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Modulation of Fetomaternal Crosstalk through Chronic Helminth Infection: Sustained Alterations to T Cell Responses and DC Functionality

Matthew John James Lacorcia

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Vorsitz: Prof. Dr. Jürgen Ruland

Prüfende/-r der Dissertation:

1. apl. Prof. Clarissa Prazeres da Costa

2. Prof. Dr. Dietmar Zehn

3. Prof. Dr. Roland Lang

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Abstract

Prenatal exposure to infections can impact the development of the immune system. Maternal infection and other environmental disturbances in early life can play a decisive role in educating the growing immune system, predisposing susceptibility towards inflammatory disorders and training future immune responses. Infection with the helminth *Schistosoma mansoni* has demonstrated effects of bystander immunity that modify responsiveness, driving immune heterogeneity in terms of co-infection, allergy and atopic disease, and vaccine efficacy in endemic populations. Early life exposure to maternal schistosomiasis, with no direct transmission of infection, can result in transmaternal priming effects on immune responses to bystander antigenic challenges, with studies in humans showing interactions with susceptibility to atopic skin disorders and indications of modified vaccine efficacy. Murine models have clarified a role for maternal schistosomiasis in sensitivity to experimental asthma, with modulations that hint at widespread immunomodulation to bystander antigens as well as cognate exposure to schistosomes. The work contained in this thesis provided a thorough examination of the effects of chronic maternal schistosomiasis in a closed murine experimental system. Initial findings clarify that suppressed allergic airway inflammation in helminth-exposed offspring is associated with modified lung CD4+ inflammatory responses, which subsequently indicate altered T and B cell responses to allergic sensitization. We also identified altered activation within the APC compartments, with the suggestion of a skewed IL-4/B-cell-dominant response to antigenic challenge and also present in the steady state, including increased activation of B cells, persistence of anti-parasite antibody titres, and of offspring-derived IL-4-producing CD44+ memory CD4+ T cells. Further investigation of the relationship of this skewing to vaccine efficacy revealed differential CD4+ responses, and alterations in induction of CD8+ T cell responses during immunization, which are modulated particularly dependent upon vaccine formulation. The modified CD8+ responses of schistosome-exposed offspring had a functional impact upon the efficacy of vaccination against viral infection in a murine Hepatitis B virus model.

Although priming differences were identified in the CD4+ memory T cell compartment, the CD44- naïve CD4+ CD8+ responses did not show functional nor transcriptional differences after removal of memory cells, supporting the concept of maternal priming-induced changes to pathways that activated and regulate T cell responses, rather than developmental priming of the naïve, antigen-inexperienced T cell pool. Through adoptive transfer of transgenic T cells, in vitro priming with offspring-dervied APCs, and steady state analysis, this study reveals an altered dendritic cell phenotype in schistosome-primed offspring, sustained into adulthood. These modified DCs serve as a link between altered T cell responsiveness to heterologous antigens and the multilayered regulatory network of immune processes primed via fetomaternal crosstalk during helminth infection.

Zusammenfassung:

Der pränatale Kontakt mit Infektionen kann die Entwicklung des Immunsystems des Nachwuchses beeinflussen. Dabei kann die Anfälligkeit für entzündliche Erkrankungen erhöht, sowie zukünftige Immunantworten verändert werden. Die Infektion mit dem Helminthen *Schistosoma mansoni* kann die Immunität gegenüber Körper-fremden Stimulationen verändern und die Reaktionsfähigkeit modifizieren, was die Immunheterogenität auf Ko-infektionen, Allergien und atopische Erkrankungen und die Wirksamkeit von Impfstoffen in endemischen Populationen fördern. Eine frühe mütterliche Bilharziose-Infektion, ohne direkte Übertragung der Würmer, kann somit zu transmütterlichen Priming-Effekten führen, die die Immunantwort auf fremde Antigen-Herausforderungen modifizieren. Vorangegangene Studien konnten zeigen, dass Priming-Effekte die Anfälligkeit für atopische Hauterkrankungen erhöhen, sowie die Impfstoffwirksamkeit verändern können. In Mausmodellen konnte gezeigt werden, dass eine mütterliche Schistosomiasis-Erkrankung die Anfälligkeit für die Entwicklung von experimentellem Asthma in den Nachkommen verringern kann. Die damit verbundenen Modulationen zeigten zudem breitflächige Immunmodulationen auf körperfremden Antigenen sowie auf eine verminderte Reaktion auf die erneute Infektion mit Schistosoma mansoni. In dieser Arbeit wurden die Auswirkungen einer chronischen mütterlichen Schistosomiasis in einem geschlossenen experimentellen System bei Mäusen eingehend untersucht. Erste Ergebnisse verdeutlichen, dass eine unterdrückte allergische Atemwegsentzündung bei Helminthen-exponierten Nachkommen mit veränderten CD4+-Entzündungsreaktionen in der Lunge einhergeht, die entsprechend auf veränderte T-und B-Zellen-Reaktionen auf allergische Sensibilisierung sowie auf eine veränderte Aktivierung innerhalb der APC-Kompartimente hinweisen. Dabei gibt es Hinweise auf eine korrelierende IL-4/B-Zell-dominanten Antwort auf die Immun-Challenge mit dem Antigen, die auch im Steady-State vorhanden ist, einschließlich erhöhter Aktivierung von B-Zellen, Persistenz von Anti-Parasiten-Antikörpertitern und von IL-4-produzierenden CD44+ Gedächtnis-CD4+-T-Zellen der Nachkommen. Die weitere Untersuchung des Zusammenhangs dieser Schieflage mit der Wirksamkeit des Impfstoffs ergab differentielle CD4+-Antworten und eine veränderte Induktion von CD8+-T-Zell-

