helminth parasites in murine models of infection, with particular reference to the induction and impact of regulatory mechanisms.

H. polygyrus infection alters the phenotype of the DC populations in the gut. DCs are crucial for the priming of the adaptive arm of the Th2 response against *H. polygyrus*, while innate cells (such as AAMs, eosinophils and ILC2s) are still activated when DCs are depleted.[73] Several studies showed an increase in the CD11c^{lo} CD45RB[mid] DC population that promoted the induction of Tregs and IL-10 production during chronic infection with *H. polygyrus*, while the CD11c^{hi} DC population decreased.[127, 128] This decrease in the CD11c^{hi} DC population was thought to be through the blocking of migration of mucosal DCs to the MLN.[128]

ILC2s are rapid responders as they are activated early on in a Th2 response by alarmins, cytokines and inflammatory mediators such as TSLP, IL-25 and IL-33 and when activated, will produce high levels of IL-5, IL-9 and IL-13.[129, 130] In an ovalbumin (OVA)-*Alternaria*-induced airway inflammation model, it was shown HES was able to suppress IL-33 secretion, which supressed ILC2 activation resulting in a lower number of ILC2s present in the lung and reduced secretion of cytokines such as IL-5 and IL-13. Furthermore, administration of recombinant IL-33 was shown to negate the suppression of ILC2s by HES.[123]

Another way that helminth parasites influence the immune system is through the release of exosomes. Exosomes are vesicles used by cells to communicate with, and transport proteins and micro RNAs (miRNAs) into, other cells.[131] Exosomes secreted by *H. polygyrus* are transferred to host cells and have been shown to contain miRNAs that are able to suppress the local innate immune response. Through downregulation of the IL-33 receptor on ILCs, downstream effects on the production of cytokines IL-5 and IL-13 and recruitment of eosinophils was demonstrated.[132, 133]

Through suppression of these early responders in the Th2 response, *H. polygyrus* is directly able to suppress not only the adaptive arm of the immune response, but also the innate arm, thereby dampening the whole Th2 response and ensuring prolonged survival and reproduction.

1.4.3 Three-way relationship between host, parasite and gut microbiota

The gut microbiota is essential for the development and homeostasis of the immune system in humans and mice.[134] *H. polygyrus* is localized in the gut throughout its lifecycle within the host and alters the composition of the gut microbiota, e.g. increasing the abundance of the *Lactobacillaceae* family, through the secretion of antimicrobial peptides present in HES and through changes in the gut environment.[135-137] The abundance of *Lactobacillus* correlated with the presence of total numbers of Tregs in the MLN and transfer of the gut microbiota from infected mice to uninfected mice was enough to induce protection.[137, 138] Together, these results suggest there is a mutual promotion between certain microbes and helminth infections, ensuring both organisms survive within the host while the altering of the microbiota by helminth infections is an important step to indirectly modulate the immune response.[136, 138, 139]

In conclusion, *H. polygyrus* has been shown to modulate the immune response against it through the induction of a variety of regulatory mechanisms (figure 1.3), which may also act beneficially to dampen concurrent allergic and autoimmune responses.

1.5 Atopic dermatitis and the allergic march

1.5.1 The skin immune system

The skin is the first line of defence against pathogens. As such, there are many types of immune cells present in the layers of the skin, both resident and circulating from the skin to draining LNs. They guard against pathogens whilst also maintaining homeostasis to prevent autoimmunity and chronic inflammation by controlling the extent of the immune response.[140]

The skin consists of two main components: the dermis and the epidermis. The epidermis (the outer layer) contains cells such as keratinocytes, melanocytes, Langerhans cells and memory CD8^{+} T cells (mainly in proximity to the Langerhans cells).[140] Keratinocytes are crucial for sensing danger signals when the skin barrier is disrupted and pathogens enter the host, as they express several toll-like receptors (TLRs) and inflammasome machinery on their surface.[141, 142] When keratinocytes are activated, they can secrete a variety of pro-inflammatory cytokines, chemokines and antimicrobial peptides to activate an appropriate immune response.[140]

The dermis is more complex in immune cell diversity. The main APC populations are dermal DC populations, plasmacytoid DCs and macrophages.[140] Dermal DCs can migrate rapidly into the draining LNs to activate T cells, and the CD11b^{+} and triple negative (TN, CD326^{-}CD11b^{-}CD103^{-}) DC populations have been shown to induce Th2 responses in a murine model of AD.[143] Both classically-activated macrophages (CAMs) and AAMs are present in the skin, with CAMs having a broad function in inflammatory responses and AAMs having a regulatory role with the secretion of IL-10 and TGFβ.[144]

There are other innate cells present in the skin, such as basophils, mast cells and ILCs. Basophils are involved in allergic inflammation and immunity against parasites in the skin. They are able to secrete Th2 cytokines such as IL-4 and TSLP, which is believed to be important for the development of AAMs and activation of ILC2s.[145] Mast cells have granules containing a variety of pro-inflammatory molecules and mediators that can be released upon activation, and can selectively release pro-inflammatory cytokines. Mast cells also have anti-inflammatory

functions and have been shown to selectively release cytokines such as IL-2 and IL-10 inhibiting chronic skin allergy and contact dermatitis *in vivo*, and they have been shown to directly interact with Tregs *in vitro*.[146-148] All three subsets of ILCs (ILC1s, ILC2s and ILC3s) are present in the skin. Dermal ILC2s constitutively secrete IL-13 and can interact with mast cells, to suppress their secretion of cytokines. IL-2-activated ILC2s were seen to promote inflammation through enhanced secretion of IL-5, eosinophil recruitment and mast cell activation, demonstrating ILC2s can have both pro-inflammatory and regulatory functions in the skin.[149]

Apart from innate cells, there are also diverse populations of T cells present in the dermis. Skin-resident T cells consisting of innate like T cells (such as γδ T cells and natural killer T (NKT) cells), and memory CD4^{+} and CD8^{+} T cells have been shown to be present in low numbers.[150] These are thought to be major players in homeostasis, wound healing and in the inflammatory response where they are the first line of defence against pathogens in the skin.[151] Adaptive CD4^{+} T cells that are recruited to the skin by DCs are mainly Th1, Th2 or Th17 cells.[140]

1.5.2   Atopic dermatitis

Skin disorders are associated with the dysregulation of the immune system in the skin. AD, or atopic eczema, is a chronic, relapsing Th2 inflammatory skin condition characterized by pruritus (itching) and inflammatory skin lesions.[152, 153] Over the past 30 years, prevalence of this disease has been increasing in developed countries and now seems to have plateaued at 10-20% of the population.[154] In more than half of the cases (around 60%), onset of this disease is within the first year of life, and most of the time it greatly improves or resolves later in childhood.[152] However, the disease can appear at any age, and when it appears to be outgrown, patients will still have sensitive hyper-reactive skin and might even have recurrences.[152] Furthermore, studies have shown onset of early AD can often lead to increased susceptibility to food allergies, asthma or other airway allergies later in life, a phenomenon called the atopic or allergic march (figure 1.4, discussed below).

The cause of disease is not fully understood, however it seems to be multifactorial through interactions between environmental and genetic factors. The highest risk factor seems to be family history of AD, of which the most well characterised genetic factors are null mutations in the *Filaggrin* gene, an important structural molecule involved in barrier function and immune mechanisms such as innate cell signalling and T cell activation.[155, 156] Well-known environmental factors include a Western diet (high sugar and fat, low fibre diet), small households, and an urban lifestyle.[152, 153]

The main mechanisms of AD are abnormal functioning and structure of the epidermis (such as disrupted skin barrier function, increased abundance of *Staphylococcus aureus* and increased water loss) and an inappropriate immune response to antigens in the skin causing allergic inflammation.[152]

Experiments using *in vivo* models have started to unravel important mechanisms in the onset and persistence of AD. Barrier disruption is an important factor in the induction of skin inflammation with alarmins such as TSLP, IL-33 and IL-25 secreted by keratinocytes after disruption of the skin barrier.[157] These alarmins activate innate cells such as ILC2s, DCs, mast cells and basophils.[52, 53] DCs, after activation, migrate to the skin draining LNs, activating the adaptive arm of the immune response. Of the adaptive arm, Th2 cells and IgE-producing B cells are mainly associated with a Th2 response. Th2 cells migrate to the skin and secrete IL-4, IL-5, IL-9 and IL-13, while Th2 cells in the LN mainly secrete IL-4.[157]

Mast cells are primarily activated through FcεRIII by binding of IgE, but can also be directly activated by alarmins such as IL-33.[158] Mast cells are located close to nerve endings, and have been suggested to have a role in the neurogenic components in the itching response of AD through the tryptase/protease-activated receptor 2 (PAR2) pathway.[153] ILC2s accumulate in AD skin lesions, and are the main source of IL-13.[159] Basophils also accumulate in AD skin lesions, where they are a main source of IL-4 and eotaxin, promoting infiltration of eosinophils.[145]

Tregs have also been shown to be important in regulating inflammation in atopy such as AD.[99, 160] It was shown depletion of forkhead box protein 3 (FoxP3)+ Tregs in

an *in vivo* model increased skin inflammation through DC and T cell activation and proliferation, and increased IgE serum levels.[160] Furthermore, they found that inducing airway allergy after sensitization and challenge through the skin route, Treg depletion increased lung immune cell infiltration, and higher infiltration of CD4$^+$ T cells and eosinophils in the BALF.[160] These results indicate Tregs are important in regulating allergic responses in the skin and preventing development of subsequent airway allergy.

1.5.3 Mouse models of atopic dermatitis

Murine models for AD can be divided into three categories: epicutaneous application, transgenic mice, and mice spontaneously developing AD-like skin lesions.[161]

Epicutaneous application of haptens

Many chemicals have been used as a topical treatment, mimicking the constant exposure to external factors, to effectively induce AD in mice. Two examples are described below:

Fluorescein isothiocyanate (FITC) has been shown to induce contact hypersensitivity (CHS), which is a common form of AD, mostly mediated by Th2 cells. Dibutyl phthalate (DBP) is used as a solvent, and it has been shown to enhance the potency of allergens such as FITC.[162] Epicutaneous application of DBP-FITC was shown to induce TSLP production, activating DCs and inducing Th2 inflammation and AD-like skin lesions.[143] As various chemicals are able to induce CHS upon repeated exposure to the skin in humans, topical application of DBP-FITC has proved to be an effective model to study AD in mice.[162]

MC903 (calcipotriul; Dovonex) is a low-calcemic analogue of vitamin D3 (1α,25-(OH)2D3) and used for the treatment of psoriasis in humans.[163] MC903 has been seen to effectively induce AD like symptoms and is used to study AD in mice. It is dissolved in ethanol and applied topically on the ears of mice and induces AD-like symptoms such as increased skin thickness, dry scaly skin and scratching behaviour suggesting pruritus.[163] MC903 induces TSLP production by keratinocytes, and an

increase in Th2 inflammation was seen in MC903-treated ears.[163, 164] This demonstrates MC903 induces a skin condition mimicking human AD.

Transgenic mice

Transgenic mice are used to study the importance of specific components of the immune system on the development and progression of AD, such as cytokines, chemokines and specific genes found in genome-wide association studies (GWAS).[161, 165]

Transgenic mice overexpressing IL-4 spontaneously develop pruritus at 4 months and have impaired wound healing with enhanced angiogenesis and Th2 inflammation, symptoms closely resembling AD.[161, 166]

Transgenic mice overexpressing TSLP develop Th2 inflammation closely resembling AD. When these mice were crossed with mice lacking T cells (TCRβ$^{-/-}$ mice), AD still developed, demonstrating the onset of disease is not dependent on T cells. [161, 167]

Flaky tail (FLG^{ft}) mice have reduced expression of filaggrin, which has shown to result in dry, flaky skin which resembles skin lesions in AD, and enhanced susceptibility to sensitization with epicutaneous antigens.[168] As loss-of-function mutations in filaggrin are seen in up to 50% in patients with AD, it is shown to be an important factor in the development of the disease, although not solely responsible.[169]

Spontaneous development

Nc/Nga mice develop spontaneous AD-like lesions under conventional conditions, but not under specific-pathogen free (SPF) conditions, demonstrating an additional environmental factor involvement (the microbiota). Scratching behaviour, erythema and haemorrhaging is seen after 8 weeks and is followed by development of dry and scaly skin, oedema, and retarded growth.[170] The skin lesions are characterised by infiltration of CD4$^+$ T cells, eosinophils, mast cells, high levels of Th2 cytokines, and the mice have elevated serum levels of IgE.[153, 161, 171] The frequency of developing skin lesions in mice kept under SPF conditions varies greatly between laboratories, therefore in some groups Nc/Nga mice also receive some form of epicutaneous treatment to induce AD.[153]

1.5.4 The allergic march

The allergic march is described as the progression from AD in early childhood to other allergies such as food allergy, asthma and allergic rhinitis later in childhood, although this can occur at any age.[172] Evidence has supported this relationship between allergic diseases, and it has been estimated around one in three patients with AD develop asthma later on in life, and two in three patients develop allergic rhinitis.[172, 173] One mechanism through which the allergic march may occur is through the defective skin barrier. Due to the activated state of immune cells in the skin, further sensitization to other allergens may occur more readily in AD patients, thus making them more susceptible to increased allergic responses in early life. (figure 1.4).[173]

It has been demonstrated experimentally that sensitization through the skin can indeed result in allergic responses in distal tissues. Where AD-like responses were defective in mice lacking TSLP specifically in the skin, airway inflammation after intradermal (ID) sensitization and intranasal challenge was also attenuated.[174] These results not only demonstrate the importance of TSLP in the development of allergic responses, but also the importance of the sensitization stage in the skin for developing allergic airway inflammation. Double mutant flaky tail ($Flg^{ft/ft}$) mice that develop spontaneous AD, developed spontaneous lung inflammation later in life.[175] $Rag1^{-/-}$ $Flg^{ft/ft}$ mice still developed AD (although skin pathology was less severe) in the absence of B and T cells, but did not develop lung inflammation, demonstrating the adaptive arm of the Th2 response could be crucial for the initiation of the allergic march.[175]

It seems disrupted skin barrier and sensitization in the skin is crucial for the initiation of the allergic march. Therefore, preventing the onset of AD would be highly beneficial for the health of patients.

1.6 Aims of this project

H. polygyrus effectively suppresses allergic inflammation in the guts and lungs through the induction of regulatory mechanisms such as Tregs.[13, 15, 16] Tregs have also been shown to be important for regulating inflammation in the skin during AD, and Treg depletion led to increased inflammation through DC activation of Th2 cells.[160] This suggests Tregs induced by *H. polygyrus* might also be able to suppress allergic responses in the skin. However, there is a limited number of publications addressing the effect of helminth parasites during skin allergy in both epidemiological studies and murine models, and there is a need for further investigation in this area.[176, 177] Furthermore, suppression of Th2 inflammation in AD lesions by helminth parasites may also prevent initiation of the allergic march, and therefore, prevent the onset of other allergic diseases later in life.

Therefore, the overall aim of this project is to study if the gut dwelling mouse parasite *H. polygyrus* is able to suppress Th2 inflammatory responses induced in skin tissue.

Specific goals:

1) To determine if *H. polygyrus* alters immune cell and cytokine profiles in the skin tissue, via use of 4C13R (IL-4 and IL-13) dual reporter mice to study expression profiles of the canonical Th2 cytokines IL-4 and IL-13 by immune cells in various tissues by flow cytometry.

2) To assess if *H. polygyrus* is able to suppress Th2-mediated inflammation induced by two different allergy models, i.e. treatment via an ID injection of house dust mite (HDM) or topical application of the chemical hapten DBP-FITC in the ear skin tissue.

3) To identify if *H. polygyrus* induces $CD4^+$ $FoxP3^+$ Tregs in the auricular LNs and ear skin tissue.

4) To characterize differences in the Th2 inflammatory response following treatment of *H. polygyrus*-infected mice in comparison to uninfected mice.
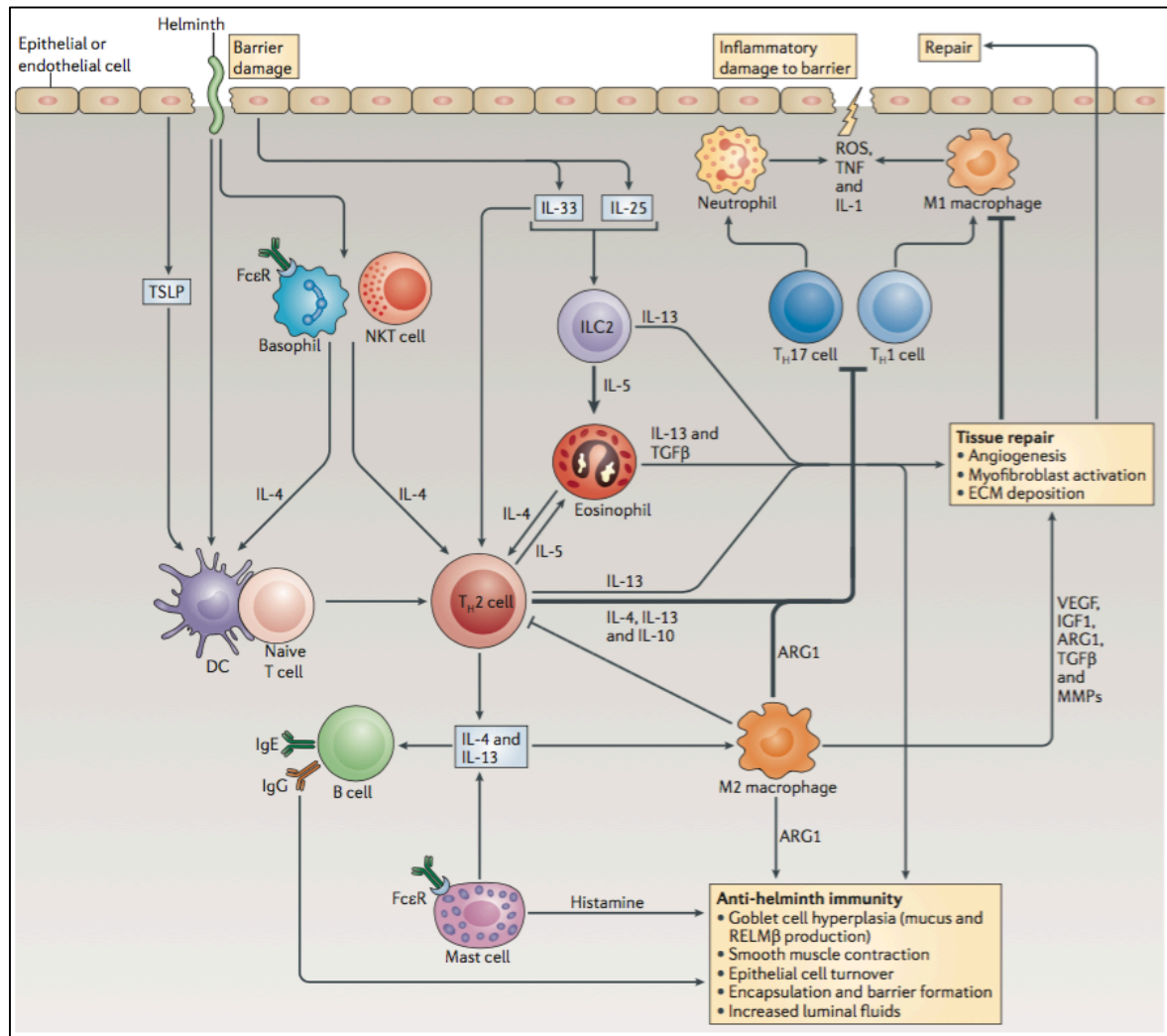
Figure 1.1: Th2 immunity and wound repair