Antworten während der Immunisierung, die insbesondere in Abhängigkeit von der Impfstoffformulierung moduliert werden. Die veränderten CD8+-Antworten von Schistosomen-exponierten Nachkommen hatten einen funktionellen Einfluss auf die Wirksamkeit der Impfung gegenüber viralen Infektionen in einem Maus-Hepatitis-B-Virus-Modell.

Obwohl Priming-Unterschiede im CD4+ Gedächtnis-T-Zell-Kompartiment identifiziert wurden, zeigten die CD44- naiven CD4+ CD8+ Antworten weder funktionelle noch transkriptionelle Unterschiede nach Entfernung der Gedächtniszellen. Das zeigt, dass mütterlich induziertes Priming zu einer Veränderung der Signalwege führt, wodurch es zur Aktivierung und Regulierung von T-Zell-Antworten kommt. Zugleich beweist es, dass nicht das entwicklungsbedingte Priming des naiven, Antigen-unerfahrenen T-Zell-Pools dafür verantwortlich ist. Durch den Transfer von transgenen T-Zellen, in vitro Priming mit APCs, die vom Nachkommen stammen, und Steady-State-Analyse zeigt diese Studie einen veränderten Phänotyp dendritischer Zellen in Schistosomen-geprimten Nachkommen, der bis ins Erwachsenenalter erhalten bleibt. Diese veränderten DCs dienen als Bindeglied zwischen der veränderten T-Zellen-Ansprechbarkeit auf heterogene Antigene und dem vielschichtigen regulatorischen Netzwerk von Immunprozessen, die durch den Mutter-Kind Crosstalk im Uterus während der Helmintheninfektion ausgelöst werden.

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2. Abbreviations and Glossary

AAI, allergic airway inflammation

APCs, antigen presenting cells

BAL, bronchoalveolar Lavage

BFA, Brefeldin A

BMDC, bone marrow-derived dendritic cells

ChIP, chromatin immunoprecipitation

CFA, Complete Freund's Adjuvant

cDC, conventional dendritic cell

DC, dendritic cell

DGE, differential gene expression

EMA, ethidium monoazide bromide

HBcAg, Hepatitis B core antigen

HBsAg, Hepatitis B surface antigen

igLN, inguinal lymph nodes

i.p, intraperitoneal

i.v, intravenous

MDS, multidimensional scaling

MVA, Modified Vaccinia virus Ankara

s.c, subcutaneous

Tfh, follicular helper T cells

OVA, ovalbumin

PMA, phorbol 12-myristate 13-acetate

1. INTRODUCTION

1.1 Developmental Origins of Skewed Immune Responses

1.1.1 Project Overview and Goals

Environmental cues are increasingly recognised as driving significant physiological deviations to the proper functioning of the immune system. Changes to what has been termed the "exposome", the sum of environmental factors facing an organism, can impact the immune system at any age. However, the period of early life, including the important "first 1000 days" in humans, has been designated as a pivotal "window of opportunity" for effecting profound change to immune development, including the seeding of effects driving non-communicable diseases (such as inflammatory conditions), or conversely healthy maturation associated with appropriate responses in later life (as discussed by Schaub and Prescott in (Wahn and Sampson 2016)). Environmental factors during early life, such as maternal and paternal stress, diet, and other practices (such as antibiotic usage) that effect microbiota, have demonstrated impact on the development of conditions including immune disorders, allergy, as well as neurodevelopmental disorders such as autism and schizophrenia (Eshraghi et al. 2018) (Macpherson, de Aguero, and Ganal-Vonarburg 2017). It is during early life that developing body systems are highly vulnerable to environmental changes, and disturbances, or even lack of sufficient stimuli at these times may shift the predisposition towards disease states.

Maternal health during pregnancy has been shown to have far-reaching effects upon future capacity to effectively manage infectious challenges and other heterogeneity within immune responses, such as appropriate inflammatory responses. Prenatal exposure to infections, from viral (Choi et al. 2016), bacteria (Kyburz et al. 2018), and parasitic agents (Straubinger et al. 2014), as well as other stimuli such as stress and pollution (Bolton et al. 2013), can modify the developing foetal immune system. Maternal dietary changes (particularly in association with changes to microbiome and metabolite profile) (Thorburn et al. 2015) are also linked to immune heterogeneity of differential inflammatory outcomes observed between individuals later in life. Aside from the obvious context of infection during pregnancy,

these effects, observed in the presence or absence of such agents, also point to regulatory switches that underlie proper versus dysregulated immune development and functioning.

Theories on the origins of immune disease, including allergy and inflammatory disorders, point to these conditions as dysregulated states involving immune pathways that have been otherwise selected through evolution under the pressures of immune challenge. This thesis addresses this relationship using a murine model of enhanced early life immune exposure. Here, chronic maternal parasite infection, previously documented to modify allergic susceptibility in murine offspring, is revealed to drive persistent immune priming effects that modify immune responses to challenge in later life. This thesis explores the cellular and molecular networks involved in this shift, outlining points of intervention towards for generating shifts in immune predisposition.

1.1.2 Background and Scope of Thesis

The link between helminthic worms and allergy has become a major element of the hygiene hypothesis for over two decades (Yazdanbakhsh, Kremsner, and van Ree 2002). The body reacts to these parasites with type 2 immune responses, including antibody-mediated and granulocytic effector functions to induce clearance of these extracellular foreign bodies. Similar processes direct the clearing of toxins such as snake or bee venom, IgE induction and associated mast cell degranulation (Metz et al. 2006). Type 2 responses, which target extracellular parasites and toxic molecules, can be contrasted to major type 1 (directed against intracellular microbes and viruses) and type 3 responses (directed against extracellular microbial or fungal agents respectively) (the distinctions between all three are reviewed in (Annunziato, Romagnani, and Romagnani 2015)). To mask their presence, evade host defence, and permit mutual long-term survival of host, parasites manipulate these responses and further induce supplementary anti-inflammatory responses, skewing the immune response towards a regulatory character that supports chronic infection. The host defences involved in the clearance of such parasites share parallels with those type 2 inflammatory processes that underlie allergic responses. This is not by chance and instead may be intrinsically linked, in the sense that these responses evolved under the pressure of parasites and toxins, with potential allergens being otherwise harmless molecules that

through context of exposure, or particular molecular features (such as proteolytic activity or binding to pattern recognition receptors, and therefore providing similar signals as per venoms or parasites), are able to trigger such immune "clearance" responses in susceptible individuals.

Allergic susceptibility itself has been theorised to stem from multiple factors, but it is well documented that environmental cues can play a major role aside from genetic factors (von Mutius 2016). The "old friends" aspect of the hygiene hypothesis contends that such susceptibility is increased in the absence of frequent exposure to such infectious agents. Such unexposed individuals may be missing the regulatory feedback loops induced through management of infection, as one aspect of the changes that human populations have undergone during processes of industrialisation. The resulting hyperresponsiveness to inflammatory or allergic stimuli enhances negative immune outcomes compared to those with immune systems balanced by an appropriate amount of "educative" pre-exposures.

The work in this thesis uses a murine system of developmental exposure to chronic maternal infection with the parasitic helminth *Schistosoma mansoni* to explore how early life immune stimuli modify priming of the immune system to alter inflammatory outcomes, specifically as relate to activation of antigen-specific T cell responses. Exploring the immunological priming effects of maternal helminth infection during pregnancy, this study investigates transgenerational imprinting effects upon $CD8^+$ as well as $CD4^+$ T cell functionality, using models that expand on the antigen-specific priming events that underlie not only allergic sensitisation and inflammation, but also vaccination and effective management of infection. Mechanistically, this thesis then profiles the cellular changes involved in determining what ultimately presents as a shifted regulatory network, across distinct immune compartments including T cells, B cells, and APCs including dendritic cells, that contribute to an altered immune phenotype sustained into adulthood. The subsequent changes to activation of T cell responses and their polarisation in terms of cytokine production is linked to inflammatory outcomes in allergy, vaccination, and infection.

Current paradigms in clinical approaches to treating allergic and inflammatory disorders largely rely upon immunomodulators that are essentially drug-based anti-inflammatory in nature. This treatment relies on direct delivery of immunosuppressive agents is therefore often very short lived or requires

perpetual treatment. Long-established interventions include glucocorticoids, including cortisone, prednisolone and dexamethasone (Stahn et al. 2007), still in widespread usage today, and recently shown to have a marked impact in reducing the inflammatory sequelae of viral infection stemming from acute infection with COVID-19 (Theoharides and Conti 2020).

More recent developments in this field include JAK/STAT inhibitors, including ruxolitinib and tofactinib (as reviewed in (Damsky and King 2017)), as well as monoclonal antibodies against specific immune molecules, such as cytokines or their receptors, to block their effects. The selection of these agents allows for a narrowed focus on which inflammatory pathways are inhibited, but still rely on direct drug-mediated immunosuppression, with the likelihood of return to disease states upon cessation, alongside potentially severe consequences of long-term usage.

This thesis instead focuses on the ways prior exposure can induce regulatory pathways that down modulate subsequent immune challenge, through priming-based induction of enhanced regulatory circuits. These therefore serve as methods of inducing immunoregulation through exogenous exposure that serve as alternatives to drug-induced immunosuppression. The potential of environmentally manipulating these pathways has been seen in work on tolerogenic immunotherapy, through induction of regulatory responses through antigen-specific exposure (Palomares et al. 2017). What is a particularly novel about the work presented in this current thesis, however, is that immunomodulation driven by maternal schistosomiasis is able to drive bystander immune modulation that does not require prior specific antigen exposure. This is consistent with previous research on the effects of maternal and early life exposures to particularly microbial agents in particular, including high profile studies examining the effects of farm exposure (and associated exposure to particular bacteria found in these environments, notably *Acinotobactar lwoffi*) which are documented to drive lower susceptibility to allergic asthma.