When helminth parasites infect the host, epithelial barriers such as those in the skin, lungs or gut are disrupted. In the skin, damage initiates secretion of alarmins by keratinocytes such as TSLP, IL-25, and IL-33 that induce a Th2 response. This response acts to simultaneously repair tissue damage and mediate killing and expulsion of the worm. ILC2s, activated by IL-25 and IL-33, are the main source of IL-5, which activates eosinophils, and IL-13, which plays a role in both helminth expulsion from the host (e.g. goblet cell hyperplasia, smooth muscle contraction, epithelial cell turnover) and tissue repair (e.g. angiogenesis, myofibroblast activation and extracellular matrix (ECM) deposition). TSLP activates DCs, which migrate to the draining LNs to activate T cells and drive Th2 differentiation. Basophils are also thought to be an important source of IL-4 for Th2 differentiation. Th2 cells produce IL-5, IL-13, and IL-4, which can induce B cell class-switching to IgE and IgG1, and induce M2 macrophages. Activated B cells produce parasite-specific antibodies which activate basophils and mast cells. Mast cells and M2 macrophages secrete effector molecules to mediate parasite killing and wound repair. Tissue repair is also induced by suppressing tissue damaging Th1- and Th17-mediated immune responses through production of IL-4, IL-10 and IL-13 from Th2 cells and Arg1 from M2 macrophages. (Gause, W.C., Wynn, T.A. & Allen, J.E. Type 2 immunity and wound healing: Evolutionary refinement of adaptive immunity by helminths. *Nat Rev Immunol* **13**, 607-614 (2013)).[44]
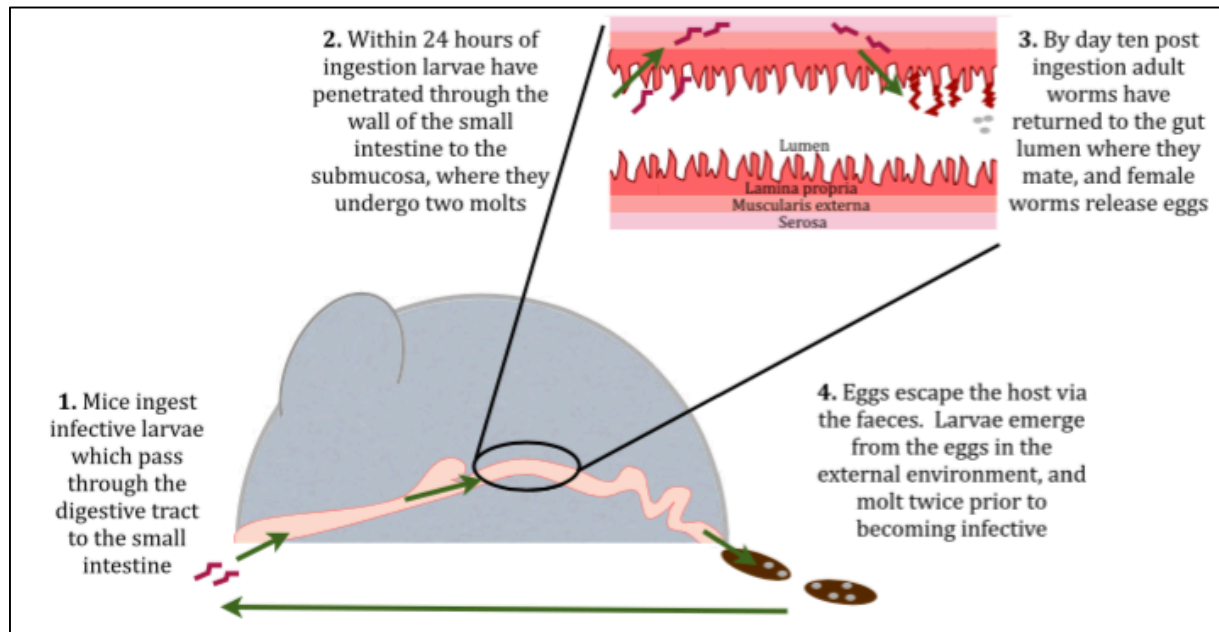
Figure 1.2: The lifecycle of *H. polygyrus* in mice

Infective L3-stage larvae are ingested by the mice (or administered by oral gavage in an experimental setting) and pass to the small intestine where they penetrate the wall and undergo two moults whilst encysted in the submucosa. Adult worms (8-10 days post-infection) return to the gut lumen where they coil around the villi and mate, with the eggs released in the host faeces. Once outside the host, the eggs hatch and the larvae undergo two moults to become infective larvae (Reynolds, L.A., Filbey, K.J. & Maizels, R.M. Immunity to the model intestinal helminth parasite *Heligmosomoides polygyrus*. *Semin Immunopathol* **34**, 829-846 (2012)).[79]

Figure 1.3: Immunomodulation by helminth parasites

Helminths induce a Th2 response consisting of Th2 cells, B cells, eosinophils, mast cells, neutralizing antibodies and Th2 cytokines IL-4, IL-5, and IL-13. However, they also actively induce the regulatory mechanisms of the Th2 response consisting of AAMs (or M2 macrophages), Tregs and regulatory B cells. M2 macrophages secrete molecules implicated in wound healing. Tregs secrete IL-10 and TGFβ, and regulatory B cells secrete IL-10 and inhibitory antibodies. The induction of these cells and regulatory molecules leads to suppression of inflammatory immune responses and prolonged worm survival and reproduction. Allergic inflammation presents a similar phenotype to the anti-helminth immune response, and in the lungs (as seen in allergic asthma) can lead to mucus production, accumulation of eosinophils, bronchoconstriction, smooth muscle contraction and airway remodelling. This allergic inflammation is likely to be suppressed due to bystander effects from regulatory mechanisms induced by helminth infection (Daniłowicz-Luebert, E., O'Regan, N.L., Steinfelder, S. & Hartmann, S. Modulation of specific and allergy-related immune responses by helminths. *J Biomed Biotechnol* **2011** (2011)).[17]

Figure 1.4: The allergic march

The proposed pathway illustrating the allergic march and the development of asthma and other airway allergies from childhood eczema. A mixture of genetic and environmental factors creates a defective skin barrier allowing entry of, and epicutaneous sensitization by, allergens. The migration of Th2 memory cells from the skin to other lymphoid tissues, such as the nasal and bronchial lymphoid tissue, sensitises the airways, leading to asthma or allergic rhinitis after re-exposure to the same allergen. Furthermore, trauma to the skin barrier triggers the release of TSLP which enhances further allergic responses (Dharmage, S.C. *et al.* Atopic dermatitis and the atopic march revisited. *Allergy* **69**, 17-27 (2014)).[172]

Chapter 2.   Material and Methods

2.1 Materials

2.1.1 Labware

Product	Supplier
Beads (stainless steel, 5mm) suitable for tissue lyser	Qiagen
Clear flat-bottom (Nunc) immuno nonsterile 96-well plates	Thermofisher Scientific
Eppendorf® Safe-Lock microcentrifuge tubes 2ml	Sigma
Falcon™ 1ml Tuberculin syringes	In vitro Technologies
Falcon™ 70 μm cell strainer	In vitro Technologies
Falcon™ conical polystyrene sterile centrifuge tubes, 15 and 50 ml	In vitro Technologies
Falcon™ polystyrene round bottom tubes 5ml	In vitro Technologies
Falcon™ polystyrene serological pipet 2, 5, 10 and 25ml	In vitro Technologies
Falcon™ polystyrene sterile clear flat bottom well plates 6, 12, 24 wells	In vitro Technologies
Falcon™ polystyrene sterile clear round bottom well plate 96 wells	In vitro Technologies
MicroAmp® optical 96-well reaction plate with barcode	Applied Biosystems
Microtome blades MX35	Thermofisher Scientific
Plate adhesive seals suitable for qPCR	Applied Biosystems
PrecisionGlide needles 25G	BD Biosciences
Stainless steel feeding needle 22G	Total lab systems
Sterile microtubes 0.2 and 1.7ml clear	Axygen Scientific Inc.

Sterile pipet tips (certified RNAse free) 10, 20, 200 & 1000μl	Axygen Scientific Inc.
Sterile screw top tubes 5ml	Thermofisher Scientific
Sterile striptubes 0.2ml clear	Axygen Scientific Inc.
Superfrost plus microscope slides	Thermofisher Scientific
Surgical blade number 10	Swann-Morton
Tissue-Loc cassettes	Thermofisher Scientific
Transfer pipette, sterile individually wrapped 1ml	Interlab
Ultrafine insulin syringe needle 29G 0.3ml	BD Biosciences

2.1.2 Reagents

Reagents	Catalog number	Supplier
Accutase	SCR104	Chemicon
Acetone	AC03112500	Scharlau
Bovine serum albumin (BSA)	A7888-100G	Sigma
Brefeldin A	B5936-200UL	Sigma
Collagenase I	17018029	Roche
Collagenase IV	C5138-1g	Sigma
Compensation beads anti-mouse	552843	BD CompBead
Compensation beads anti-rat/anti-hamster	552845	BD CompBead
Crushed up whole house dust mite (*Dermatophagoides pteronyssinus*)	B84	Greer
Cytofix/Cytoperm kit	554714	BD Biosciences
DEPC-treated H_2O	AM9906	Ambion
Di-n-butyl phthalate (DBP)	31244	Alfa Aesar
Dispase II	04942078001	Roche
DNAse 1	1010415900	Roche
Dulbecco's phosphate-buffered saline (DPBS)	14190-250	Gibco
Ethanol (absolute)	N1008622-5A	Pure Science
Ethylenediaminetetraacetic acid (EDTA)	15575-038	Invitrogen
Fetal Bovine Serum (FBS)	10091-148	Gibco

Fluorescein isothiocyanate (FITC)	F7250	Sigma
FoxP3/ Transcription Factor Staining Buffer Set	00-552300	eBioscience
Hank's Balanced Salt Solution (HBSS) with Phenol Red and Glucose	14170-161	Gibco
High Capacity RNA-to-cDNA kit	4387406	Applied Biosystems
Ig - HRP secondary antibody	1010-05	Southern Biotech
Ionomycin	I3909-1ML	Sigma
Iscove's Modified Dulbecco Medium (IMDM) with Phenol Red, GlutaMAX™, 25 mM HEPES buffer and 3.024 mg/L NaHCO$_3$	31980-097	Gibco
Isopropanol	I95-16-500ML	Merck
Liberase TM	05401127001	Roche
Penicillin-streptomycin	15140122	Gibco
Phorbol 12-myristate 13-acetate (PMA)	524400	Calbiochem
Phosphate-buffered saline (PBS) powder	21600-069	Gibco
Red blood cell (RBC) lysing buffer	R7757-100ml	Sigma
RNAlater	AM7021	Invitrogen
Roswell Park Memorial Institute (RPMI) 1640 Medium with L-glutamine and Phenol Red	11875-119	Gibco
Sodium azide (NaN$_3$)	S8032-25G	Sigma-Aldrich
Sodium chloride (NaCl)	S9888-1KG	Sigma-Aldrich
Sphero Accucount beads	ACBP-50-10	Spherotech

Sulfuric acid (H_2SO_4)	VWRC102765G	BDH Chemicals (Analar)
Taqman gene expression master mix	4352042	Applied Biosystems
TMB substrate	555214	BD OptEIA
Trizol	15596026	Invitrogen
Trypan Blue	15250-061	Gibco
Tween-20	P1379-500ML	Sigma-Aldrich

2.1.3 Buffers and solutions

Buffers and solutions	Composition
Anaesthetic working solution	DPBS 10x stock solution ketamine/xylazine (1:10 dilution in DPBS) provided by the Biomedical Research Unit at the Malaghan Institute
Complete HBSS media (cHBSS)	HBSS 500ml 10% FCS (50ml) 1% Pen-Strep (5ml)
Complete IMDM media (cIMDM)	IMDM 500ml 10% FCS (50ml) 1% Pen-Strep (5ml)
Complete RPMI media (cRPMI)	RPMI 500ml 10% FCS (50ml) 1% Pen-Strep (5ml)
Digestion mix 1: Ear Tissue	Accutase Dispase II - 3U/ml Liberase TM - 100µg/ml
Digestion mix 2: Ear Tissue	HBSS Collagenase IV - 2mg/ml DNAse 1 - 100µg/ml
Digestion mix 3: Lymph Node	DPBS Collagenase IV - 1mg/ml DNAse 1 - 100µg/ml

Digestion mix 4: Lung tissue	cIMDM
	Collagenase I - 2.5mg/ml
	DNAse 1 - 120µg/ml
DBP-FITC	0.5% FITC
	1:1 DBP/Acetone
ELISA blocking buffer	1x PBS
	2% BSA
ELISA coating buffer	500ml dH$_2$O
	1.5g Na$_2$CO$_3$
	3g NaHCO$_3$
	Adjust pH to 9.6
ELISA wash buffer	1x PBS
	0.05% Tween-20
FACS buffer	1x PBS 500ml
	0.5% BSA (2.5g)
	0.05% NaN$_3$ (250µl)
Perm buffer	dH$_2$O
	10x Permeabilization buffer from FoxP3/ Transcription Factor Staining Buffer Set (1:10 dilution)
Perm wash	dH$_2$0
	10x Cyto-permeabilization buffer from Cytofix/Cytoperm kit (1:10 dilution)
Stop buffer 1	cHBSS
	5mM EDTA

Stop buffer 2	DPBS
	5mM EDTA
Saturated salt solution	1L dH$_2$O
	400g NaCl

2.1.4 Mice

2.1.4.1 Mouse strains

C57BL/6J mice (Breeding pairs obtained from The Jackson Laboratory): These mice are unable to expel *H. polygyrus* in a primary infection.

4C13R dual reporter mice: these mice express fluorescent reporters for IL-4 (AmCyan) and IL-13 (DS-Red). (Obtained from Dr. William E. Paul, National institute of Allergic Diseases, National Institute of Health)

2.1.4.2 Maintenance and ethical approval

All mice (female and 6-10 weeks of age) were bred in the Biomedical Research Unit at the Malaghan Institute of Medical Research. All procedures were carried out as approved by the Victoria University of Wellington (protocol: 2014R15M).

2.1.5 Flow cytometry:

Antibodies were titrated and the optimal antibody dilution was determined after purchase. Antibody mixes were made up in FACS buffer or perm buffer and vortexed just before use.

2.1.5.1 List of used antibodies

Antibody	Conjugate	Company	Clone
Bcl6	AF647	BD Pharmingen	K112-91
CD3	BUV395	BD Pharmingen	145-2C11
	BV786	BD Pharmingen	145-2C11
CD4	PerCP	BD Pharmingen	RM4-5
	APC-Cy7	BD Pharmingen	GK1.5
	FITC	BD Pharmingen	RM4-5
	BV605	BD Pharmingen	RM4-5
	BUV737	BD Pharmingen	GK1.5
CD8	FITC	Made in house	2.43
CD8α	BV786	BD Pharmingen	53-6.7
CD11b	BV650	BD Pharmingen	M1/70
	FITC	BD Pharmingen	M1/70
	APC-Cy7	BioLegend	N418
	BUV395	BD Pharmingen	M1/70
CD11c	FITC	BioLegend	N418
	PE-Cy7	BD Pharmingen	HL3
	BUV395	BD Pharmingen	HL3
CD19	APC-H7	BD Pharmingen	ID3

CD25	FITC	BD Pharmingen	7D4
	BV421	BD Pharmingen	7D4
CD44	APC	BD Pharmingen	IM7
CD45	BV510	BD Pharmingen	30.F11
	BUV395	BD Pharmingen	30.F11
	APC	BD Pharmingen	30.F11
	APC-Cy7	BD Pharmingen	30.F11
CD45R (B220)	BV786	BD Pharmingen	RA3-6B2
	FITC	BD Pharmingen	RA3-6B2
	BV650	BD Pharmingen	RA3-6B2
	PE-CF594	BD Horizon	RA3-6B2
CD49b (DX5)	PE-Cy7	eBioscience	DX5
	FITC	BioLegend	DX5
CD62L	FITC	BD Pharmingen	MEL-14
CD64	APC	BioLegend	X54-5/7.1
CD69	PE-Cy7	BD Pharmingen	H1.2F3
CD86	PE	BD Pharmingen	GL1
CD90.2	BV605	BD Pharmingen	53-2.1
CD103	APC	eBioscience	2E7
CD117 (Ckit)	BV421	BD Pharmingen	2B8
CD278 (ICOS)	BV421	BioLegend	C398.4A
	AF647	BioLegend	C398.4A

CD326	BV711	BD Pharmingen	G8.8
F4/80	FITC	eBioscience	BM8
FcgIII/IR (block)		Made in house	2.4G2
FceRIα	Biotin	eBioscience	MAR-1
	FITC	eBioscience	MAR-1
	AF647	eBioscience	MAR-1
FoxP3	PE	eBioscience	NRRF-30
GATA3	AF647	BD Pharmingen	L50-823
	BV421	BD Pharmingen	L50-823
I-A/I-E (MHCII)	Pacific Blue	BioLegend	M5/114.15.2
IL-4	BV421	BD Pharmingen	11B11
IL-10	PE	BD Pharmingen	JES5-16E3
IL-13	eF660	eBioscience	eBio13A
KLRG1	APC	BD Pharmingen	2F1
Ly6G	BV711	BD Pharmingen	1A8
NK1.1	FITC	BD Pharmingen	PK136
Relmα	Biotin	AbCam	rabbit pAb
Siglec F	PE-CF594	BD Horizon	E50-2440
ST2	PerCP-eF710	eBioscience	RMST2-2
Streptavidin	BV605	BD Pharmingen	
	PE-Cy7	BD Pharmingen	
TCRβ	BV605	BD Pharmingen	H57-597

2.1.5.2 Viability dyes

4,6-diamidino-2-phenylindole, dihydrochloride (DAPI), purchased from Invitrogen, was prepared in H_2O at a concentration of 200µg/ml. DAPI was added to the samples just before analysis at a dilution of 1:5,000 in PBS.

Live/dead fixable blue, purchased from Invitrogen, was reconstituted in 50µl DMSO from the same kit and diluted 1:1000 in PBS. 200µl was added per well and cells were incubated for 20 minutes in the dark at 4°C.

2.1.6 qPCR primers

Gene	Dye	Assay ID	Company
Foxp3	FAM-MGB	Mm00475162_m1	Thermofisher Scientific
Gapdh	VIC-MGB	NM_008084.2	Thermofisher Scientific
Il4	FAM-MGB	Mm00445259_m1	Thermofisher Scientific
Il10	FAM-MGB	Mm01288386_m1	Thermofisher Scientific
Il13	FAM-MGB	Mm00434204_m1	Thermofisher Scientific
Il33	FAM-MGB	Mm00505403_m1	Thermofisher Scientific
Tgfβ	FAM-MGB	Mm01178820_m1	Thermofisher Scientific
Tslp	FAM-MGB	Mm01157588_m1	Thermofisher Scientific

2.2 Methods

2.2.1 Mouse treatments

2.2.1.1 Intragastric (IG) administration of infective L3 H. polygyrus larvae

Viability and concentration of L3 *H. polygyrus* larvae were assessed by light microscopy. Mice were given 200 L3 larvae in 200µl H_2O via oral gavage using a stainless steel 22G feeding needle.

2.2.1.2 Intradermal injection of mashed-up whole HDM

Mice were anaesthetized with 200µl of anaesthetics working solution by intraperitoneal (IP) injection with a 25G needle. Mice were given an ID injection in both ears with a 29G insulin needle containing 200µg of mashed up HDM in 30µl sterile PBS.

2.2.1.3 Contact hypersensitivity model of DBP-FITC

Mice were anaesthetized with 200µl anaesthetic working solution by IP injection with a 25G needle. The hair on the lower part of the abdominal skin was removed by shaving with a number 10 surgical blade, one week before sensitization. Mice were sensitized on the abdominal skin on days 0 and 2 by epicutaneous application of 40µl of DBP-FITC. On day 6, baseline ear thickness was measured with a micrometer (Micromaster, Capa System) and baseline trans-epidermal water loss (TEWL) was measured with TEWL equipment from Dermalab (Dermalab TEWL open chamber units), followed by challenging the mice with 20µl of DBP-FITC on one ear and 20µl of vehicle (DBP) on the other ear. From day 7-13, ear thickness and TEWL was measured and the change from baseline was calculated. On day 8, 2 mice from each group were sacrificed and ear tissue was harvested for histology. On day 14, remaining mice were sacrificed.

2.2.1.4 Topical application of DBP-FITC for DC migration

Mice were anaesthetized with 200μl anaesthetic working solution by IP injection with a 25G needle. Mice were treated with 20μl of DBP-FITC on both ears on day 0. On day 2, mice were sacrificed and auricular lymph nodes from all mice, and guts from infected mice were harvested.

2.2.1.5 Topical application of DBP-FITC for T cell responses

Mice were anaesthetized with 200μl anaesthetic working solution by IP injection with a 25G needle. Mice were treated with 20μl of DBP-FITC on both ears on day 0 and day 2. Mice were sacrificed and auricular lymph nodes from all mice, and guts from infected mice were harvested on day 7 and 9.

2.2.1.6 Euthanasia

Mice were sacrificed by lethal injection of anaesthetic (200μl 10x stock solution) and cervical dislocation, or CO_2 asphyxiation and/or cervical dislocation.

2.2.2 Tissue harvest and processing

2.2.2.1 Lymph nodes

Auricular LNs were carefully removed from euthanized mice and placed in DPBS until processed.

Generating single cell suspensions from lymph nodes for analysis of immune cells
Isolated LNs were mashed through a 70μm cell strainer using the rubber end of a 1ml syringe. The strainer was washed with 5ml cRPMI. Samples were centrifuged at 1300 rounds per minute (RPM) for 5 minutes; the supernatant was discarded and the pellet was resuspended with 1ml cRPMI for counting.

Generating single cell suspensions from lymph nodes for analysis of dendritic cells

Isolated LNs were cut in half and digested in 1ml of digestion buffer (digestion mix 7 for LNs) for 30 min at room temperature (RT) on a rotator. After digestion 2ml of DPBS with 5mM EDTA was added to stop any further digestion. LNs were mashed through a 70μm cell strainer using the rubber end of a 1ml syringe, centrifuged at 1300 RPM for 5 minutes and resuspended in 1ml of DPBS with 5mM EDTA for counting.

2.2.2.2   Ear tissue

Ears that were harvested from mice were kept either in DPBS for use in flow cytometry, or in RNAlater for use in quantitative PCR (qPCR).

Digestion mix 1

Ears used for flow cytometry were split into dorsal and ventral halves and placed dermis side down in a 12-well plate in 1ml of digestion enzyme mix per ear. Ears were incubated for 20 min in a shaking incubator (100 RPM) at 37°C. After incubation ears were chopped up into small pieces and incubated for another 20 minutes in a shaking incubator (100 RPM) at 37°C. After incubation, ear tissue was filtered through a 70μm cell strainer using the rubber end of a 1ml syringe and washed with 10ml of cRPMI.

Digestion mix 2

Ears used for flow cytometry were split into dorsal and ventral halves and immediately chopped up into small pieces in 5ml tubes and incubated for 30 minutes in a shaking incubator (150 RPM) at 37°C with 1ml digestion buffer per ear. After incubation, 1ml HBSS stop buffer per ear was added to stop digestion. Ear tissue was filtered through a 70μm cell strainer using the rubber end of a 1ml syringe and washed with 10ml of cRPMI.

After digestion, cells were centrifuged for 10 minutes at 1300 RPM, supernatant was removed and cells were resuspended at 1ml for counting. After counting cells, 100μl

of the cell samples was kept in a 1.7ml microtube for counting by flow cytometry, the rest of the samples was used for analysis by flow cytometry

2.2.2.3 Parasitology

The small intestine and cecum were harvested from euthanized mice and placed on ice until processed.

Life cycle and maintenance

Mice are orally infected with infective L3 larvae. After infection, the larvae first move into the gastric mucosa of the stomach. The larvae emerge after 36 hours and proceed to the duodenum and burrow close to the longitudinal muscle layer of the gut. Adult parasites emerge after 8 days into the lumen after which they mate and produce eggs 9 days after infection, which can last for several months.[178]

H. polygyrus larvae lifecycles were maintained by the Malaghan Institute according to protocol.[178] Infective L3 larvae were kept in H_2O at 4°C at a concentration of 200 L3 larvae per 200µl.

Adult worm count

The small intestine was sliced open longitudinally and hung over a parafilm strip in a 50ml falcon tube filled with PBS and placed overnight in a half-filled 37°C water bath creating a thermal gradient. Viable adult worms actively migrate from the gut to the bottom of the 50ml tube. The next day adult worms were collected and counted under a dissecting microscope.

Egg count

The contents of the cecum were transferred to a 5ml tube and dissolved in 2ml dH_2O. After a few hours, 2ml of saturated salt solution was added and eggs were counted under a light microscope using a McMaster egg counting chamber.

2.2.2.4   Serum

Mice were euthanized through a lethal dose of anaesthetics. Whole blood was obtained through brachial bleed and the blood was collected in a 1.7ml microtube. Blood was left to clot overnight at 4°C and then centrifuged for 10 minutes at 2000 RPM. Serum was collected in a new tube and centrifuged again for 10 minutes at 2000 RPM to remove all RBCs. Serum was collected in a new tube and frozen at -70°C until used for analysis by enzyme-linked immunosorbent assay (ELISA).

2.2.2.5   Whole blood

Mice were euthanized through a lethal dose of anaesthetics. Whole blood was obtained through brachial bleed and the blood was collected in a sterile FACS tube with 1ml DPBS with 10mM EDTA and mixed thoroughly. Blood was lysed twice for 4 minutes with 5ml RBC lysing buffer and resuspended in media for analysis by flow cytometry.

2.2.2.6   Lung

Mice were euthanized by cervical dislocation. Lungs were harvested and digested using the digestion mix 8 for lung tissue. Lungs were chopped up into small pieces in a 6-well plate and 5ml of digestion mix was added. Plates were incubated in a 37°C shaking incubator for 1 hour at 100 RPM. The cells were filtered through a 70µm cell strainer using the rubber end of a 1ml syringe. The strainer was washed with 10ml cIMDM. Samples were centrifuged at 1400 RPM for 5 minutes; supernatant was discarded and the cells were lysed in 4ml RBC lysing buffer for 4 minutes. Cells were washed with 10ml cIMDM and centrifuged at 1300 RPM for 5 minutes and resuspended in 5ml cIMDM for cell count.

2.2.2.7 Cell count

Cells were counted under a light microscope using trypan blue for exclusion of dead cells. Samples were diluted 1:1 in Trypan blue dye and pipetted onto a haemocytometer for counting. RBCs and cells that had taken up the dye were excluded from the cell count. Samples are centrifuged and resuspended to a concentration between $10 \times 10^6 - 25 \times 10^6$ cells/ml.

2.2.3 Cell staining

2.2.3.1 Cell surface staining

$1 \times 10^6 - 5 \times 10^6$ cells/well were added to a round bottom 96-well plate, centrifuged for 2 minutes at 1300 RPM and washed twice in PBS. Cells were incubated in 50µl of 1:400 dilution of Fc block (2.4G2) in FACS buffer for 10 minutes at 4°C. After incubation, the cells were washed once in 150µl of FACS buffer. 30µl of cell surface antibody mix made up in FACS buffer was added per well, and the cells were incubated in the dark at 4°C for 20 minutes. Cells were washed twice with FACS buffer, and incubated for another 20 minutes at 4°C with the secondary antibody and/or resuspended in 200µl FACS buffer and transferred to microtubes for analysis by flow cytometry.

2.2.3.2 Intracellular staining of transcription factors

After staining with cell surface antibodies, cells were permeabilized with transcription factor fix/perm kit for 30 min at RT or ON at 4°C. Cells were washed twice in perm buffer and 30µl of intracellular antibody mix made up in perm buffer was added per well. Cells were incubated ON (if permeabilized for 30 minutes at RT) or for 20 minutes at 4°C (if permeabilized ON at 4°C). Cells were washed twice in perm buffer, resuspended in 200µl of FACS buffer, and transferred to microtubes for analysis by flow cytometry.

2.2.3.3 Intracellular cytokine staining

Isolated lymphocytes were stimulated for 4 hours at 37°C in cRPMI with PMA (50ng/ml), Ionomycin (1µg/ml), and Brefeldin A (10µg/ml). After stimulation, cells were stained with cell surface staining mix. After washing, cells were permeabilized for 30 minutes at 4°C in Cytofix/Cytoperm solution (BD Biosciences). Cells were left overnight in perm wash at 4°C and stained the next day with intracellular cytokine staining mix in perm wash for 20 minutes at 4°C. Cells were resuspended in 200µl FACS buffer for analysis by flow cytometry.

2.2.3.4 Viability dye staining

DAPI was added either just before analysis in a 1:5000 dilution, or live/dead fixable blue staining was added in a 1:1000 dilution for 20 minutes before staining with cell surface antibodies.

2.2.4 Flow cytometry

2.2.4.1 Controls

Controls used for experiments included: unstained cells, live/dead single stained cells, fluorescence minus one (FMO) stained cells, and single stained compensation beads.

2.2.4.2 Acquisition and analysis

Samples were acquired on either the LSRII or the Fortessa using FACSdiva software. At least 100,000 events were recorded for lymph node and skin samples. At least $1x10^6$ events were recorded for DC experiments. Data was analysed using FlowJo software.

2.2.4.3 Counting cells by flow cytometry

After cell count, 100μl of cell samples was placed in a 1.7ml microtube. Cells were centrifuged at 1300 RPM for 3 minutes and resuspended in 100μl FACS buffer for flow cytometry. Just before analysis, 50μl of Sphero Accucount beads (containing about 50,000 beads) were added to the mix and 5μl of 1:1000 dilution of DAPI. One sample containing only beads was added as a control. At least 100,000 events were collected on the LSRII.

2.2.5 Quantitative PCR

2.2.5.1 RNA extraction

Ear tissue was removed from RNAlater and placed in a cliplock tube in 500μl Trizol. Ears were roughly chopped and either frozen in -70°C freezer or used immediately for RNA extraction. One stainless steel bead (5mm) was added to the tubes and samples were homogenized 2x 2 minutes in a tissue lyser (Tissue Lyser II, Qiagen). Another 500μl Trizol was added after homogenization. The samples were centrifuged at 12,000g for 10 minutes at 4°C and the supernatant was transferred to a sterile 1.7ml microtube. The samples were left to rest for 5 minutes at RT, after which 200μl of chloroform was added per sample. The samples were shaken and incubated for 2-3 minutes at RT. Samples were centrifuged at 12,000g for 15 minutes at 4°C and the aqueous phase was transferred to a new sterile 1.7ml microtube. 500μl of isopropanol was added to the aqueous phase and the samples were incubated for 10 minutes at RT. Samples were centrifuged at 12,000g for 10 minutes at 4°C and the supernatant was removed leaving a RNA pellet at the bottom of the tube. The samples were washed with 1ml 70% ethanol, briefly vortexed and centrifuged at 7500g for 5 minutes at 4°C. The supernatant was removed and the pellet was left to air dry for 5-10 minutes. The RNA pellet was resuspended in 20μl DEPC-treated H_2O and incubated in a heat block set at 60°C. RNA concentrations were measured using a spectrophotometer (ND-1000, Nanodrop). Samples were stored at -70°C.

2.2.5.2 RNA to cDNA conversion

The high capacity RNA-to-cDNA kit (Applied Biosystems) was used for RNA to cDNA conversion, following the manufacturer's protocol. Per sample: 10µl of 2x RT buffer, 1µl of 20x enzyme mix, and up to 9µl of RNA sample (up to 2µg of RNA) was added to 0.2ml striptubes, DEPC-treated H_2O was added when necessary to increase the volume to 20µl. The tubes were spun down briefly to remove air bubbles and the tubes were placed in a thermo cycler (Veriti 96-well Thermo Cycler, Applied Biosystems) and incubated for 60 minutes on 37°C, 5 minutes on 90°C to stop the reaction and held at 4°C. cDNA was diluted to a concentration of 25ng/ml in DEPC-treated H_2O and stored in the freezer at -20°C.

2.2.5.3 Quantitative PCR

4µl of cDNA sample (100ng) was added to Optical 96 plates with barcode. Samples were run in duplicate including an H_2O sample in duplicate as a control. PCR reaction mixes were prepared: per reaction 1µl of 20x Taqman gene expression primers were added with 1µl of *Gapdh* as housekeeping gene, 10µl of 2x Taqman Gene Expression Master Mix and 4µl of DEPC-treated H_2O. 16µl of this reaction mix was added per well. The plate was covered with a plate adhesive seal and spun down briefly to remove air bubbles. PCR was run and analysed on Applied Biosystems' Quantstudio 7 (Applied Biosystems).

2.2.6 Serum ELISA

2.2.6.1 House dust mite specific total Ig

Clear 96-well ELISA plates (NUNC) were coated with 1µg/ml mashed up HDM in pH 9.6 carbonate buffer (50µl per well) and incubated overnight in the fridge. The next day, plates were washed 5x in wash buffer and moisture flicked off. Plates were blocked with 200µl per well of 2% BSA in PBS for 2 hours in a 37° incubator. Serum was defrosted slowly on ice and 1:50 dilutions were made up in block. Samples were serially diluted in 3-fold dilution steps across 7 wells and plates were incubated

overnight in the fridge. The next day, plates were washed again and 50μl block with αIg detection antibody in a 1:6000 dilution. Plates were incubated for 60 minutes in a 37°C incubator. Plates were washed 5x in wash buffer, and another 5x in PBS without Tween-20. 100μl TMB substrate was added per well, and the reaction was stopped by adding 50μl of 1M H_2SO_4 per well. ELISA plates were read at 450nm using an ELISA plate reader (Infinite M1000PRO, Tecan). Titre was calculated as the reciprocal serum dilution at which the dilution curve passed 50% of the maximum optical density (OD) of the whole plate.

2.2.7   Statistical analysis

All statistical analyses were performed using Prism 7 (Graphpad Software Inc.). First, a Shapiro-Wilk test for normality was used to determine normal distribution of samples within a group. If samples passed the Shapiro-Wilk test for normality, for the comparison of 2 groups, an unpaired T-test was used. If samples did not pass the Shapiro-Wilk test for normality, for the comparison of 2 groups, a Mann-Whitney test was used. When more than two groups were compared, a one-way ANOVA was used with a Bonferonni's multiple comparison test. $P<0.05$ was considered as significant.

Symbols represent individual samples and error bars represent mean ± the standard error of the mean (SEM).

Chapter 3.   The effect of *H. polygyrus* infection on systemic Th2 responses in mice

3.1 Introduction

H. polygyrus induces a local Th2 response in the gut resulting in expansion of immune cells in the draining LN throughout infection. While *H. polygyrus* has an entirely enteric lifecycle within the host, previous studies have shown an expansion of IL-4^{+} Th2 cells in other tissues such as blood, spleen and lung.[179] Furthermore, there was also an increased IL-4^{+} population of innate cells such as eosinophils and mast cells.[179]

In the skin, overexpression of IL-4 cannot induce an AD phenotype by itself, but is seen to alter skin homeostatic conditions and induce some processes also seen in AD.[166, 180] These include increased collagen deposition, keratinocyte hyperproliferation and mast cell accumulation leading to loss of dermal fat tissue, and impaired migration of Langerhans cells.[180] However, under these conditions no influx of pro-inflammatory cells such as Th2 cells, eosinophils or neutrophils was noted.[180] Furthermore, overexpression of IL-4 had a negative influence on wound healing, and wounds in IL-4 transgenic mice showed increased expression of inflammatory cytokines/chemokines and infiltration of inflammatory cells.[166] However, IL-4 also induces differentiation of AAMs through STAT6, which suppresses Th1/Th17 responses as well as Th2 cell function through expression of PD-L1, secretion of anti-inflammatory cytokines IL-10 and TGFβ, and enhancing differentiation of Tregs through a retinoic acid dependent mechanism.[44, 110-112] These studies indicate that IL-4 can affect skin in the steady-state and induce AAMs with anti-inflammatory properties, but by itself cannot induce AD.

IL-13 is a pro-inflammatory Th2 cytokine and is constitutively expressed in the skin by intraepithelial cells such as ILC2s. While IL-13 is implicated in the pathogenesis of AD, it is also important for maintaining skin homeostasis and lack of IL-13 leads to decreased skin barrier integrity and impaired wound healing.[159, 181] This demonstrates IL-13 also has a protective function in the skin under homeostatic conditions.

For this section, 4C13R dual-reporter mice were used (originally provided by Dr. W.E. Paul, NIH) to investigate the effect of *H. polygyrus* infection on the activation

of IL-4 and IL-13 producing immune cells in the skin. These mice were genetically constructed via insertion of a bacterial artificial chromosome and express the fluorescent protein AmCyan under the control of IL-4 and DS-Red under the control of IL-13.[149, 182] The use of these mice allows for the detection of canonical Th2 cytokines IL-4 and IL-13 without the need for *in vitro* stimulation and intracellular cytokine staining.

3.2 Aims

Previous studies have shown that *H. polygyrus* infection leads to the expression of IL-4 by T cells and innate cells in the spleen and lung. However, there is no data demonstrating this in skin tissue. Furthermore, as IL-13 is an important cytokine in the Th2 response and also has a protective role in the skin, it could be increased after helminth infection. Therefore, this chapter summarises experiments investigating the effects of *H. polygyrus* in mice on immune cell and cytokine profiles in organs distal to the gut.

Specific aims:

1) To assess if there is an induction of IL-4 in various tissues (MLN, whole blood, lung tissue and ear skin tissue) during infection with *H. polygyrus*, and to assess what immune cells (macrophages, eosinophils, neutrophils, B cells, T cells or ILC2s) are producing IL-4.

2) To assess if there is an induction of IL-13 in these tissues (MLN, whole blood, lung tissue and ear skin tissue) during infection with *H. polygyrus*, and to assess what immune cells (macrophages, eosinophils, neutrophils, B cells, T cells or ILC2s) are producing IL-13.

3.3 Results

For this section, 4C13R mice were used to look at IL-4 and IL-13 expression. Mice were infected with 200 L3 infective larvae 14 days before harvesting tissues. To determine the effects of *H. polygyrus* in mice in various organs, four different tissues were studied: MLN, whole blood, lung tissue and ear skin tissue.

H. polygyrus infection caused expansion of immune cells in the MLN but not in the other tissues assayed (data not shown). Different innate and adaptive immune cells were identified within CD45$^+$ cells (supplementary figure 1) by flow cytometry: macrophages (CD11b$^+$ CD64$^+$), eosinophils (CD11b$^+$ Siglec F$^+$), neutrophils (CD11b$^+$ Ly6G$^+$), ILC2s (Lin$^-$ B220$^-$ CD4$^-$ ICOS$^+$), B cells (B220$^+$) and T cells (TCRβ$^+$ CD4$^+$). Lineage markers used to negatively gate for ILC2s were: CD8, CD11b, CD11c, F4/80, DX5 and FcεRI. For these immune subsets, the frequency of cells producing IL-4 and/or IL-13 was determined by flow cytometric analysis of the specific reporter (supplemental figure 2, figure 3.1).

In the MLN, IL-4 production was significantly increased in infected mice of which the main producers are T cells (figure 3.1A). Although macrophages and eosinophils also produced IL-4 in this site, the difference between naive and *H.* polygyrus-infected groups was not significant. IL-13 production in the MLN was not significantly altered in infected mice, although IL-13 expression by ILC2s was slightly increased (figure 3.1B).

In the blood, the proportions of IL-4 producing macrophages and T cells were increased in *H. polygyrus*-infected mice compared to naïve (figure 3.1C). IL-13 production was also increased in macrophages of infected mice. This difference, however, was not significant (figure 3.1D).

In the ear skin tissue, IL-4 production was slightly increased in eosinophils (although not significantly), and significantly increased in T cells in *H. polygyrus*-infected mice compared to their naive counterparts (figure 3.1E). The proportion of cells in the skin producing IL-13 was not significantly altered by *H. polygyrus* infection, however there was a trend for increased IL-13 production by ILC2s (figure 3.1F).