1.2 Review of Major Relevant Concepts

1.2.1 Rising Incidence of Allergy and Immune Disorders.

The 20th century saw consolidated industrialisation, with movement away from traditional, rural lifestyles in favour of urbanisation. These changes to human living conditions were accompanied with

decreased rates of common infectious diseases, and an associated dramatic rise in the incidence of immune disorders and diseases of chronic inflammation in developed countries (Bach 2002). These include autoimmune conditions, such as type 1 diabetes or multiple sclerosis, or allergic conditions such as asthma, atopy, hay fever, and atopic dermatitis (eczema), and chronic pathologies including type 2 diabetes, metabolic syndromes or inflammatory bowel disease. These conditions are tied through shared underlying inflammatory processes with environmental components, as uncovered by GWAS and SNP-based genetic analysis alongside monozygotic twin studies (Farh et al. 2015), and have been further characterised by insufficient immune regulation, such as mediated through regulatory T cells (Lu, Barbi, and Pan 2017). Environmental factors considered as potential drivers for increased incidence of immune disorders include: dietary changes, psychosocial stress, xenobiotics and pollutants, alterations to the commensal microbiome, and changes to infectious burden (Strachan 2000) (Bach 2002).

1.2.2 Immunology of Allergy

Allergy is defined as a disproportionate inflammatory response to what are generally considered otherwise harmless molecules. Allergic inflammation, characteristic of the type 1 hypersensitivity response as seen in atopic asthma, involves type 2 immune responses, with innate and adaptive components. Initial triggers include innate sensing of foreign particles alongside tissue damage, including of epithelial cells, and the ensuing triggered release of alarmins, such as IL-25 and IL-33 and TSLP. Type 2-driving innate cells, including ILC2s and basophils are activated signals during initial responses (and have been postulated as providing the initial IL-4 needed to polarise T cells). These are involved in driving the initial priming of antigen-specific immune responses, including activation of antigen-specific CD4+ and their polarisation, which amplify and direct the innate response, including further production of type 2 cytokines, such as IL-4 which supports activation and proliferation of B cells, including the maturation and class-switching of plasma cells to produce for example, IgG1 and also the more pro-allergic IgE. Recognition of allergen by IgE can trigger downstream effector functions, for instance when bound via its constant region to Fcε receptors on granulocytes including mast cells and basophils, with this crosslinking providing the signal to stimulate degranulation, releasing preformed inflammatory mediators including histamine, leukotrienes and prostaglandins. Further

classical type 2 cytokines include IL-5 that drives granulocyte proliferation and activation, especially of eosinophils, and IL-13 which can induce changes at the barrier surface including goblet cell hyperplasia and mucous production as enhancing positive feedback loops. Chronically these processes lead to tissue remodelling and the clinical symptoms of asthma, but these are also same immune processes that disable, clear, and ultimately can drive protective immunity to parasites.

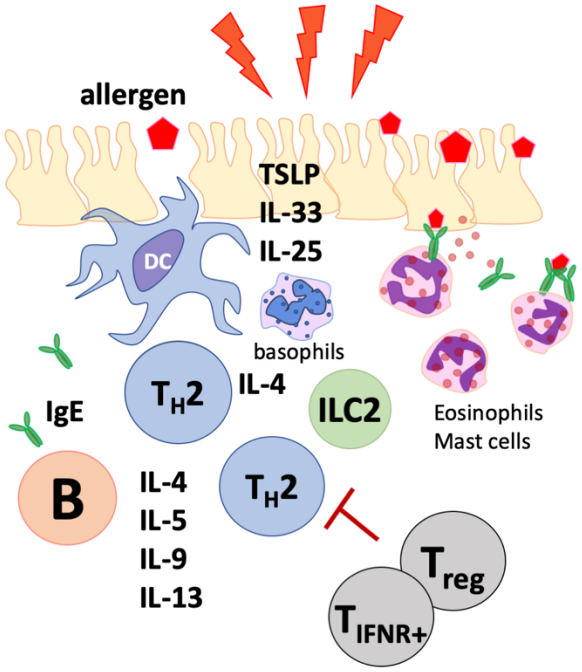


Figure 1.1-1 Mechanisms of Allergy
Graphical depiction of allergy induction via contact with allergen. This triggers downstream innate and adaptive changes leading to activation of T_H2 cells, production of IL-4, IL-5, IL-9, and IL-13, class switching of B-cells to produce allergen-specific IgE, and stimulation of granulocytic mast cells and eosinophils via cross-linking of allergen and IgE. These are processes of sensitization and subsequent memory responses that instigate effector functions upon re-exposure, and where pathological responses lack appropriate regulatory elements e.g: Tregs with necessary specificity and function, such as IFNR phenotypes. Adapted from (Boyman et al. 2015) and (Yazdanbakhsh, Kremsner, and van Ree 2002).