Finally, IL-4 production in lung tissue was significantly increased in B cells and T cells, however this difference was not biologically significant in B cells as the difference was very small (0.004% in uninfected versus 0.022% in infected mice) (figure 3.1G). IL-13 production in the lung was slightly increased in T cells, although not significantly (figure 3.1H).
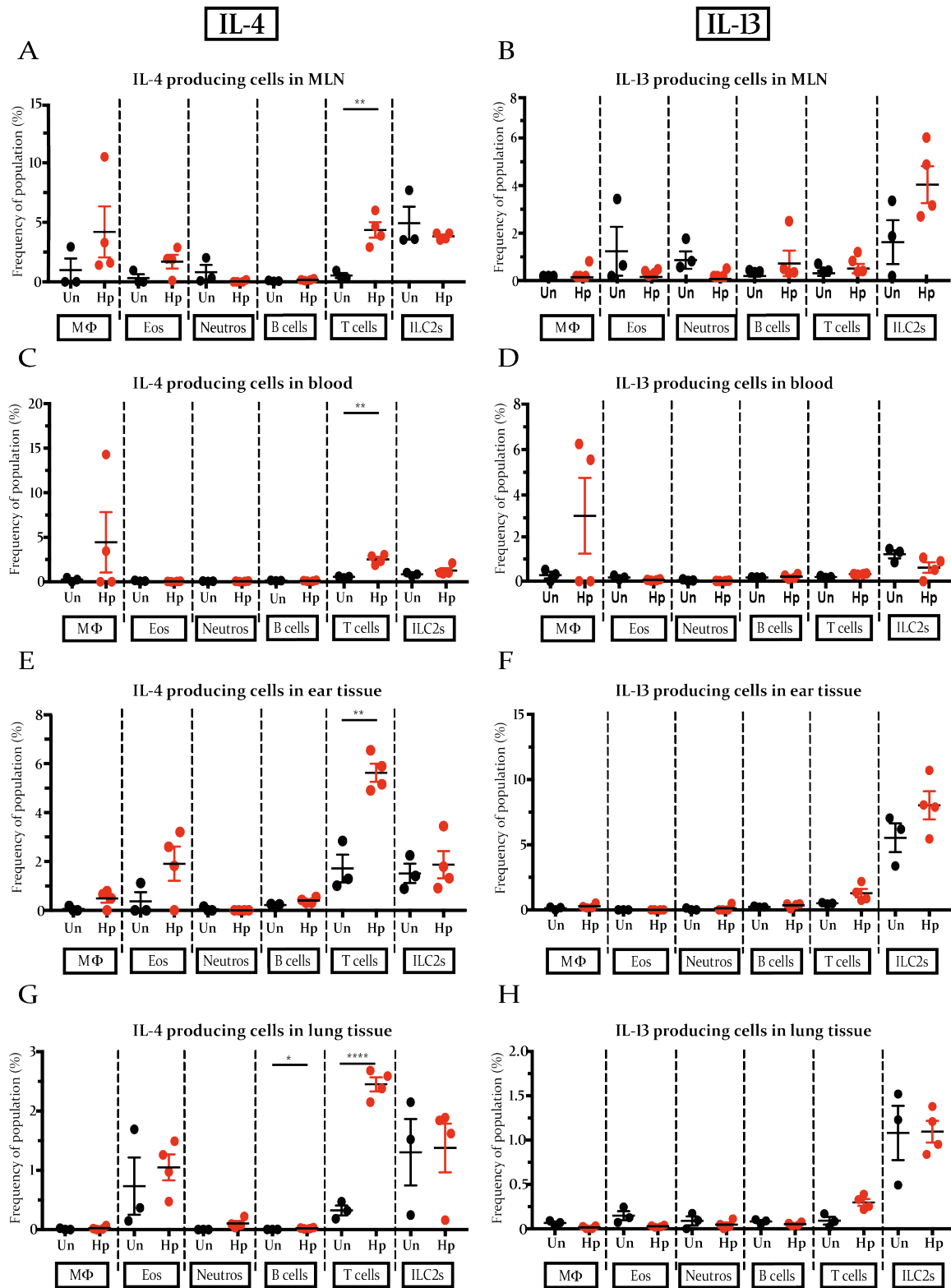
Figure 3.1: The effects of *H. polygyrus* infection on systemic Th2 responses.

Isolated cells from various tissues (MLN, whole blood, ear tissue and lung tissue) from uninfected mice (black) or *H. polygyrus* infected mice (red) analyzed by flow cytometry. IL-4 (A, C, E, G) and IL-13 (B, D, F, H) cytokine expression were assessed in various immune cells (macrophages, eosinophils, neutrophils, B cells, T cells, ILC2s). Y-axis depicts frequency of IL-4 or IL-13 producing cells within each population. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. MLN – mesenteric lymph nodes, Mφ - Macrophage, Eos – eosinophils, Neutros – neutrophils.

3.4 Discussion

Previous studies found that *H. polygyrus* induces an IL-4^{+} Th2 cell population in various organs, including blood, spleen, and lung.[179] The results above coincide with these previous studies and supplement them by demonstrating this population of IL-4 producing T cells is also present in ear skin tissue in *H. polygyrus*-infected mice. IL-4 has been shown to alter skin homeostasis, but is not by itself able to induce inflammation or an AD phenotype.[180] Furthermore, IL-4 induces AAMs, which have anti-inflammatory properties and are important for wound healing mechanisms.[44, 110-112] While the innate arm of the immune system is enough to induce AAMs in various tissues including the lungs and the skin, the adaptive arm (Th2 cells) is needed to maintain the presence of AAMs.[183, 184] Innate cells such as eosinophils were found to produce IL-4, although this increase in *H. polygyrus*-infected mice was not statistically significant above naive levels, and a larger sample size would be needed to determine if this difference was significant. The main source of IL-4 production in the skin of *H. polygyrus*-infected mice seems to be from T cells, making it likely that the stimulus for both induction and maintenance of AAMs is present in the skin of infected mice.

One of the immunomodulatory effects of *H. polygyrus* is the induction of AAMs, cells which can suppress Th1/Th17-mediated responses and Th2 cell function and induce wound healing mechanisms.[44, 85] If these AAMs are indeed induced in the skin of *H. polygyrus*-infected mice, they could lead to suppression of skin inflammation and atopy, and to faster wound healing. Further study into AAMs in the setting of skin atopy would therefore be beneficial.

While IL-13 is mainly known for its pro-inflammatory and anti-parasitic properties, earlier this year a study indicated that IL-13 is important for maintaining homeostasis of the skin barrier.[181] IL-13 is constitutively expressed by ILC2s in the skin, which coincides with the results above in uninfected mice. Infected mice seemed to have slightly elevated levels of IL-13 expressed by ILC2s, although this difference was not statistically significant. A larger sample size would be needed to determine if this difference is significant.

3.5 Conclusions

Key points:

1) IL-4 is induced by *H. polygyrus* in various organs, including the MLN, blood, ear skin tissue, and lung tissue, and the main source of this IL-4 is CD4$^+$ T cells.

2) Innate cells such as eosinophils also produce IL-4, although there was no recorded difference in *H. polygyrus*-infected mice.

3) IL-13 is constitutively produced by ILC2s in the skin of mice, yet was not significantly altered by *H. polygyrus* infection.

Chapter 4.   *H. polygyrus* suppresses house dust mite-induced Th2 inflammatory responses in the ear

4.1 Introduction

HDM is one of the most common environmental allergens causing airway inflammation in humans and is said to affect at least 15-20% of the population.[185, 186] The most common species of HDM are *Dermatophagoides pteronyssinus* and *D. farinae* and more than 20 allergens that induced specific IgE antibodies have been identified from these two species (named Der p or Der f followed by a number).[187]

4.1.1 HDM contains a variety of allergens that induce allergic responses

HDM allergens are divided into four families: proteases, non-proteolytic enzymes, lipid-binding proteins, and non-enzymatic components.[186] Many allergens have enzymatic properties, e.g. proteases (Der p 1, 3, 6, and 9) are able to cleave tight junctions, weakening the epithelial barrier and contributing to sensitization of allergic responses.[188]

Mites are also contaminated with microbial pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) and β-glucan that act as adjuvants in the priming of an immune response.[189] These mite allergens and microbial PAMPs stimulate TLRs, C-type lectin receptors (CLR) and NOD-like receptors (NLR) on immune cells and epithelial cells and induce Th2-mediated inflammation.[190-193] LPS through TLR4 signalling, was found to be a determining factor in the initiation of an immune response: low doses of LPS initiated Th2 responses with higher eosinophil recruitment, while high doses of LPS initiated stronger Th1 responses with higher neutrophil infiltration.[194]

4.1.2 HDM induces Th2 inflammation in the lungs

HDM is often used in an experimental setting in mice to induce allergen-induced lung inflammation and airway hyper-responsiveness, mimicking asthma, as humans with asthma are often also allergic to HDM.[195] This inflammation is characterized by increases in Th2 cytokines, innate cells including DCs, ILC2s, eosinophils, neutrophils, AAMs and basophils, and adaptive cells such as Th2 cells, and IgE producing B cells.[186]

Inhalation of HDM in a murine model leads to TLR4-dependent recruitment of basophils, eosinophils and DCs to the lung draining LNs. It was found DCs are required for the initiation of the Th2 response, and basophils and eosinophils are the main innate sources of IL-4 for the induction of inflammation. [196] HDM induces damage to the airway epithelial cells, causing release of alarmins such as IL-33, which contribute to the initiation of innate cell infiltration and activation such as ILC2s.[74, 186, 190] Mice deficient in ST2 exhibit reduced airway inflammation, demonstrating the importance of innate cells and stimuli in HDM-induced airway hyperresponsiveness.[197]

HDM-induced lung inflammation leads to differentiation of Th2 cells.[190] In the lymph nodes, it was found T follicular helper cells (Tfh) are an important source of IL-4, and induce class switching of B cells to IgG1 and IgE.[198] In the lung tissue, Th2 cells were an important source of Th2 cytokines IL-4 and IL-13, mediating Th2 inflammation.[198, 199] B cells also migrate to the lung tissue, locally releasing IgE antibodies, which activate mast cells.[198]

4.1.3 *H. polygyrus* suppresses HDM-induced Th2 inflammation in the lung

H. polygyrus was found to suppress Der p 1-induced inflammation in the lung, with decreased infiltration of immune cells including eosinophils in the lung and decreased IL-5 and IL-13 production.[13, 123] This suppression of inflammation coincided with increased numbers of Tregs present in the lung and elevated levels of IL-10 and TGFβ.[13] Furthermore, this protection was ablated when Tregs were depleted, and adoptive transfer of Tregs from *H. polygyrus*-infected mice to uninfected mice also conferred protection.[13]

Another study showed an expansion of a suppressive CD23^{hi} B cell population in the MLN of *H. polygyrus* infected mice.[14] After adoptive transfer to uninfected mice, these cells were able to suppress Der p 1-induced asthma in an IL-10 independent manner, with decreased lung eosinophilia, airway pathology and IL-5 production.[14]

Together, these results indicate *H. polygyrus*-induced regulatory mechanisms are able to suppress murine lung inflammation models induced by HDM allergens.

4.1.4 The use of HDM in skin inflammation models

HDM has been used in a murine model to induce strong Th2-mediated skin inflammation by ID injection in the ear skin tissue.[200] This inflammation is characterized by an increase in IL-4$^+$ CD4$^+$ T cells in the auricular LNs, which peaked between day 5-8, and production of HDM-specific IgG1 antibodies from B cells.[200] Furthermore, innate cells including eosinophils, basophils and mast cells increased in the ear skin tissue following HDM injection.[200]

HDM allergens have also been implicated in the development of AD, and the major mite protease allergens Der p 1 and Der f 1 have been found on human skin.[201] HDM protease allergens present on the skin tissue were seen to weaken the skin barrier, stimulate keratinocytes, and diminish epidermal barrier recovery in humans and mice.[202, 203]

These results demonstrate HDM-induced Th2 inflammation and impaired skin barrier function by topical application of mite allergens mimic components of AD.

4.2 Aims

For this section, an ID injection of HDM was used to induce Th2 inflammation in the skin.[200] There is limited information available on the immune cells that are activated during this HDM-induced inflammation in the ear skin tissue, and more research is needed.

Furthermore, *H. polygyrus* has been shown to suppress HDM-induced lung inflammation, however, it is currently not known if it is also able to suppress HDM-induced skin inflammation in the ear skin tissue. Therefore, more research is needed to determine the suppressive effects of *H. polygyrus* on ear skin tissue, using HDM to induce strong Th2 inflammation.

Specific goals:

1) To determine what immune cells are present in the ear draining LN and ear skin tissue during HDM-induced inflammation, and to investigate if this changes over time.

2) Investigate if *H. polygyrus* can suppress HDM-induced Th2 inflammation in the auricular LNs by inducing Tregs.

3) To determine if *H. polygyrus* can suppress HDM-induced Th2 inflammation in the ear skin tissue by inducing Tregs.

4) To determine if a 28-day *H. polygyrus* infection is more suppressive in the ear skin tissue than a 14-day *H. polygyrus* infection.

4.3 Results

4.3.1 ID injection of HDM in the ear induces substantial Th2 inflammation

The previous chapter revealed there was an induction of IL-4$^+$ T cells in the ear skin tissue after infection with *H. polygyrus*. To determine the effect IL-4 could have on HDM-induced Th2 inflammation, it is important to know what cells will be present over time, especially during the peak of inflammation.

For this experiment, mice were injected ID with whole HDM at different time points (3 days, 6 days and 9 days) before the endpoint at day 0 (figure 4.1A). Both the auricular LNs and one ear from each mouse was taken for analysis by flow cytometry. CD45$^+$ immune cells isolated from the LN (supplementary figure 1) were analysed for the presence of ILC2s (lin$^-$ CD4$^-$ CD90$^+$ KLRG$^+$ ICOS$^+$ ST2$^+$), T cells (CD3$^+$CD4$^+$), and ST2$^+$ T cells as a marker for IL-33 responsive Th2 cells. Lineage markers for negatively gating on ILC2s were: B220, CD8, CD11b, CD11c, FcεRI, and NK1.1. From the ear skin tissue, CD45$^+$ immune cells (supplementary figure 1) were stained for the presence of innate cells including eosinophils (CD11b$^+$ Siglec F$^+$), neutrophils (CD11b$^+$ Ly6G$^+$), basophils (FcεRI$^+$ DX5$^+$ c-kit$^-$) and mast cells (FcεRI$^+$ c-kit$^+$ DX5$^-$).

Previous studies showed an increase in eosinophils, basophils and mast cells in the ear skin tissue, and IL-4$^+$ CD4$^+$ T cells in the auricular LNs peaking between day 5-8.[200] In the auricular LN, there was an increase in total live immune cells after HDM injection, which peaked between day 6 and day 9 (figure 4.1B). There were no ILC2s detected in the LN (data not shown), and total CD4$^+$ T cells increased in number over time (figure 4.1C). The numbers of CD4$^+$ T cells expressing ST2 increased slightly over time, however this difference was not significant (figure 4.1D).

In the ear skin tissue, there was an increase in total live immune cells, which peaked at day 6 post-HDM injection (figure 4.1E). The eosinophil population was apparent in all samples, but peaked at day 9 (figure 4.1F). On the other hand, neutrophils made up 50% of immune cells present in the ear skin tissue at day 3 and 6, and dropped back to just above naive baseline at day 9 (figure 4.1G). There were no clear populations of basophils or mast cells present in the skin, nor did these populations

appear to increase during HDM-induced inflammation (data not shown). These results supplement data from Camberis *et al.*[200] demonstrating the presence of neutrophils early on in the immune response after HDM injection as well as eosinophils, and T cell expansion during the Th2 response.

4.3.2 *H. polygyrus* suppresses HDM-induced Th2 inflammation in the skin, coinciding with a slightly increased proportion of Tregs in the LN

Next, to determine if *H. polygyrus* is able to suppress HDM-induced inflammation, C57BL/6 mice were infected with 200 L3 larvae via oral gavage at day -14, injected ID with HDM on day 0, and analysed on day 3, 7, and 10 (figure 4.2A). Both the auricular LNs and one ear from each mouse was taken for analysis by flow cytometry. CD45$^+$ immune cells isolated from the LNs (supplementary figure 1) were analysed for the presence of B cells (B220$^+$), T cells (CD3$^+$ CD4$^+$), and Tregs (CD3$^+$ CD4$^+$ FoxP3$^+$). Furthermore, serum was extracted from whole blood to determine the levels of HDM-specific antibodies through ELISA.

First, in the LN, the total number of live immune cells present in HDM-only treated mice peaks at day 7, confirming the results in section 4.3.1 above (figure 4.2B) *H. polygyrus* infection alone did not result in a change in cell number in the auricular LNs compared to naive levels. At day 7, the number of total immune cells in the LNs of infected mice after HDM injection is significantly decreased (threefold difference) in comparison to uninfected mice (figure 4.2B). As the peak of inflammation was at day 7, and there was a significant difference between uninfected and infected mice after HDM injection, further analysis has focused on data from this time point.

Proportions of B cells in the auricular LNs did not differ in proportion between uninfected or infected mice after HDM injection (figure 4.2C). However, B cell numbers and HDM-specific antibodies are decreased (although not significantly) in *H. polygyrus*-infected mice (figure 4.2C, D).

There was no difference in T cell proportions between infected and uninfected mice with HDM injection, although T cells numbers did seem to be decreased (figure 4.2E).

Finally, as expected Treg proportions were increased in all *H. polygyrus*-infected mice in comparison to uninfected mice (figure 4.2F), however the actual number of Tregs is still decreased in *H. polygyrus*-infected mice with HDM injection, reflecting the overall size of the auricular LNs. These results suggest there is an overall suppression of immune cells present in the auricular LNs by *H. polygyrus* infection, which coincides with an increase in Treg proportions in the LNs.

4.3.3 *H. polygyrus* suppresses HDM-induced Th2 inflammation in the skin, coinciding with a slightly increased proportion of Tregs in the ear skin tissue

$CD45^+$ immune cells isolated from ear skin tissue (supplementary figure 1) were analysed for the presence of T cells ($CD3^+$ $CD4^+$), Tregs ($CD3^+$ $CD4^+$ $FoxP3^+$), eosinophils ($CD11b^+$ Siglec F^+), and AAMs ($CD11b^+$ $CD64^+$ $Relm\alpha^{hi}$). Using the same experimental protocol (figure 4.3A) as for the auricular LNs, in the ear skin tissue there was no significant difference in total live immune cells between groups and even over time (figure 4.3B). There was a slight difference on day 7, where uninfected mice after HDM injection had the highest number of immune cells, which coincides with data from the auricular LNs. This points to day 7 as the peak of the inflammation after ID injection of HDM, and further analysis in this section will focus on data from this time point.

In both groups containing infected mice, $CD4^+$ T cells proportions were increased, although this difference was not significant, and absolute numbers did not differ (figure 4.3C). Proportions of $FoxP3^+$ Tregs were also increased in both groups of infected mice, suggesting the increase of $CD4^+$ T cells is most likely due to the increased presence of Tregs in the skin (figure 4.3D).

Eosinophils were recruited to the ear skin tissue after HDM injection, and although not significant, there is a trend towards a lower proportion of eosinophils present in the ear skin tissue in infected mice (figure 4.3E).

Finally, infected mice without HDM injection showed an increase in AAM population in the ear skin tissue. Both groups of mice injected with HDM (uninfected and infected) showed an increase in this AAM population in comparison to naive mice, however there was no significant difference between these two groups (figure 4.3F).

4.3.4 Chronic infection with *H. polygyrus* suppresses HDM-induced Th2 inflammation further

H. polygyrus can establish a chronic infection within several strains of mice.[92] Treg numbers peak at 28 days post-infection in the gut-draining LNs and have a strong regulatory phenotype.[16] Therefore, a 28-day infection with *H. polygyrus* was used to determine if there was a stronger suppression of HDM-induced skin inflammation in the ear tissue at 28 days, compared to the 14-day model.

For this section, 4C13R mice were used to determine the expression of the Th2 cytokines IL-4 and IL-13. Mice were infected with 200 L3 larvae at day -28, and injected ID with HDM at day 0. The mice were sacrificed at day 7, and the auricular LNs were taken for analysis by flow cytometry (figure 4.4A).

Coinciding with previous data from the 14-day model (figure 4.2), total numbers of live immune cells present in the auricular LNs are increased in uninfected mice after HDM injection, and *H. polygyrus* infection significantly reduces these numbers (figure 4.4B).

Previous data showed a decreased absolute number of B cells in infected mice after HDM injection. After 28-day infection with *H. polygyru*s, there is a significant reduction not only in B cell number, but also in B cell proportion (figure 4.4C),

suggesting a 28-day model of *H. polygyrus* infection is more suppressive than a 14-day model.