There are numerous factors that have been postulated to contribute to exacerbated allergic sensitivity, as drivers of atopic conditions. One predominant factor is a family history of atopic disorders, associated with high levels of IgE (Liu et al. 2009), with associated SNPs found at a range of gene loci, including those involved in triggering or regulating related immune pathways such as IL-33, TSLP, IL-10, and in antigen presentation, including HLA-DR (Kim and Ober 2019). Other significant factors relate to environmental exposures, and include history of infection, antibiotic usage, smoking and contact with

vehicle exhaust, contact with pets and other animals, with strong evidence for a role of maternal nutrition and health during pregnancy (reviewed in extensively in (Krusche, Basse, and Schaub 2020)). These factors, among others and in combination with individual genetic variation, represent the complex set of challenges that can modulate immune set points, from sensitizing triggers to more general factors that alter the regulation of antigen-specific immune responses, and as such sharing pathways and potentially underlying aetiologies with other immune disorders.

1.2.3 Immune Regulation and Resolution

Activation of immune responses, including those involved in allergy and more broadly in antigen-specific adaptive expansion, trigger reciprocal inhibitory responses that fine-tune inflammation. These reactions often occur within feedback loops due to the interconnected and complex nature of the immune system and its signalling networks. This involves a multilevel system of negative regulation, as occurs on a molecular level, in the counter-induction of suppressive transcription factors, negative feedback loops suppressing transcription of overemployed inflammatory mediators, or on a cellular level through the induction of specialised regulatory cells, including antigen-specific adaptive cells such as Tregs and Bregs, as well as innate cells including tolerogenic changes to dendritic cells and other APCs including monocytes, and less classical immune cells including stromal cells and epithelia.

The initial drivers of diversity in building the immune repertoire is random recombination in the variable regions of T cell receptor and B cell receptors. Through selection processes these already become regulated within a system that achieves a balance between eradicating excessive pro-inflammatory responsiveness and at the same time developing an arsenal of cells armed with antigen-specific receptors capable of detecting pathogens, toxins and abnormal structures such as those associated with cancer to protect the host and maintain a homeostatic balance of health. Beyond this initial direct regulation of the repertoire, a finely tuned balance is reached through the myriad regulatory processes present throughout the body to dampen, resolve, and shape the immune response in a series of checks and balances to maintain order.

Chronic inflammatory conditions arise through positive feedback loops, when appropriate inhibitory responses do not dampen sufficiently, and as such are indicative of faults in regulatory mechanisms that remain key areas of research unmet clinical need. The inflammatory switches that drive allergic responses, including alarmin-based IL-25, TSLP, IL-33 signals associated with epithelial damage and loss of barrier integrity, as well as the allergen-specific crosslinking of Ig bound to effector cells that occurs on re-exposure. The induction of tolerance towards allergic conditions involves the expansion of antigen-specific regulatory T and B cells. These operate through mechanisms mediated by the soluble mediators, including cytokines such as IL-10 and TGFb, as well as cell surface molecules such as CTLA-4, LAG-3, PD-1, which, as reviewed in (Palomares et al. 2017), are induced within allergen-specific immunotherapy (AIT), which remains a major recent advance in the treatment of existing allergy. Recent studies utilising advances in single-cell sequencing technology continue to shed light on the specific factors that dampen allergic inflammation versus those that contribute most essentially to increased pathogenic states. In one recent study using single-cell transcriptomic approaches, IL-9-producing TH2 cells were a subset found particularly associated with allergic asthmatic responses (Palomares et al. 2017), with IL-9 signalling previously recognised as a determining factor enhanced in subjects with active peanut allergy (i.e.: with inflammation) versus those only showing sensitisation (Brough et al. 2014). Further, helper and regulatory T cells with interferon response signatures (IFNR+) were found in non-allergic subjects, including enrichment of tumour necrosis factor-related apoptosis-inducing factor (TRAIL) (Palomares et al. 2017), which has been shown to dampen T helper cell activation via inhibition of TCR signalling, and block associated inflammation (Chyuan et al. 2019). Continued adoption of such advancements in technology to more clearly describe the basic processes associated with allergic conditions, and their resolution, will further aid specific approaches to addressing, for example, their modulated response to environmental triggers.

1.2.4 Vaccination and Immune Training

There are clear parallels between the antigen-specific processes involved in allergy and the proper induction of necessary immune responses, including immunological memory, as required for effective vaccination. Both are based in the induced sensitivity towards exogenous foreign antigens, with the

processes underlying these responses subject to differences within individuals. Factors including genetics, gender, socio-economic and environmental background, and previous infections have all been suggested to play a role in both the susceptibility to allergy and immune disease, but also as factors that underlie heterogenous responses to vaccination that can in some contexts threaten to compromise sufficiently protective effects on immunisation.

The basis of vaccination was built upon the observation that prior exposure to one more agreeable immune agent, such a weaker related virus or its elements, could induce protective responses against more serious pathogens. As understandings of memory within the immune system have developed, it is becoming clearer that there are both major and more subtle effects whereby a lifetime of immune exposures shape the immune system beyond genetic baseline. These training effects have been shown to involve adaptive and innate immune cells.