Finally, CD4$^+$ T cell numbers are significantly reduced in *H. polygyrus*-infected mice after HDM injection in comparison to uninfected mice after HDM injection (figure 4.4D). Of these CD4$^+$ T cells, there is an increased proportion and number of IL-4$^+$ T cells in uninfected mice with HDM injection compared to naive mice, and this is decreased in the *H. polygyrus*-infected group (figure 4.4D).
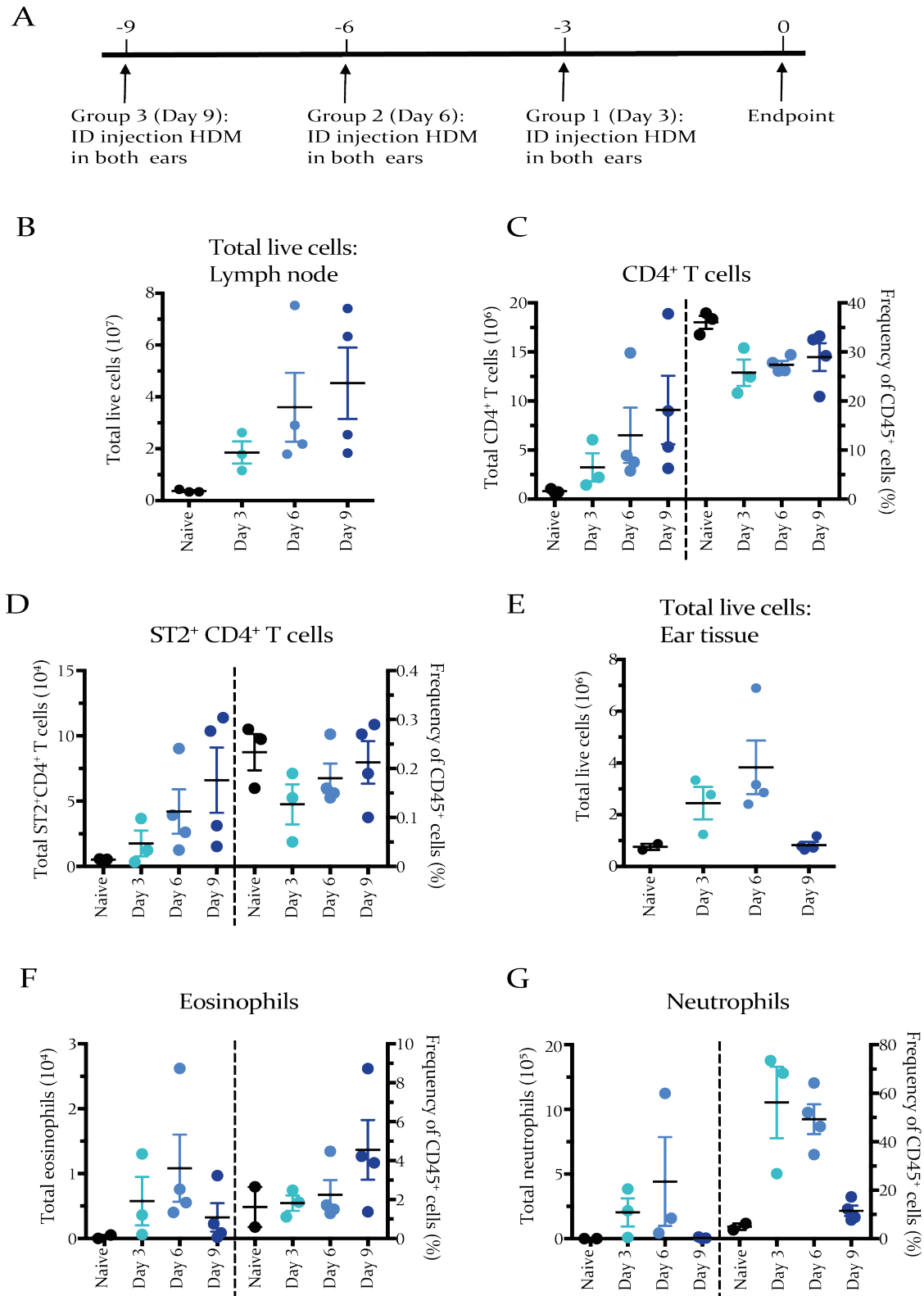
Figure 4.1: Time course of ID injected HDM-induced Th2 inflammation.

Cells from LN and ear skin tissue were stained and analysed by flow cytometry A) Timeline of the experiment. B) Total live cells, C) CD4$^+$ T cells, and D) ST2$^+$ CD4$^+$ T cells present in the auricular LNs of mice in naive and HDM injected mice during different time points over the time course. E) Total live cells, F) eosinophils, and D) neutrophils present in the ear skin tissue of mice in naive and HDM injected mice during different time points over the time course. Graphs show mean ±SEM.

Figure 4.2: *H. polygyrus* **suppresses HDM-induced inflammation in the auricular LN and induces Tregs.**

A) Model for *H. polygyrus* infection and ID injection of HDM (numbers depict the day) B) Total live cells present in the auricular LNs at day 3, 7 and 10. Flow cytometry analysis at day 7 in the LN C) total live B cells, E) total CD4$^+$ T cells and F) CD4$^+$ FoxP3$^+$ Tregs. D) HDM-specific total Ig measured by ELISA in the serum. Titre is defined as the reciprocal serum dilution at which the dilution curve passes 50% of the maximum OD of the whole plate. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * p<0.05
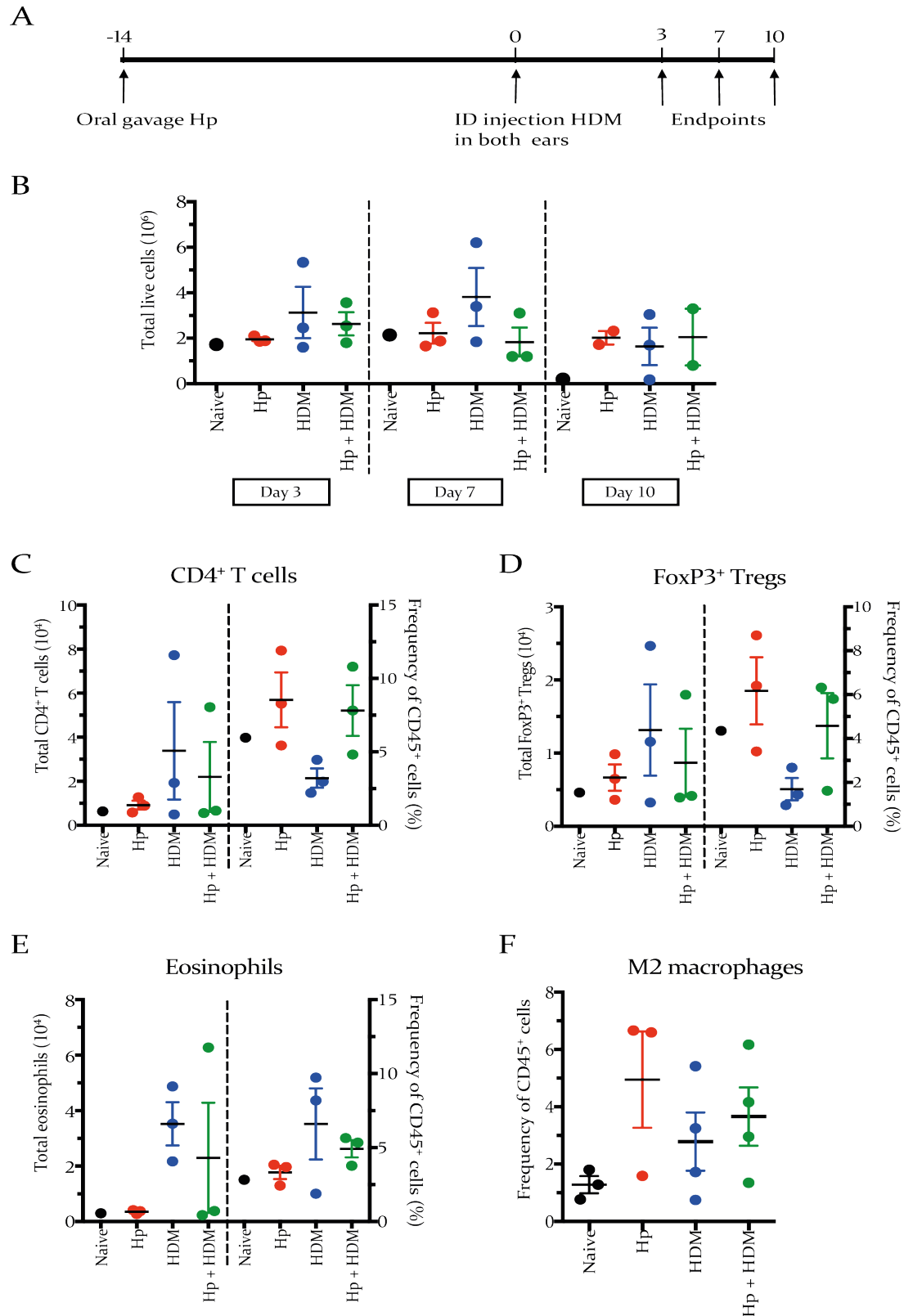
Figure 4.3: *H. polygyrus* **affects HDM-induced inflammation in the ear skin tissue and induces Tregs and M2 macrophages/AAMs.**

A) Model for *H. polygyrus* infection and ID injection of HDM (numbers depict the day) B) Total live cells present in the ear skin tissue at day 3, 7 and 10. Flow cytometry analysis at day 7 in the ear skin tissue C) CD4$^+$ T cells, D) CD4$^+$ FoxP3$^+$ Tregs, E) total live eosinophils, and F) total live M2 macrophages/AAMs. Graphs show mean ±SEM
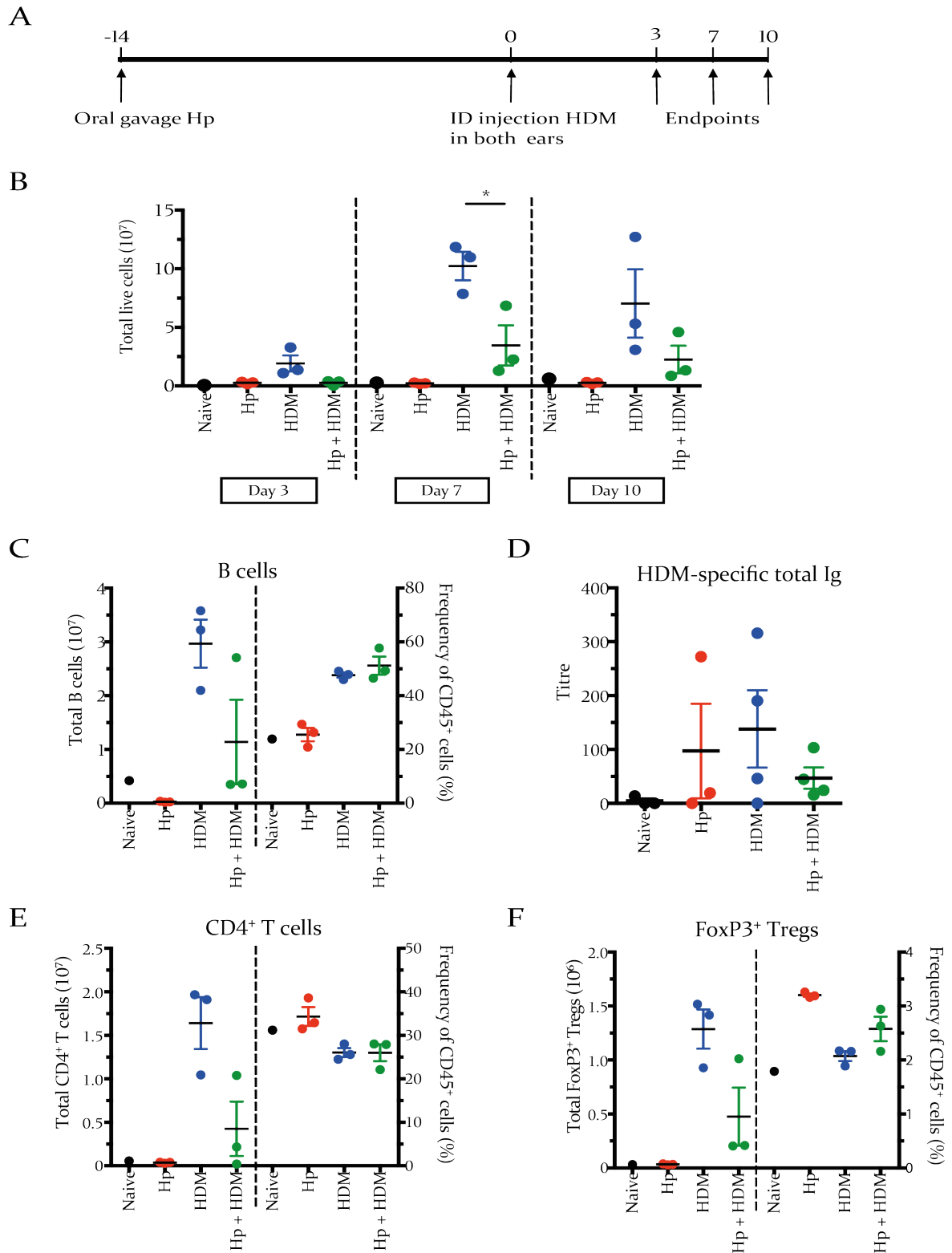
Figure 4.4: *H. polygyrus* suppresses HDM-induced inflammation in the auricular LNs during chronic infection.

A) Model for *H. polygyrus* infection and ID injection of HDM (numbers depict the day) B) Total live cells present in the auricular LNs. Flow cytometry analysis in the LN C) total live B cells, D) total CD4^{+} T cells and AmCyan^{+} (IL-4) CD4+ T cells. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * p<0.05, ** p<0.01

4.4 Discussion

Previous studies have shown HDM induces strong inflammation in the lung, mainly through TLR-dependent mechanisms and cleaving of tight junctions of epithelial cells leading to secretion of alarmins such as IL-33.[188, 190, 191] While there is limited data available on the initiation of a Th2 inflammatory response by ID injection of HDM, it has been shown to be sufficient in activating DCs without the presence of TLSP.[143]

The results above demonstrate ID injection of HDM induces strong inflammation in the auricular LN and ear skin tissue as seen by the significant increase in total live immune cells during the peak of the inflammation on day 7. This inflammation is characterized by the presence of innate cells such as eosinophils and neutrophils in the skin and CD4$^+$ Th2 cells in the LN.

Previous studies have also shown *H. polygyrus* was able to suppress HDM-induced lung inflammation through the induction of Tregs.[13] Concurring with these studies, it was found *H. polygyrus* suppressed HDM-induced Th2 inflammation in the skin characterized by decreased numbers of immune cells present in the auricular LN and ear skin tissue. In the auricular LNs, a reduced number of B cells and CD4$^+$ T cells were present, although a larger sample size would be needed to determine if this difference was significant. Coinciding with a lower number of B cells, *H. polygyrus*-infected mice had less HDM-specific antibodies present after HDM injection than uninfected mice. Interestingly, one infected mouse that did not receive an HDM injection had HDM-specific antibodies in the serum, suggesting there might be some cross-reactivity between mite allergens and parasitic antigens. This phenomenon has recently been demonstrated in a paper comparing the molecular structures of common allergens and parasitic antigens.[204]

In the ear skin tissue, there was no significant difference found in total immune cells present in the tissues after HDM injection or *H. polygyrus* infection. As expected for Th2-driven inflammation, eosinophils were recruited to the ear skin tissue after HDM injection, and there was a trend towards a lower number and proportion of eosinophils in the ear skin tissue of infected mice. Again, a larger

sample size would be needed to determine the significance. However, there were issues with the digestion enzyme cocktails used in these experiments (primarily concerning CD4-cleavage from the surface of cells with some enzymes), and optimisation of digestion conditions was ongoing throughout the experimental time-frame.

H. polygyrus-infected mice had an increased proportion of Tregs in the auricular LNs, which coincides with previous studies by Wilson *et al.*[13] and is most likely a key mechanism to the suppression of inflammation in the ear skin tissue. Furthermore, *H. polygyrus* infection caused an increase in T cells in the ear skin tissue with a larger proportion of these being FoxP3$^+$ Tregs. While Tregs are evident in the skin in these experiments, they are absent in the skin of patients with AD.[205] This suggests Tregs present in the ear skin tissue after HDM-injection are likely induced by *H. polygyrus* infection. Further studies using depletion of Tregs in *H. polygyrus*-infected mice, and transfer of Tregs from *H. polygyrus*-infected mice to uninfected mice, would demonstrate the importance of Tregs in the suppression by *H. polygyrus*.

Finally, in the previous chapter it was hypothesized that the release of IL-4 in the skin of infected mice could lead to induction of AAMs. AAMs are one of the key regulatory cell types induced by *H. polygyrus*.[113] *H. polygyrus*-infected mice did seem to have an increased proportion of AAMs present in the ear skin tissue, however after HDM injection, there did not seem to be a difference between uninfected and infected mice. These results suggest AAMs are also induced after HDM-injection, and suppression of HDM-induced skin inflammation by *H. polygyrus* is not dependent on AAMs. Depletion of AAMs (using clodronate-coated liposomes), blocking of their effector molecules (such as arginase-1), or use of mice in which depletion of AAMs is possible (LysMCre or CD11b-DTR), may shed more light onto the role of AAMs in this model.

After more chronic infection with *H. polygyrus*, Tregs peak at day 28 and have a strong modulatory profile, characterized by CD103 expression, antigen-specific IL-10 production and increased TGFβ on the cell surface of CD4$^+$ T cells.[16] Using a 28-day chronic infection model with *H. polygyrus*, total number of live immune cells in the

auricular LNs were decreased in comparison to uninfected mice after HDM injection. The 28-day model was more suppressive than the 14-day model, as *H. polygyrus* infection suppressed expansion of B cell and IL-4$^+$ T cell proportion as well as absolute numbers. These IL-4$^+$ T cells are most likely Tfh cells, as they have been shown to be important for the class switching of B cells, which would coincide with a lower HDM-specific antibody production in the serum and decreased proportion of B cells in the LN.[198] While immune cells in the auricular LNs seem to be more suppressed with a 28-day infection before HDM injection, a 14-day infection is sufficient for *H. polygyrus* to significantly suppress HDM-induced skin inflammation.

Future experiments, as mentioned before, would include determining the importance of Tregs in *H. polygyrus*-mediated suppression of HDM-induced Th2 inflammation in the ear skin tissue. Furthermore, due to issues with digestion enzyme cocktails, more research is needed to accurately determine the suppression of immune cells present in the ear skin tissue by *H. polygyrus*. Another enzyme cocktail could be used to study T cells and FoxP3$^+$ Tregs in the ear skin tissue. Furthermore, culturing these Tregs *in vitro* with other lymphocytes would determine if Tregs induced by *H. polygyrus* distal to the gut are more suppressive than Tregs induced in uninfected mice.

4.5 Conclusions

Keypoints:

1) ID HDM injection into the ear skin tissue induced strong Th2 inflammation characterized by Th2 cells, B cells, eosinophils and neutrophils.

2) *H. polygyrus* suppressed HDM-induced Th2 inflammation in the ear skin tissue and auricular LN, characterized by decreased number of immune cells present.

3) *H. polygyrus* infection increased the proportions of Tregs in LNs distal to the gut, i.e. the auricular LNs, and in the ear skin tissue. However, this difference was not significant.

4) *H. polygyrus* induced AAMs in the ear skin tissue under homeostatic conditions, and after HDM injection, there was no difference between uninfected and infected mice.

5) A chronic 28-day infection with *H. polygyrus* was more suppressive than a 14-day *H. polygyrus* infection, and resulted in a decreased number and proportions of immune cells such as B cells and IL-4$^+$ T cells in the LN.

Chapter 5.   *H. polygyrus* suppresses hapten-induced skin allergy

5.1 Introduction

One of the most common forms of AD is CHS, which is mediated by T cells after activation by APCs such as DCs. This skin inflammation is characterized by dermal infiltration of immune cells such as Th2 cells, mast cells, eosinophils, increased IL-4 production, and increased IgE levels.[161, 206, 207] Furthermore, as with AD patients, mice exhibited increased epidermal hyperplasia and a decreased expression of filaggrin and tight junction proteins, leading to impaired skin barrier function and increased TEWL, which was found to correlate with AD severity.[161, 206-209]

In an experimental setting, CHS is often induced by topical application of chemical haptens, such as oxazolone, MC903 or FITC. Chemical haptens are small molecules, which induce an immune response after binding to soluble or cell-associated proteins.[162] While most haptens induce an acute Th1 response in the skin, multiple challenges to the skin over a longer period will induce a shift from a Th1 response towards a Th2 immune response that is similar to AD in humans.[161, 207] This shift towards a Th2 response after repeated sensitization by chemical haptens, demonstrates the importance of environmental factors for the onset of AD, and demonstrates hapten-induced skin inflammation can further amplify damage to the skin barrier.[62, 161]

Application of FITC, in combination with DBP, although an acute model, induces Th2-dominated CHS with high levels of IL-4 and IL-10 seen in the skin even though it is an acute model.[162, 210] DBP is used as a solvent for FITC, and also acts as an adjuvant to enhance the potency of the hapten through induction of TLSP by keratinocytes.[211, 212] TSLP is important for the initiation of Th2-mediated inflammation via suppression of IL-12 production by DCs, expression of co-stimulatory molecules, and upregulation of chemokine ligands (CCL) 17 and CCL22.[55, 213, 214] Through these processes DCs are activated towards a Th2-activating phenotype. As elevated levels of TSLP produced by keratinocytes have also been seen in humans with AD, DBP-FITC-induced CHS looks to be a suitable model to study AD in mice.[213, 215]

Research using this model of AD to induce skin allergy has found specific subsets of dermal DCs, namely the CD11b^{+} DCs and TN DCs, to be responsible for the activation of Th2 cells in the skin-draining LN.[143] These TN DCs were highly responsive to TSLP, and not activated in the absence of this cytokine.[143] Another study showed there was an increase in ear thickness, eosinophil infiltration, and IgE levels after DBP-FITC application to ear skin.[215] Furthermore, anti-IL-4, anti-IL-5, and mast cell depletion reduced ear thickness, and major histocompatibility complex II (MHCII) KO mice and STAT6 KO mice were protected from DBP-FITC-induced CHS.[215] These results demonstrate the importance of MHCII-dependent DC activation of CD4^{+} Th2 cells to initiate Th2 inflammation, and that innate cells such as eosinophils and mast cells contribute to inflammation in the skin.[215]

5.2 Aims

The previous chapter demonstrated *H. polygyrus* was able to suppress HDM-induced skin inflammation, coinciding with an increase in Treg proportions in the auricular LNs and ear skin tissue. However, as TSLP is not detected in this model, and has been demonstrated to be elevated in humans with AD, it is not entirely suitable as a model for AD.[143, 200, 213] DBP-FITC has been shown to mimic the human CHS phenotype more accurately.[213, 215] As yet, it is not known if *H. polygyrus* is able to influence DBP-FITC-induced CHS. Therefore, for this section, a murine model of DBP-FITC-induced Th2 inflammation was used to study the effect of *H. polygyrus* on skin allergy.

Specific goals:

1) Determine if *H. polygyrus* is able to suppress DBP-FITC-induced CHS through measuring ear thickness as a read-out for inflammation, and TEWL as a measure for skin barrier function.

2) Determine if *H. polygyrus* is able to influence DC phenotype or numbers after DBP-FITC treatment on the ear skin tissue.

3) Determine if *H. polygyrus* is able to suppress CD4$^+$ T cells subsets, and cytokine production of lymphocytes, by inducing Tregs in the auricular LNs.

4) Determine what cytokines are being produced in the ear skin tissue and if *H. polygyrus* is able to influence the production of these cytokines

5.3 Results

5.3.1 *H. polygyrus* suppresses skin inflammation in DBP-FITC-induced CHS

While the previous chapter demonstrated HDM-induced skin inflammation was suppressed by *H. polygyrus*, further research is needed to determine if *H. polygyrus* is also suppressive in a more suitable model of AD.

For this experiment, mice were infected with 200 L3 larvae via oral gavage at day -14, the flanks of the mice were shaved at day -7 so no irritation from the shaving would remain during sensitization by DBP-FITC on the flanks (on day 0 and 2), and finally, mice were challenged with DBP-FITC on the left ear and the vehicle DBP alone on the right ear on day 6 (figure 5.1A). Before challenge and every day after challenge, ear thickness was measured to determine the level of inflammation, and TEWL was measured to determine if skin barrier function was compromised.

While the vehicle DBP alone did not increase ear thickness in mice, challenge with DBP-FITC increased the ear thickness of uninfected mice and the peak of inflammation was around day 1 and 2 after challenge (figure 5.2B). Infected mice challenged with DBP-FITC had significantly reduced ear thickness at day 2 after challenge (figure 5.2B). However, there was no difference in TEWL (figure 5.2C). These results suggest *H. polygyrus* is able to suppress DBP-FITC induced skin inflammation, but does not affect skin barrier function.

5.3.2 *H. polygyrus* suppresses inflammation early in the immune response but does not alter DC phenotype in the skin

DCs have been shown to be crucial for the initiation of DBP-FITC induced CHS.[215] Furthermore, *H. polygyrus* infection induces a tolerogenic phenotype in intestinal CD11c^{lo} DCs and blocks the migration of CD11c^{hi} DCs to the MLN, but it is still not known if *H. polygyrus* infection influences DCs in other tissues, particularly the skin.[128] Additionally, Tregs are able to influence the phenotype of DCs through CTLA-4 binding to CD86.[100]

Earlier results indicated HDM-induced inflammation seemed to be suppressed at day 3 in infected mice (figure 4.2B). Therefore, it was hypothesized Th2 inflammation in the skin could be suppressed through the alteration of DC phenotype or the blocking of migration to the auricular LNs.

For this experiment, mice were infected with 200 L3 larvae via oral gavage at day -14, and treated once with DBP-FITC on the ear skin tissue of both ears on day 0 (figure 5.2A). At day 2, both LNs were harvested and processed according to protocol. Immune cells isolated from the LNs (supplementary figure 1) were stained and analysed using flow cytometry, and 5 DC subsets were surveyed for the cell numbers, frequency of cells that were FITC$^+$ (i.e. DCs that had taken up the treatment), and CD86 median fluorescence intensity (MFI) as a measure of activation (figure 5.2B).

Previously, Ochiai *et al.*[143] showed that after one treatment of DBP-FITC on the ear skin tissue of mice, CD11b$^+$ DCs and TSLP-dependent TN DCs were essential for induction of Th2 inflammation in this model. These subsets had expanded most, had the highest frequency of FITC$^+$ DCs and had the highest CD86 expression.[143] Coinciding with this study, one application of DBP-FITC caused inflammation (increased total cell number) in the auricular LNs (figure 5.3A). Furthermore, there was a significant reduction of total live immune cells present in the auricular LNs of infected mice in comparison to uninfected mice after DBP-FITC treatment. In addition, CD11b$^+$ and TN DC subsets showed the highest numbers of FITC$^+$ cells (figure 5.3B). However, infected mice only showed a slight reduction in FITC$^+$ DCs in each subset, which did not reach significance.

Finally, while the TN DC subset had the highest fold increase of CD86 expression, coinciding with the results from Ochiai et al,[143] there was no difference between uninfected mice and infected mice (figure 5.3C).

5.3.3 *H. polygyrus* suppresses inflammation and induces IL-4-producing Th2 cells and Tregs in the auricular LNs

Tregs have been shown to suppress Th2 inflammation in tissues such as the lung, and to be important for the regulation of inflammation in AD.[13, 160] As Tregs were induced after HDM-induced skin inflammation in the auricular LN and ear skin tissue, it is likely that a similar phenotype would be seen during a different AD model.

To determine if *H. polygyrus* is able to suppress lymphocytes in the auricular LNs after DBP-FITC treatment, C57BL/6 mice were infected with 200 L3 larvae via oral gavage at day -14, treated with DBP-FITC on day 0 and day 2, and analysed on day 7 (figure 5.4A). Immune cells isolated from both auricular LNs (supplementary figure 1) were taken for analysis by flow cytometry and one ear was taken for RNA extraction for analysis of gene expression by qPCR. Immune cells isolated from the LNs were analysed for the presence of B cells (CD19$^+$), CD8$^+$ T cells (CD3$^+$ CD8$^+$), CD4$^+$ T cells (CD3$^+$ CD4$^+$), Tregs (FoxP3$^+$ CD4$^+$ T cells), and Th2 cells (GATA3$^+$ CD4$^+$ T cells).

Coinciding with previous results, DBP-FITC induces expansion of immune cells in the auricular LNs, and there is a significant reduction of immune cells present in *H. polygyrus*-infected mice (figure 5.4B). This significant reduction of immune cells also extends to numbers of B cells (figure 5.4C), CD8$^+$ T cells (figure 5.4D), and CD4$^+$ T cells (figure 5.4D), although these cell proportions are unchanged (data not shown).

While the proportion of CD4$^+$ T cells in the LN are unchanged by infection after DBP-FITC treatment, proportions of T cell subsets have changed. FoxP3$^+$ Tregs (figure 5.5A), and GATA3$^+$ Th2 cells (figure 5.5B) are significantly increased in proportion in infected mice, although this increase in Tregs does not translate to absolute numbers and is still significantly lower in *H. polygyrus*-infected mice, reflecting the size of the LNs. These results indicate that CD4$^+$ T cells have various subsets that change in proportion in *H. polygyrus*-infected mice after DBP-FITC

treatment and there might be other subsets of T cells that are influenced by *H. polygyrus*.

Previous results have shown that *H. polygyrus* induces IL-4$^+$ T cells in various tissues, including in the ear skin tissue (figure 3.1), while *H. polygyrus* suppresses IL-4$^+$ T cells (likely Tfh cells) in the auricular LNs after ID HDM injection (figure 4.4D).

For this section, immune cells isolated from the auricular LNs were cultured and stimulated for 4 hours in the presence of Brefeldin A, PMA, and Ionomycin. B cells and CD4$^+$ T cells were analysed for their expression of Th2 cytokines IL-4, IL-10, and IL-13.

IL-4 was produced by both B cells and CD4$^+$ T cells in the LN, and in contrast to the HDM model, cells producing IL-4 were significantly increased in proportions in H. *polygyrus*-infected mice (figure 5.5C, E). IL-10 was also produced by both B cells and T cells (figure 5.5D, F), and while proportions were no different, absolute numbers were significantly decreased in infected mice. This suggests suppression of inflammation by *H. polygyrus* is not dependent on IL-10, which corresponds with previous data from Wilson *et al.*[13, 14] IL-13-producing lymphocytes were not present in the auricular LNs (data not shown), and more likely were present in the ear skin tissue during the peak of inflammation.

5.3.4 *H. polygyrus* suppresses *Il13* gene expression during peak of inflammation

Previous studies have shown that *H. polygyrus* suppressed HDM-induced lung inflammation and pro-inflammatory cytokine expression in the lungs.[13] Furthermore *H. polygyrus* was able to block IL-33 expression in a model of OVA-*Alternaria*-induced lung inflammation, suppressing expansion of and cytokine production by ILC2s.[123] While *H. polygyrus* suppresses inflammation and expansion of lymphocytes in the auricular LNs, cytokine profiles in the ear skin tissue might be altered, as illustrated by intracellular cytokine staining results above. Therefore, from the same experiment RNA was extracted from the ear skin tissue of mice at

day 7 post-DBP-FITC treatment, according to protocol for gene expression analysis by qPCR.

Tslp expression, which had been demonstrated to be crucial for the initiation of the Th2 response,[214] was increased after DBP-FITC treatment as expected, although was only slightly increased during the peak of inflammation (figure 5.6A), suggesting an earlier time point would be needed to determine *Tslp* levels directly after DBP-FITC treatment. Although *Tslp* levels are decreased in *H. polygyrus*-infected mice, this difference did not reach significance (figure 5.6A). Previous results have demonstrated *H. polygyrus* increases IL-4$^+$ T cells in the ear skin tissue. However, after DBP-FITC treatment, *Il4* expression increased profoundly and *H. polygyrus* did not alter this high level (figure 5.6B). Conversely, while *Il13* expression increased after DBP-FITC treatment, it was significantly decreased in *H. polygyrus*-infected mice (figure 5.6C), suggesting *Il13* gene expression is more susceptible to the suppressive effects of *H. polygyrus* than *Il4*.

Intriguingly, expression of the Treg marker *Foxp3* was decreased in *H. polygyrus*-infected mice compared to uninfected mice after DBP-FITC treatment (data not shown), and no difference was found in expression of anti-inflammatory cytokines *Il10* and *Tgfβ* (data not shown). These results suggest during the peak of the inflammation there is no increase in Tregs or anti-inflammatory cytokines in *H. polygyrus*-infected mice, and an alternative mechanism of suppression may be induced by the parasite, at least in this setting.
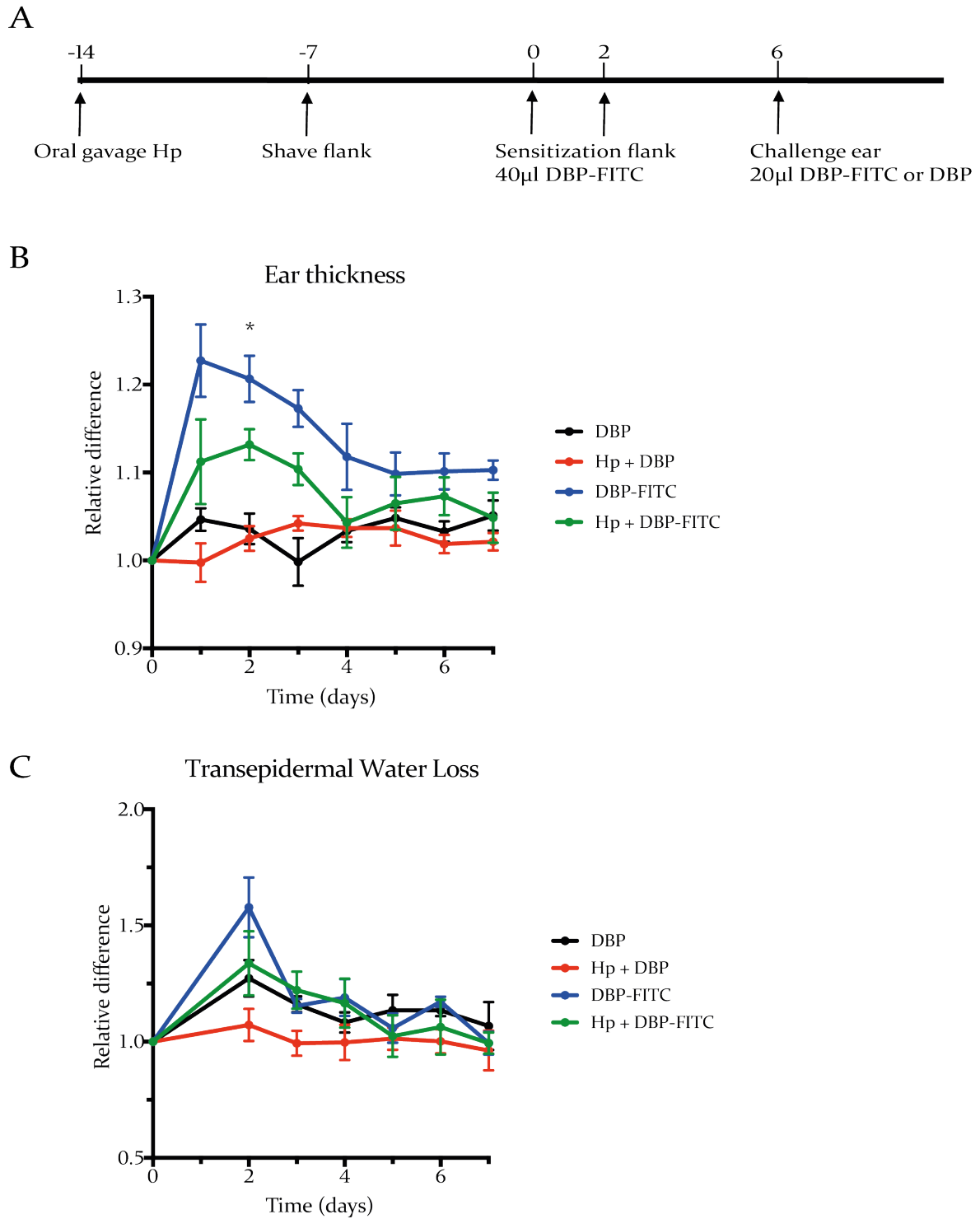
Figure 5.1: *H. polygyrus* **suppresses inflammation in a model of DBP-FITC-induced CHS.**

A) Model for *H. polygyrus* infection, sensitization with DBP-FITC and challenge with DBP-FITC or DBP (numbers depict the day). Relative increase of B) ear thickness and C) TEWL in comparison to baseline (before challenge on ear skin tissue) Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * $p < 0.05$.
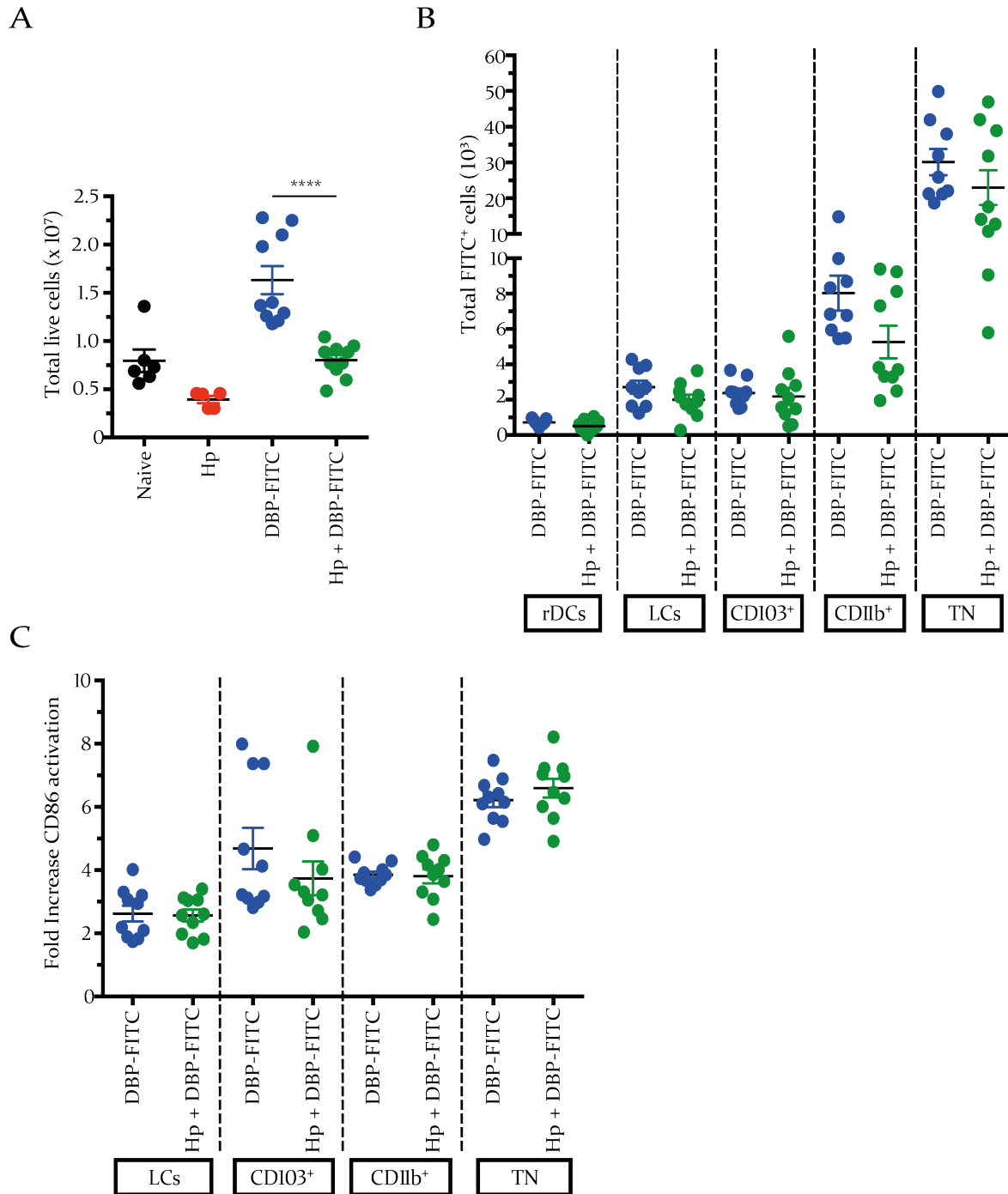
Figure 5.2: Model and gating strategy for analysing DC subsets after treatment with DBP-FITC.