The developing field of innate memory is showing how training effects can be found not only in the adaptive expansion of antigen-specific lymphocytes, but also within the skewed polarization of the sentinel cells of the immune system. These include monocytes, macrophages, and NK cells, which retain training effects that lead to enhanced or tolerised responses to subsequent interactions, as induced by infections and pathogenic stimuli, but also through damage signals (Crisan, Netea, and Joosten 2016). Recent findings also implicate adaptive training effects within APCs including the DC compartment, where subsequent protection from pathogenic challenge was transferable via DCs that had been "trained" through previous exposure, changing their inflammatory responsiveness and providing "memory-like" responses (Hole et al. 2019). Some further non-specific effects are documented to generate bystander immune enhancement for certain vaccines, notably Bacille Calmette-Guérin (BCG). These too were found be linked, among other mechanisms, to training of responsiveness in innate cells including monocytes, macrophages, and even haematopoietic progenitor cells (Uthayakumar et al. 2018). Infant immunisation is considered therefore to play a putative role for general training of immune responsiveness in later life, with a lower association with overall mortality, particularly in low-birth weight infants, as well as signs of accelerated neonatal immune development such as increased non-specific cytokine production to innate stimuli (Jensen et al. 2015).

The growing recognition of the need for personalized approaches to health based on individual differences was originally built upon an understanding of the range of genetic variation underlying these, such as the appearance of mutations and SNPs in association with particular disease states, which shed light on the underlying groupings and aetiologies that link immune diseases (Farh et al. 2015). As these approaches develop, it is becoming clearer that environmental factors play a substantial role in driving individual differences relevant to health outcomes. Gene expression itself is now understood to be greatly altered through epigenetic machinery, regulating expression in ways that are informed by external factors that mechanistically underlie gene-by-environment-driven phenomena, and can already operate during fetal life (Pfefferle, Pinkenburg, and Renz 2010). One such highlight of systems to study epigenetic effect of disturbances to the maternal environment is the "agouti" mouse model, where colour change (towards yellow) is induced via hypermethylation of the responsive agouti gene, as indicative of lasting effects on the fetal epigenome such as occurs through maternal endocrine disruption via perinatal exposure to the xenobiotic bisphenol A (BPA), commonly found in the manufacture of household plasticware (Dolinoy 2008). The earlier referenced memory-like effects in pre-exposed DCs for example, were linked to changes in epigenetic status, and their persistence was lost through chemical inhibition of sustained histone modification (Hole et al. 2019). Moving forward, considering the effects of individual immune history will inform how individual backgrounds create the differences that appear as biological heterogeneity to immune stimuli, such as disease susceptibility and allergic predisposition.

1.2.5 Infection-Induced Immunoregulation: The Hygiene Hypothesis

With regard to the impact of industrialisation on infection, sanitary practices have led to a strong decrease in particular gastrointestinal and faecal-oral infections. Association of this with hyperactive immune responses led to the formation of the so-called "hygiene hypothesis" (Strachan 1989), formally proposed by David Strachen after observations on sibling order, common infections, and allergic incidence (Strachan 2000). The "old friends" expansion of this theory idea (Rook, Martinelli, and Brunet 2003) presented a revised focus on the effects upon human evolution under the burden of such infectious agents, many of which dampen the immune system in order to ensure their survival within their host, produced a hypervigilant immune system, which, lacking these dampening checkpoints in its more

urbanized, sanitized form, is free to cause immune-mediated pathology (Rook 2009). The immune system evolved under the constant pressure of contact with infectious agents, and as such, contact with bacteria, viruses, and parasites, could be a "normal" part of the educative exposures needed by a growing immune system to develop effective regulatory pathways. Indeed, studies on the interplay between infection and immune disorders have discovered specific immunoregulatory agents such as bacteria and other microbes in farming environments, supporting inverse associations between early life farm exposure and allergy (Schaub et al. 2009).

The question then arises whether the mechanisms used by these organisms, with parasites as one example, to dampen the immune system, can inform us about the aetiology of immune-mediated diseases. Allergy itself has previously been described as an "evolutionary hangover from parasitism" (Pritchard, Hewitt, and Moqbel 1997). In particular parasitic infection is often accompanied by a range of morbidities including anaemia, stunted growth, negative pregnancy outcomes, and in some cases fatality, and therefore would generally be considered to pose no actual "benefit" and instead would be majorly outweighed by deleterious and pathological effects. Still, the modes through which parasites modulate the immune system to enable survival within the host continue to provide insight into more general operations of immunomodulation relevant to combating inflammatory disease state, and continued interest in the therapeutic potential of parasite products or even intentional live infection (as reviewed in (White, McManus, and Maizels 2020)). This thesis largely explores the axis of environmental exposure and regulation of immune disease through the lens of the expanded version of hygiene hypothesis, and focuses on helminth infection as a driver of "immune education", within a larger context of how microbial exposures and other immunological challenges during key phases of early life can lead to training effects on later immune function.