A) Model for *H. polygyrus* infection and treatment with DBP-FITC (numbers depict the day). B) Gating strategy for 5 specific DC subsets in the auricular LNs: resident DCs (rDCs), Langerhans cells (LCs), CD11b+ DCs, CD103+ DCs and TN DCs. From each DC subset frequency of FITC+ cells and median fluorescence intensity (MFI) of CD86 was determined.

Figure 5.3: *H. polygyrus* suppresses early inflammation after DBP-FITC treatment.

A) Total immune cells and B) FITC$^+$ DCs from each DC subset present in the auricular LN. C) Fold increase of CD86 MFI in FITC$^+$ DCs in relation to CD86 MFI in FITC$^-$ DCs for LCs, CD103$^+$ DCs, CD11b$^+$ DCs and TN DCs. Data was collected from two separate experiments. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
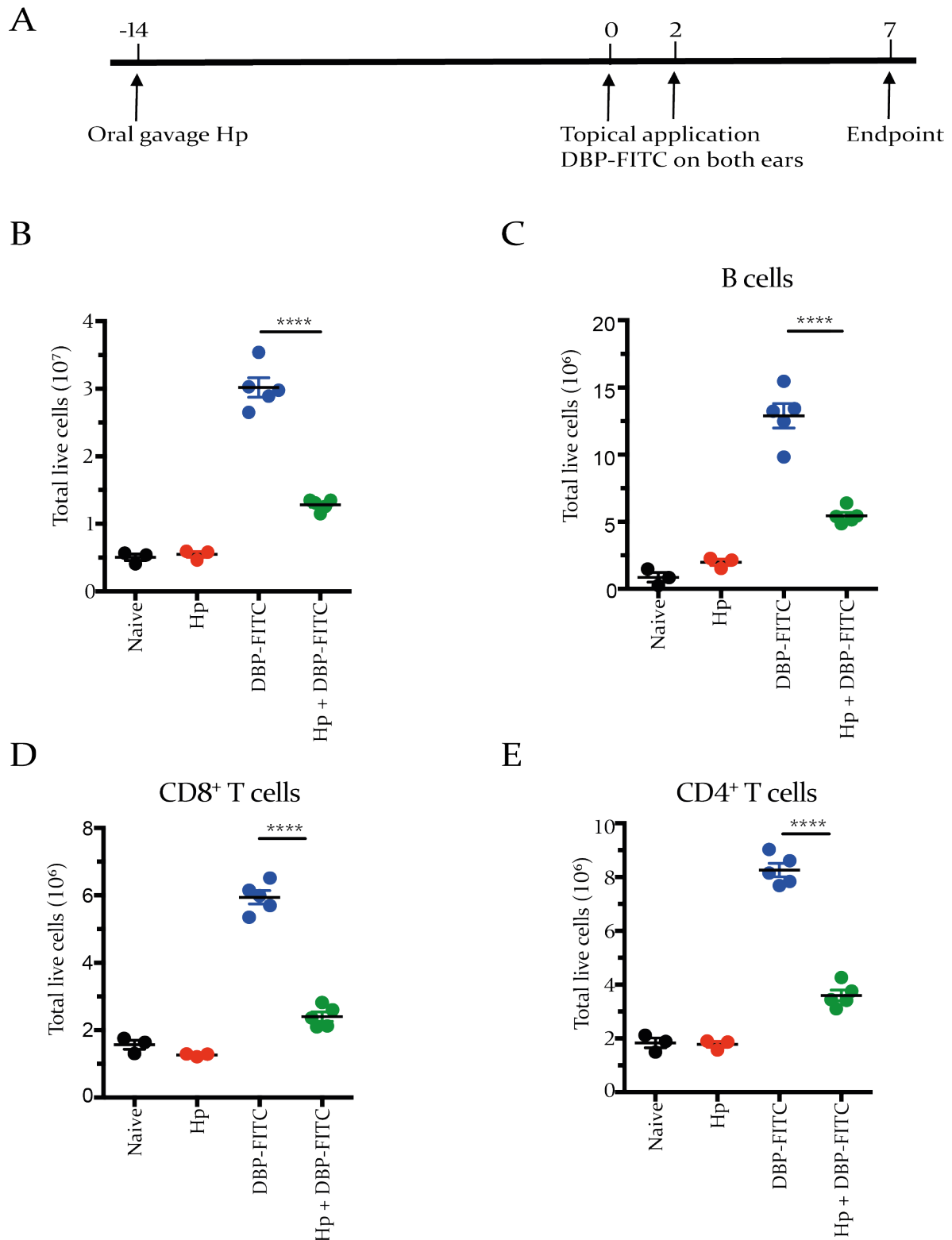
Figure 5.4: *H. polygyrus* **suppresses inflammation in the auricular LNs during the peak of inflammation after DBP-FITC treatment.**

A) Model for *H. polygyrus* infection and treatment with DBP-FITC (numbers depict the day). B) Total live immune cells, C) total live B cells, D) CD8+ T cells, and E) CD4+ T cells present in the auricular LNs. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
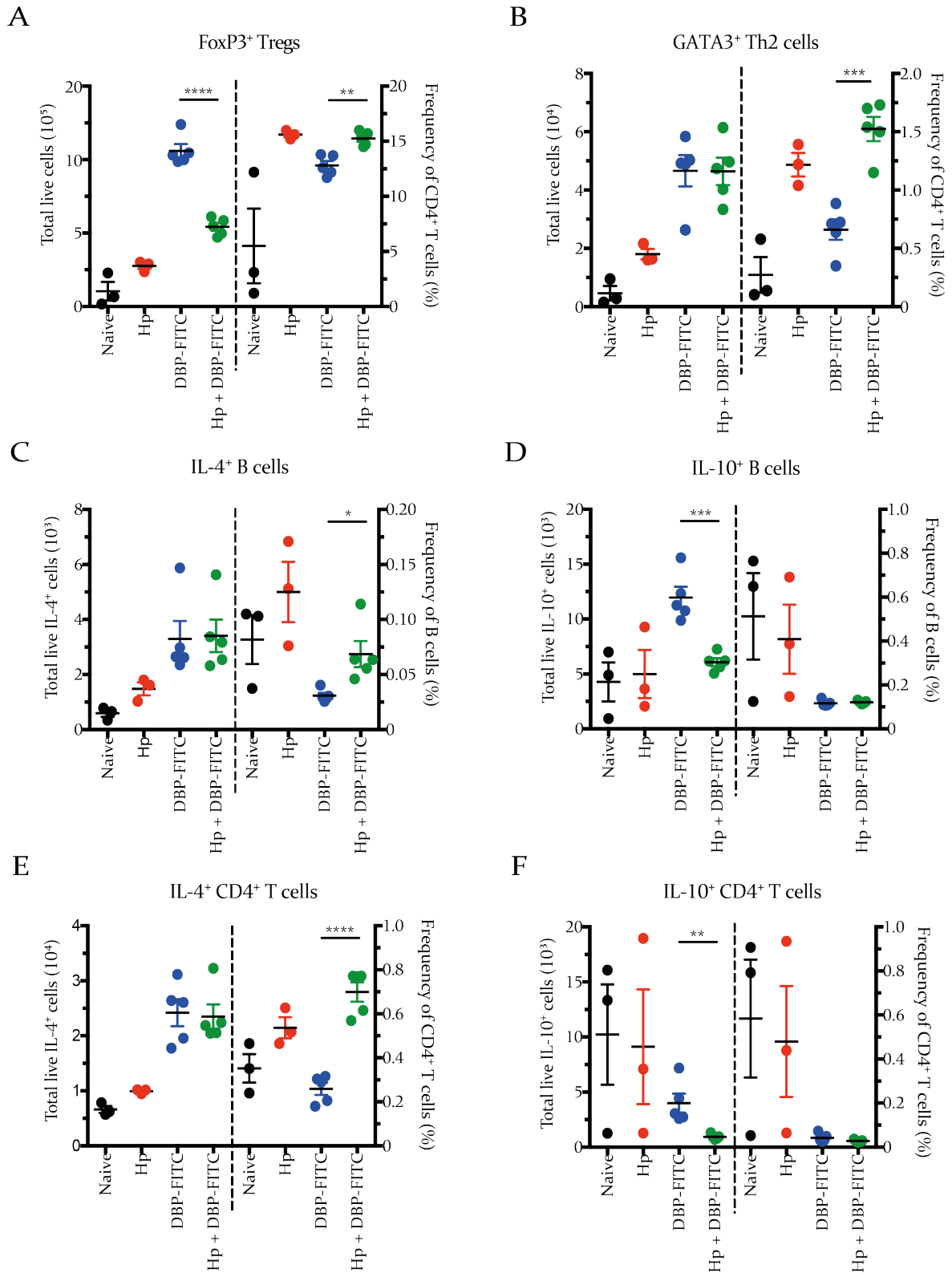
Figure 5.5: *H. polygyrus* **induces Tregs and IL-4⁺ T cells in the auricular LN after DBP-FITC treatment.**

A) Total live FoxP3⁺ Tregs and B) total live GATA3⁺ Th2 cells in the auricular LNs. C) IL-4⁺ and D) IL-10⁺ B cells in the auricular LNs. E) IL-4⁺ and F) IL-10⁺ CD4⁺ T cells in the auricular LNs. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
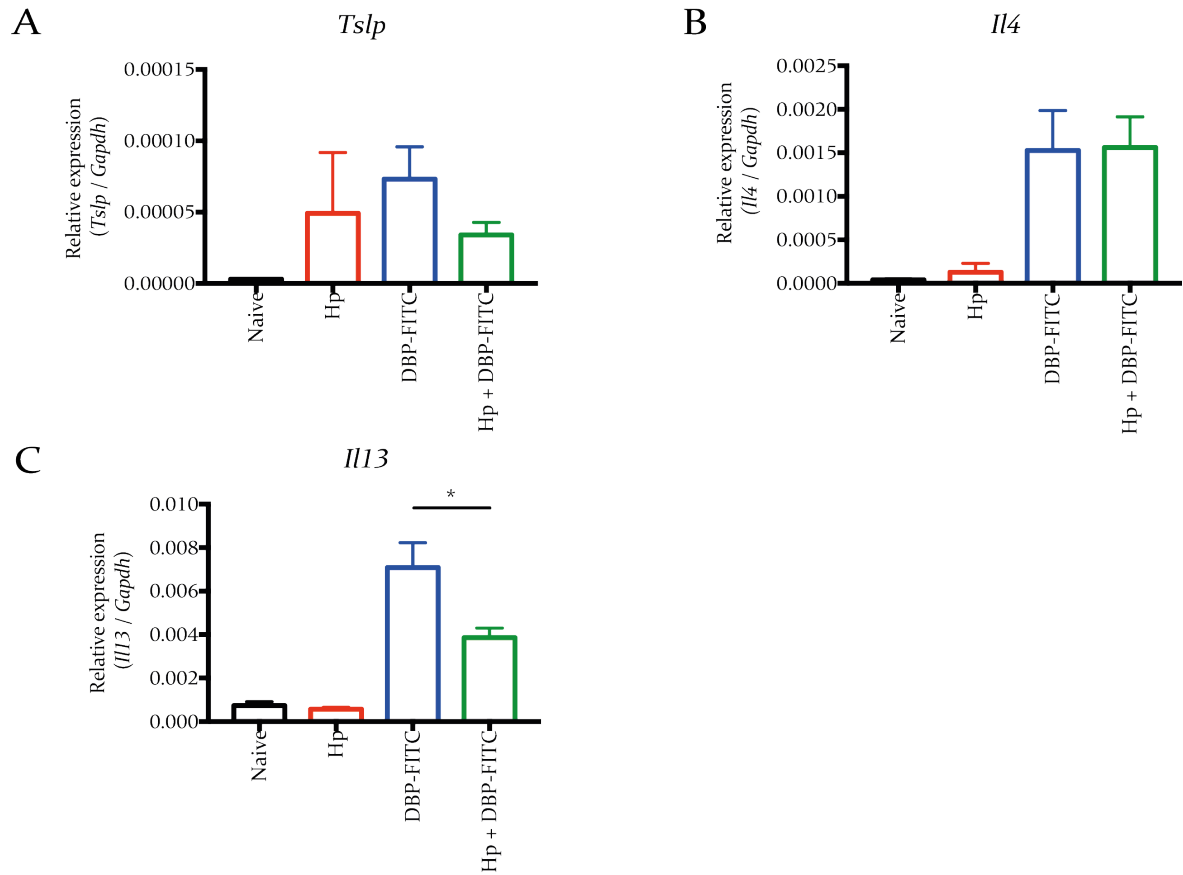
Figure 5.6: *H. polygyrus* **suppresses** *Il13* **during the peak of inflammation.**

Gene expression of A) *Tslp*, B) *Il4*, and C) *Il13* relative to that of *Gapdh*. Graphs show mean ±SEM, statistical significance was determined with a Student's unpaired T-test. * $p < 0.05$.

5.4 Discussion

Previous studies have shown DBP-FITC is a suitable model for AD, and is characterized by keratinocyte secretion of TSLP, which activates CD11b^{+} and TN subsets of dermal DCs, which migrate to the skin-draining LNs and activate Th2 cells.[143, 162, 214] The adaptive arm of the immune system has been shown to be crucial for this model and innate cells in the skin were shown to ameliorate inflammation.[215] To date, there is no data on the effects of helminth infection on DBP-FITC-induced AD.

The results above have demonstrated *H. polygyrus* is able to suppress skin inflammation in the ear tissue using a model of CHS, as ear thickness was significantly reduced during the peak of inflammation. There was no difference in TEWL, suggesting *H. polygyrus* is able to suppress inflammation in this model of AD, while it has no influence on skin barrier function. Histological staining of sections of ear tissue could be analysed to indicate specific immune cell subsets that are affected by infection. Also, analysis of specific molecules known to be involved in AD, such as filaggrin and tight junction proteins, could be undertaken to assess if helminth infection affects expression over the time course of inflammation.[156, 208, 216]

Next, as *H. polygyrus* is known to induce tolerogenic DCs in the gut and block migration of pro-inflammatory DCs to the MLNs, it was hypothesized that *H. polygyrus* might be able to influence DCs in the ear skin tissue as well.[128] Therefore, a model was used consisting of one treatment of DBP-FITC on the ear skin tissue to study migration of different DC subsets. While total live immune cells were significantly suppressed in the auricular LNs by *H. polygyrus* infection, there was no significant reduction in absolute FITC[+] DC numbers in the auricular LNs, and no change in CD86 expression. These results suggest *H. polygyrus* suppresses expansion of immune cells in the auricular LNs at an early time point, but does not alter DC phenotype according to the limited panel of markers used. Culturing these DCs *in vitro* with T cells would demonstrate if DC function is altered by *H. polygyrus*. Furthermore, *H. polygyrus* might block migration of DC into the

auricular LNs, and studying migratory markers could provide evidence for this hypothesis.

Previous results demonstrated Tregs were increased in proportion after HDM-induced Th2 inflammation during the peak of inflammation. A simplified model of DBP-FITC was used to induce Th2 inflammation in the ear skin tissue, and the auricular LNs were analysed during the peak of inflammation (day 7). In accordance with previous results, total live immune cells were significantly reduced in the auricular LNs during the peak of inflammation. While absolute CD4$^+$ T cell numbers were significantly reduced, there was no change in CD4$^+$ T cell proportion. However, within the CD4$^+$ T cell population, there were some significant changes in cell subsets in *H. polygyrus*-infected mice. Both FoxP3$^+$ Treg and GATA3$^+$ T cell proportions were significantly increased, suggesting other T cell subsets might be suppressed to compensate. Further studies including staining for other transcription factors such as Tbet for Th1 cells, and markers to look at activated or naive T cells such as CD44 and CD62L, might provide more information on CD4$^+$ T cell proportions in the LNs in *H. polygyrus*-infected mice.

Isolated immune cells from this experiment were also cultured and stimulated in the presence of Brefeldin A, PMA, and Ionomycin and stained intracellularly for the cytokines IL-4, IL-10, and IL-13. While IL-4$^+$ B cell and T cell proportions (but not absolute numbers) were significantly increased, IL-10$^+$ B cell and T cell numbers (but not proportions) were significantly decreased in *H. polygyrus* infected mice. However, as proportions of IL-4$^+$ and IL-10$^+$ cells in both B cells and T cells were very low (below 1% of B and CD4$^+$ T cells), this difference might not be biologically significant.

Finally, using the ear skin tissue, RNA was extracted for gene expression analysis by qPCR. During the peak of inflammation there was low expression of *Tslp*, and expression was slightly decreased in *H. polygyrus*-infected mice. An earlier time point may be more appropriate to assess *Tslp* gene expression in this model. As *Tslp* is important for the induction of Th2 inflammation in this model, this suggests a decrease of this cytokine could inhibit downstream activation of Th2 cells. The canonical Th2 cytokines *Il4* and *Il13* were both profoundly increased after DBP-FITC

treatment on the ear skin tissue. Earlier results showed IL-4$^+$ Th2 cells were significantly increased in ear skin tissue in *H. polygyrus*-infected mice, qPCR results here demonstrate *Il4* was slightly increased in *H. polygyrus*-infected mice, but does not reach the level of expression DBP-FITC treatment induces. While *Il4* was unchanged in *H. polygyrus*-infected mice, *Il13* was significantly decreased, which suggests *Il13* might be more susceptible to the suppressive effects of *H. polygyrus*. Surprisingly, *Foxp3* expression was significantly reduced in *H. polygyrus*-infected mice, and expression of *Il10* and *Tgfβ* was not significantly altered. These results suggest that genes of regulatory markers might be expressed before the peak of inflammation, and would require further experiments for validation. Also, other mechanisms of suppression by *H. polygyrus* cannot be ruled out.

The results above have demonstrated *H. polygyrus* is able to suppress Th2 inflammation in the ear skin tissue induced by DBP-FITC treatment (i.e. illustrated by reduced ear thickness and a decrease in total live cells in the auricular LNs). While Tregs are increased in the auricular LNs at the peak time, further study is required to validate the importance of Tregs for the suppressive effects of *H. polygyrus*. Depletion of Tregs in *H. polygyrus*-infected mice and adoptive transfer of these Tregs into uninfected mice would further demonstrate the importance of Tregs in suppression of Th2 inflammation in the ear skin tissue.

5.5 Conclusions

Keypoints:

1) *H. polygyrus* suppresses inflammation in the ear skin tissue, but does not seem to alter skin barrier function.

2) DC phenotype is not altered by *H. polygyrus* infection.

3) *H. polygyrus* suppresses inflammation and expansion of lymphocytes in the auricular LNs, coinciding with increased proportions of Tregs and Th2 cells.

4) *H. polygyrus* suppresses *Ill3* expression in the ear skin tissue, and might suppress *Tslp* expression during the peak of inflammation.

5) Further research is required to determine the importance of Tregs for suppression of Th2 inflammation in the ear skin tissue by *H. polygyrus*.