Microbes and infectious agents often bear pathogen-associated molecule patterns (PAMPs) that are recognised by the immune system, including by antigen presenting cells which are often highly specialised for sentinel functions through the expression of toll-like receptors (TLRs). There is growing evidence that immune challenges through maternal infection and microbial challenge can have a profound impact on the development of the immune system, and lasting effects in determining future

immune responses. Early exposure to maternal bacterial and virus infections during pregnancy can lead, through stimulation of TLRs, to training of innate cell populations, shifting polarisation of adaptive immune cell populations, and downstream effects in later life on immune responses and inflammatory disorders. Such effects may be based in polarization shifts within the adaptive compartments, such as the expansion of T helper subsets that antagonize future allergic responses, or the expansion of suppressor cells such as Tregs or Bregs, populations whose maintenance would reflect ongoing epigenetic landscape alterations to stabilize these effects (Pfefferle, Pinkenburg, and Renz 2010). This can involve altered dendritic cell maturation, as well as that of other APCs and innate cells, and present a role for those cells that retain innate memory, such as those within tissues as self-renewing locally resident populations, to be involved in maintaining priming effects, probably also based in molecular processes that operate on an epigenetic basis, on future immune responses initially triggered by maternal environmental cues. The absence of such stimuli, and the resultant induced-tolerogenic states, may underlie the set of processes that lead to increased allergic predisposition (Palomares et al. 2017). These further reveal molecular switches involved in appropriate immune surveillance, balancing sufficient identification and clearance of non-self pathogens, as well as the altered-self of oncogenic transformations, with limited undesirable autoimmune outcomes (Jensen-Jarolim et al. 2018).

1.2.6 Helminth Infection and Schistosomiasis

Parasite and helminth infection were common and ubiquitous immune challenges during the evolution of the mammalian and human immune system. Many elements of inflammatory and type 2 immune responses are tailored to withstand, endure, and clear these infections. Currently, over 1.5 billion people are thought to be infected with soil-transmitted helminths worldwide, and over 200 million with schistosomiasis (Organization 2019b, 2019a), including approximately 40 million women infected during pregnancy. The infectious burden of these significant Neglected Tropical Diseases (NTDs) (DALYs and Collaborators 2017), associated with deaths and disability-adjusted life years (DALYs), is amplified by their role in population heterogeneity regarding management of co-infection, allergy, immune disease, and potentially vaccination efficacy. Chronic helminth infection is established through immunomodulatory processes that allow long term survival in the host. Spillover effects upon allergic

sensitivities (Resende et al. 2018) and vaccine responses (Riner et al. 2016) (including anti-HIV vaccination in (Ondigo, Ndombi, et al. 2018)), as well as induced regulatory immune elements (Dzhivhuho et al. 2018), continue to spark interest in infectious parasites such as *Schistosoma mansoni*.

Initial infection occurs via penetration of the skin with cercariae, the larval stage of parasite released form the *Biomphilaria glabrata* intermediate snail host, and present in infected bodies of fresh water. After entering the body, these develop into the mature worms, which migrate through the circulatory system, including a passage through the thin capillaries of the lungs. During these stage, pro-clearance immune responses are largely of a type 1 pro-inflammatory nature, as evidenced by antigen-specific responses of mice during these early stage of infection between characterized by large amounts of IFNγ in response to stimulation with schistosome antigen (including those post-pregnancy in (Straubinger et al. 2014)). Adult worms ultimately reach their niche in the hepatic vasculature, including the portal vein where they are supported by the nutrient rich blood flowing from the gastrointestinal tract. Here, the male and female adult worms pair and the female begins to produce eggs. The strong type 2 responses which characterise chronic *Schistosoma mansoni* infections are largely stimulated by the parasite eggs and their soluble molecules, that also induce strong autoregulation to dampen inflammatory responses in the (Pearce and MacDonald 2002) (Layland et al. 2007) (Layland et al. 2010; Layland et al. 2013).

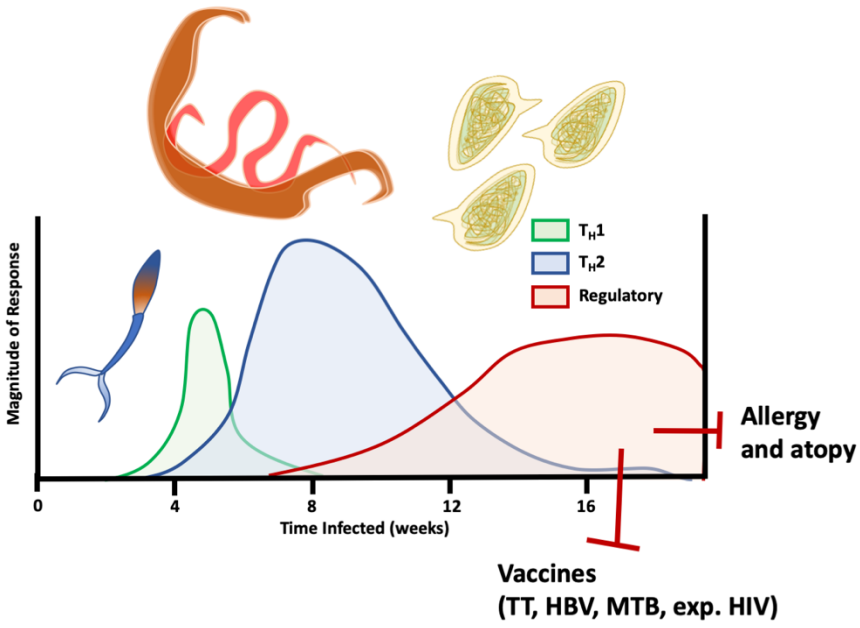


Figure 1-2 Immunology of S. mansoni infection

Graphical summary of distinct, sequential immune phases induced by each stage of infection with *S. mansoni*, from pro-inflammatory initial acute stages of response (type 1, in green, peaking in weeks 3-5) triggered by the migratory cercariae, to the peak type 2 inflammation classically induced by the mating of adult worms in the portal vasculature (blue, peaking weeks 6-10), with the ensuing accumulation of parasite eggs and induction of regulatory loops including chronic granuloma formation and activation of suppressor cells generating a mixed chronic phase dominated by a regulatory response (red, peaking week 14/16+) that can dampen specific and bystander inflammation. Adapted from (Lundy and Lukacs 2013).