Chapter 6.   Final discussion

6.1 Discussion

Helminths infect over 1 billion people in the world.[22] They cause a variety of symptoms, most importantly for quality of life being anaemia and growth deficiencies, and there are complications that arise when people are co-infected with other microbes such as HIV and Mycobacterium.[31-33] Failure to control co-infections is often due to the skewing from a Th1/Th17-mediated immune response needed for protection against many microbes, to a Th2-mediated immune response elicited by helminths, leading to impaired anti-viral and anti-bacterial immunity.[38] For these reasons, the eradication of helminths would be desirable in developing countries. However, an inverse correlation is very often found between immune disorders and helminth infections; in Western countries where people are not chronically infected with helminths autoimmune and allergic disorders are an increasing problem, while people in developing countries, where helminth infection is endemic, have a reduced risk of developing autoimmune or allergic diseases.[2, 4, 9, 12] This inverse correlation has led to the proposal of the hygiene hypothesis, which further states that early exposure to environmental allergens could also protect against the development of atopy in humans.[2, 4]

Helminths induce a strong immune response in the host mediated by Th2 cells, innate cells such as eosinophils, ILC2s, AAMs and mast cells, and Th2 cytokines IL-4, IL-5, IL-9 and IL-13. Due to many millennia of parasite:host co-evolution, it is believed the Th2 response has evolved to expel or contain helminths, while also repairing and limiting any parasite- and immune-mediated tissue damage.[24, 51] However, helminths are highly adapted to the host immune system and have been shown to induce regulatory mechanisms that suppress inflammation and limit tissue damage, ensuring prolonged survival and reproduction of the worm, but also survival of the host.[15, 24] This induction of regulatory mechanisms by helminths was also found to be protective against allergic and autoimmune diseases and currently clinical trials have found the pig whipworm *T. suis* and human hookworm *N. americanus* to be safe and effective in suppressing symptoms in IBD such as Crohn's disease, and MS.[18-21]

H. polygyrus is a gut-dwelling natural mouse parasite, and is highly adapted to the mouse immune system, making it the preferred model to study chronic helminth infection in mice. *H. polygyrus* secretes several hundred different molecules into its host, collectively termed HES.[94, 95, 217] These molecules are believed to be the underlying factors responsible for the induction of regulatory immune mechanisms, with HES alone being enough to suppress Th2 allergic inflammation, without live worm infection.[13, 15, 123] Several studies have focused on the content and function of HES, to isolate the components responsible for the worm's suppressive effects.[15, 94, 95]

Regulatory mechanisms induced by *H. polygyrus* include cells such as Tregs, Bregs and AAMs, and the secretion of anti-inflammatory cytokines, e.g. IL-10 and TGFβ (figure 1.3).[13, 14, 113, 218] Bregs are increased in the gut after infection with *H. polygyrus* and, Tregs are also induced in the lung suppressing HDM-induced lung inflammation.[13, 14] Adoptive transfer of these cells to uninfected mice transfers protection against Th2-mediated lung inflammation, while depletion of Tregs abolishes protection, suggesting Tregs are central to this helminth-induced immune suppression.[13, 14]

While *H. polygyrus* was found to suppress the adaptive arm of the immune system through inducing a tolerogenic phenotype of CD11c^{lo} DCs, which were capable of inducing Tregs and IL-10 production, while blocking migration of pro-inflammatory CD11c^{hi} DCs to the MLN.[127, 128] Furthermore, *H. polygyrus* was also able to inhibit the innate arm of the immune system in the lungs through suppression of IL-33 release from epithelial cells, which blocked expansion of ILC2s, and as ILC2s are the main source of IL-5 and IL-13 in several models of lung inflammation, recruitment and activation of downstream effector cells such as eosinophils were also suppressed.[123]

While several helminths have been shown to be protective against Th2-mediated inflammation in the lungs and guts, there is limited knowledge of the effect of helminth infections during skin allergy, and this area is in need of supplementation. AD is a chronic/chronically relapsing allergic disorder, characterized by increased TSLP production,[152, 213] and impaired skin barrier function,[173] leading to Th2-mediated inflammation and possible IgE sensitization to other allergens,[170] leading

to the allergic march.[175] The skin has been shown to be essential for the sensitization of allergens and initiation of the allergic march,[174] and the prevention of the onset of AD would also be able to prevent the onset of other allergies later in life. With this in mind, the overall aim of this project was to study if the gut dwelling mouse parasite *H. polygyrus* was able to suppress Th2-mediated inflammation in the skin tissue.

H. polygyrus infection induces IL-4$^+$ Th2 cells in the blood and various organs including the spleen and lungs.[179] However, there was no data included in previous studies on whether this also occurred in the skin tissue. Over-expression of IL-4 changes steady-state conditions in the skin, resulting in delayed wound healing, mast cell accumulation and keratinocyte hyperproliferaton.[166, 180] However, IL-4 by itself cannot induce AD.[180] Furthermore, IL-4 production in the skin tissue is sufficient for the induction of AAMs, which can secrete anti-inflammatory cytokines IL-10 and TGFβ. Another canonical Th2 cytokine IL-13 is constitutively expressed in the skin by ILC2s, and is important for the maintenance of the skin barrier and for wound repair.[149, 181] Therefore, I used 4C13R mice to study the effect of *H. polygyrus* infection on expression of canonical Th2 cytokines IL-4 and IL-13, in several tissues including the skin tissue.

Confirming research by Mohrs *et al.*[179], I found there was an induction of IL-4$^+$ T cells in tissues distal to the gut, including in the ear skin tissue (chapter 3.3). IL-4 was also produced by innate cells such as eosinophils and macrophages, although to a lesser degree. Previous studies showed that rapid production of IL-4 after tissue injury by innate cells is sufficient for the induction of AAMs, although IL-4 from Th2 cells is necessary for the maintenance and full activation of AAMs.[183] Therefore, importantly my results also suggest that the stimulus for induction and maintenance of AAMs is present in the skin of infected mice, which could lead to suppression of skin inflammation. Further studies are needed to determine if AAMs are induced in the skin after infection with *H. polygyrus* and if they are producing anti-inflammatory cytokines IL-10 and TGFβ, which could lead to induction of Tregs and further suppression of inflammation.

The first model I used to induce Th2 inflammation in the ear skin tissue utilised ID injection of whole mashed-up HDM.[200] HDM induces strong Th2-mediated inflammation through activation of TLRs, CLRs and NLRs, but in this model HDM does not induce TSLP expression by keratinocytes.[143, 190-193] I found immune cells including (IL-4 producing) T cells, B cells, eosinophils, and neutrophils were present after HDM administration, with the peak of cellular inflammation at day 7 after injection (chapter 4.3.1).

I observed *H. polygyrus* suppressed HDM-induced Th2 inflammation in the ear draining LNs and ear skin tissue, coinciding with an increased proportion of Tregs in both sites (chapter 4.3.2 and 4.3.3). Furthermore, *H. polygyrus* induced AAMs in the ear skin tissue above naive levels, although no difference was seen between uninfected and *H. polygyrus*-infected mice after HDM injection. My results suggest suppression is likely to be mediated by Tregs, which coincides with previous studies showing *H. polygyrus* suppressed HDM-induced lung inflammation.[13] Future experiments could utilise depletion of Tregs in *H. polygyrus*-infected mice or adoptively transferring them to uninfected mice to investigate if protection against HDM-induced Th2 inflammation in the ear skin tissue is indeed dependent on Tregs. Furthermore, a larger sample size would be necessary to prove if AAMs are induced and play a role in this model.

The second model I used to induce Th2 inflammation in the ear skin tissue was the topical application of DBP-FITC. DBP-FITC-induced CHS is initiated through the production of TSLP by keratinocytes.[162, 212, 214] This model has been shown to be dependent on Th2 cells activated by DCs, with innate cells such as mast cells and eosinophils in the ear skin tissue enhance inflammation.[215] My results have demonstrated that *H. polygyrus* was suppressive in this model of DBP-FITC-induced CHS with infected mice having reduced ear thickness after challenge with DBP-FITC on the ear skin tissue (chapter 5.3.1). I found no significant difference in TEWL however, indicating *H. polygyrus* suppresses ear inflammation, but does not affect skin barrier function in this model. Future experiments should include the further study of inflammation in the ear skin tissue by analysing the infiltration of different

immune cells in to the skin, and skin barrier integrity by histology and real-time PCR.

H. polygyrus induces a phenotype of tolerogenic CD11c^{lo} DCs in the gut, which are able to induce Tregs, and block migration of pro-inflammatory CD11c^{hi} DCs to the LNs.[128] As DCs are important for the initiation of Th2 inflammation in this model, I hypothesized *H. polygyrus* might be able to induce suppression of the adaptive arm through blocking DC migration to the LNs, or inducing a tolerogenic phenotype. My results indicated that CD11b^{+} DCs and TN DCs expanded most after DBP-FITC treatment and had the highest proportion of FITC^{+} DCs, i.e. DCs activated by DBP-FITC, coinciding with results found by Ochiai *et al.*[143] At this early time point, I observed *H. polygyrus* suppressed expansion of immune cells in the ear draining LNs, although the absolute DC numbers were only slightly decreased and this difference did not reach significance (chapter 5.3.2). Furthermore, there was no change in CD86 expression on any of the DC subsets, indicating that *H. polygyrus* does not affect the activation of DCs. Further studies would be needed to characterize if and how DC phenotype and surface molecule expression are affected by *H. polygyrus* infection in this DBP-FITC model. I propose further experiments, to study differentiation of T cells (e.g. induction of Tregs) using sorted DCs after activation by DBP-FITC in an *in vitro* culture with T cells, which would help to determine if tolerogenic DCs are induced by *H. polygyrus*.[127, 128] To assess if DC migration from the skin to the draining LNs might be blocked by *H. polygyrus* and therefore affect initiation of the inflammatory reaction, as was the case in previous studies, migration markers such as chemokine receptor 7 (CCR7) on their surface and chemokine expression patterns in the skin and LN could be examined.[219]

Next, I analysed lymphocytes in the auricular LNs for cytokine expression, and for the proportions of Tregs and Th2 cells. My results indicated that *H. polygyrus* suppressed inflammation in the ear draining LNs during the peak of inflammation (day 7) as lymphocyte expansion was significantly decreased (chapter 5.3.3). Within CD4^{+} T cells, FoxP3^{+} Treg and GATA3^{+} Th2 cell proportions were significantly increased in the ear draining LNs after *H. polygyrus* infection, suggesting proportions of other subsets of T cells (such as Tbet^{+} Th1 cells) might be decreased

to compensate. As with the HDM model, further studies would be needed to demonstrate the importance of Tregs for the suppression of Th2 inflammation.

Finally, I analysed cytokine expression in the ear skin tissue via qPCR. TSLP has been demonstrated to be a key cytokine in the initiation of Th2-mediated inflammation in this model, and to be upregulated in patients with AD.[213, 214] While I found no profound expression of *Tslp* during the peak of DBP-FITC-induced inflammation, *H. polygyrus*-infected mice did seem to have decreased expression of this cytokine. My results suggest TSLP is only minimally present at day 7 after DBP-FITC treatment (the peak of cellular inflammation), and an earlier time point (e.g. day 1 or day 2) would be more suitable to study the gene expression of *Tslp* and the effect of *H. polygyrus* on this cytokine. My results earlier demonstrated *H. polygyrus* increased IL-4$^+$ T cells in the ear skin tissue, but did not significantly increase IL-13 production (chapter 3.3). I observed a considerable increase in *Il4* and *Il13* gene expression after DBP-FITC treatment, and *H. polygyrus* caused a significant decrease in *Il13* expression (chapter 5.3.4). I detected no difference in *Il4* expression. My results suggest that *Il13* gene expression could be more susceptible to the suppressive effects of *H. polygyrus*, than that of *Il4*. It would be interesting to investigate in which cells this decrease in *Il13* expression is occurring (e.g. Th2 cells or ILC2s). Notably, I detected there was a significant reduction of *Foxp3* expression in *H. polygyrus*-infected mice compared to uninfected mice after DBP-FITC treatment, and expression of *Il10* and *Tgfβ* was slightly, but not significantly, decreased. As *H. polygyrus* suppresses skin inflammation as seen by decreased ear thickness and total live immune cells in the ear draining LNs, it is possible an earlier time point might show an increased expression of these regulatory markers, and would require further experiments for validation. Investigation into alternative mechanisms for immune suppression by *H. polygyrus* could also be considered.

6.2 Future directions

I propose further research will be needed to determine the effects of *H. polygyrus* on the ear skin tissue using the more physiologically relevant DBP-FITC model of AD. While ear thickness is reduced, it is not known what immune cells specifically are susceptible to the suppressive effects of *H. polygyrus* in the skin. Future studies I have planned using this model include analysis of T cells, including GATA3^{+} Th2 cells and FoxP3^{+} Tregs, in the ear skin tissue by histology, as Th2 cells in the skin tissue are a source of various Th2 cytokines, and essential in this model.[215] The presence and function of innate cells including eosinophils, mast cells and ILC2s could also be analysed by histology by also looking at cytokine production or degranulation in the ear skin tissue.

Other regulatory cells are also induced by *H. polygyrus* infection. A population of regulatory CD23^{hi} B cells were induced in the guts after *H. polygyrus* infection, and were transferred to uninfected mice to suppress allergic airway inflammation.[14] It would be interesting to investigate if this population is found in the auricular LNs of *H. polygyrus*-infected mice after DBP-FITC treatment.

AAMs were systemically induced in *H. polygyrus*-infected mice, and suppressed development of T1D in NOD mice.[113, 114] While the induction of AAMs by *H. polygyrus* have not been studied in the skin, results from the HDM model indicated they might be induced in the ear skin tissue after *H. polygyrus*-infection, making this population interesting to study further. I suggest analysis by qPCR and histology could provide more information on the presence and cytokine production by AAMs in the ear skin tissue after DBP-FITC treatment.

Research is ongoing in other laboratories into the molecular basis of helminth-induced suppression. To complement the studies done here using live helminth infection, I propose that experiments utilising the secreted molecules (HES) of *H. polygyrus* may allow further understanding of the mechanisms helminths use to downregulate immune responses.

6.3 Final conclusions

My results outlined in this thesis demonstrate a potential therapeutic use for helminths, or their products, to suppress allergic diseases in the skin, and possibly inhibit the onset of the allergic march. Considering the ever-increasing burden of allergic diseases in New Zealand, and the wider world, this area of research is important and timely.[220, 221]

To summarize, I have demonstrated:

1) *H. polygyrus* infection significantly increased the proportion of IL-4 producing CD4$^+$ T cells in various tissues of mice, including the skin tissue.

2) Both allergy models of HDM-induced and DBP-FITC-induced Th2 inflammation were suppressed by *H. polygyrus* infection, characterized by significantly decreased ear thickness after DBP-FITC-induced CHS, and significantly decreased total live immune cell numbers in the auricular LNs in both allergy models.

3) This suppression by *H. polygyrus* infection coincided with an increased Treg proportion, and further experiments I have planned include depletion of Tregs to determine the importance of these regulatory cells in the suppression afforded by *H. polygyrus*.

4) *H. polygyrus* infection significantly decreased lymphocyte numbers (B cells, CD4$^+$ T cells, and CD8$^+$ T cells) in both allergy models, and increased GATA3$^+$ Th2 cell proportions in the auricular LNs in the DBP-FITC model. Furthermore, *Il13* expression was significantly decreased in the ear skin tissue during the peak of inflammation (day 7) after treatment with DBP-FITC.

Supplemental figures
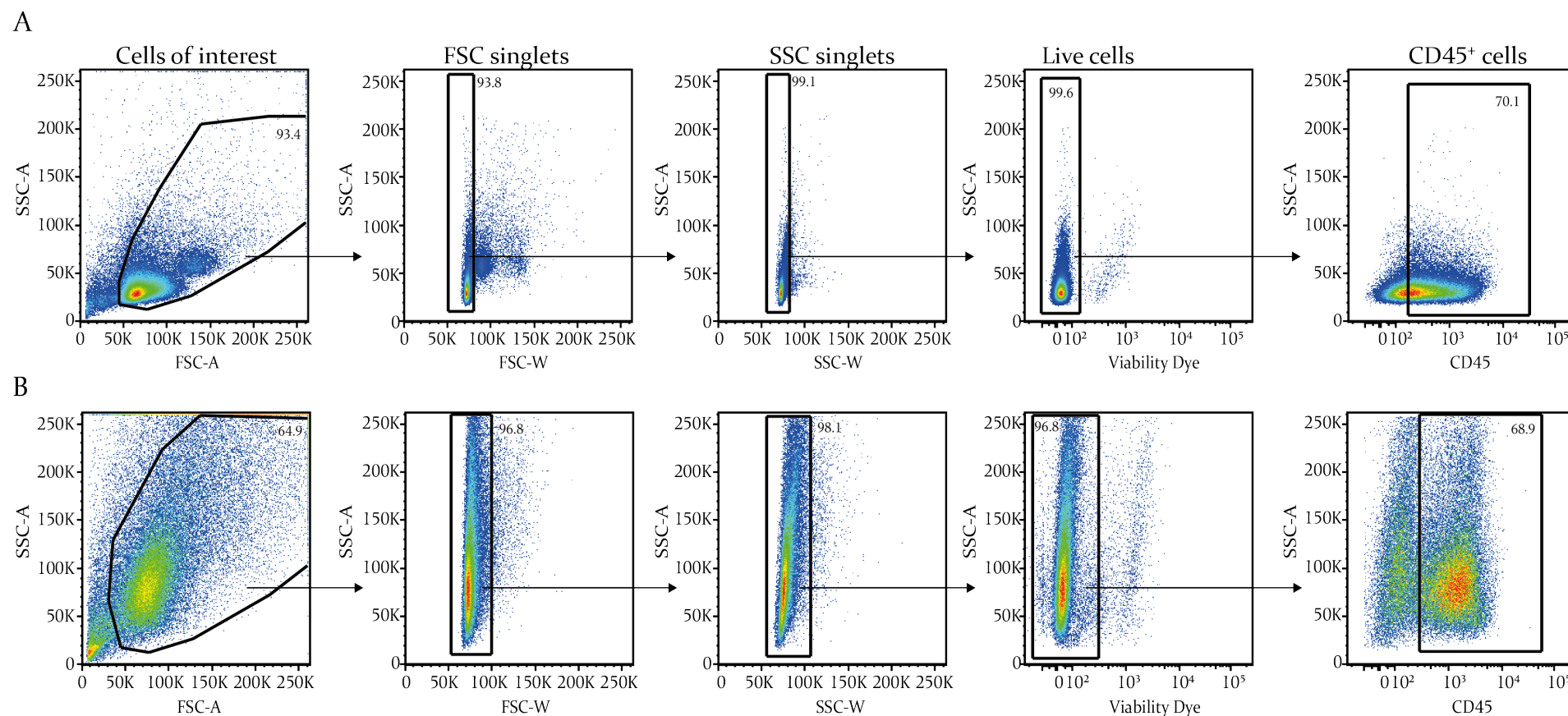
Supplemental Figure 1: Gating strategy for LN and ear tissue

Gating strategy used to identify CD45^{+} immune cells for cells isolated from A) LN or blood, and B) skin tissue or lung tissue
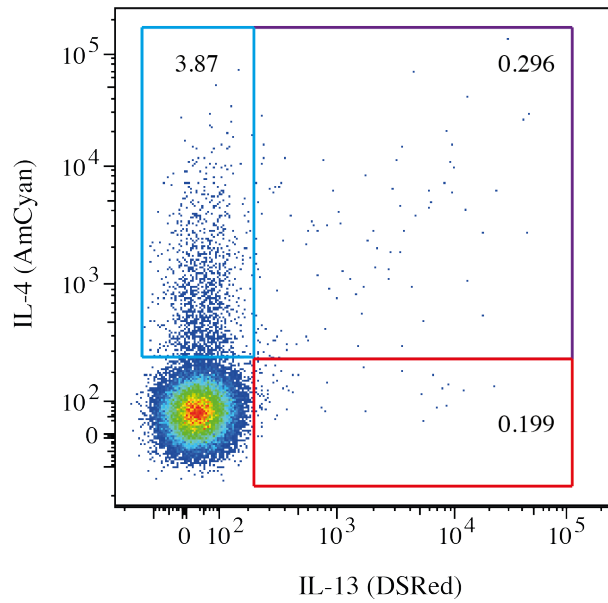
Supplemental Figure 2: Gating strategy for IL-4 and IL-13 expression in 4C13R reporter mice

Gating strategy used to identify IL-4 and IL-13 expression, by detection of the reporters AmCyan and DSRed respectively, in various immune cell populations. Blue gate: AmCyan[+] IL-4 producing cells; red gate: DSRed[+] IL-13 producing cells; purple gate: IL-4 and IL-13 double-producing cells.

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