Long-term survival of helminth parasites relies upon modulation of these processes. In chronic stages of schistosomiasis these are controlled and superseded by induced regulatory responses, including the induction of regulatory T and B cells, alternative activation of macrophages, and tolerogenic changes in dendritic cells alongside induction of myeloid derived suppressor cells and the associated increases of regulatory cytokines including IL-10 (the breadth and detail of these changes explored in (Wammes et al. 2014)). This has the spill-over effect of reducing bystander immune-responses, for example to vaccines, other infections, and allergy.

1.2.7 Schistosomes as Immunological "Old Friends"

Although various gradations of symptomatic disease clearly mark helminths as pathogenic parasites and not true "commensals" or symbionts, co-evolution may have evolutionarily assigned a type of regulatory role for these parasites upon the immune system, and their reduction or absence in human populations from the developed world, a recent phenomenon in evolutionary terms, potentially removed key immune regulatory processes. Parasitic helminth infections have a documented history of altering inflammatory disease and allergy. Such chronic infections have a demonstrated effect on suppressing bystander immune responses which has been extensively reviewed (Smits and Yazdanbakhsh 2007; Maizels and Yazdanbakhsh 2003) (Finlay, Walsh, and Mills 2014). Some of the strongest data for associations between helminth infection in humans and immunomodulation pertain to reduced allergic skin-prick testing responses. For example, a Brazilian study of people with heavy *S. mansoni* infections were on average 5-fold less reactive to skin-tests with allergens compared to matched uninfected individuals from the same region (Araujo et al. 2000). A meta-analysis of human studies showed an inverse relationship between current chronic helminth-infection and positive skin-prick reactivity to allergens

(Feary, Britton, and Leonardi-Bee 2011). In terms of active infection, one study on Ethiopian rural migrants moving to urbanised areas in Israel found they had less allergy if they harboured an *S. mansoni* infection, and displayed significant increases in allergy if they underwent antihelminthic treatment (Stein et al. 2016). On a mechanistic level, responses to Derp1 (a major dust allergen) in *S. mansoni*-infected asthmatics, compared to uninfected asthmatics, showed reduced allergen-induced IL-4 and IL-5 levels from PBMCs, while the allergen-induced IL-10 production was higher from these infected individuals (Araujo, Hoppe, Medeiros, and Carvalho 2004; Araujo, Hoppe, Medeiros, Alcantara, et al. 2004). Murine studies also support observations that *S. mansoni* reduces severity of pathology resulting from co-infections such as malaria, including progression to cerebral malaria (Waknine-Grinberg et al. 2010), as well as inflammation from autoimmune processes (La Flamme, Ruddenklau, and Backstrom 2003). Further mechanistic work has demonstrated direct inverse associations of inflammatory allergic skin responses with the IL-10 enhanced through chronic infection, particularly in the absence of pro-inflammatory IL-33 and TNF and with sufficient burden of parasite load an additional parameter determining these effects (Resende et al. 2018).

Concerning the underlying mechanisms of such interactions in immunological terms, the classical paradigm of imbalanced type 1 / type 2 immune responses might not be applicable to the effects that schistosome infection have on allergic type 2 responses, possibly due to their very dynamic immune phases and presentations. Indeed, the strong suppressive effects of helminth infection upon allergy appear to be in chronic phases where type 1 responses have largely subsided, and instead are replaced by an immune state characterised by modified type 2 immune processes coupled to suppressive, regulatory aspects such as regulatory and IL-10-producing T and B cells (Layland et al. 2007) (Haeberlein et al. 2017). As such, rather than simply modifying the type 1 / type 2 balance, the persistent challenge with immune stimuli, such as a broad spectrum of diverse microbiota, infections, and parasites, induces a regulatory network that is more equipped or primed to effectively manage challenge with novel stimuli, such as potential allergens. The apparent paradox of helminths, as strong type 2 stimuli, reducing atopic inflammation, despite association with increased sensitization to allergens such as allergen-specific IgE levels (van den Biggelaar et al. 2001), but with the presence of increased IL-10

levels, invites investigation into regulatory feedback loops induced by schistosomes that overshoot to suppress wider inflammatory responses to other antigens (Yazdanbakhsh, Kremsner, and van Ree 2002). Studies on infected mice have found reduced cytokine production and lowered T cell proliferation to heterologous antigens to be largely dependent on parasite infection-related increased IL-10 levels (Kullberg et al. 1992).

Additionally, the suppressive bystander effects from the type 2 and regulatory immune responses present during chronic helminth infections are associated with altered vaccine efficacy, including effects of chronic schistosomiasis on reducing protective type 1 responses raised against tetanus vaccines (Sabin et al. 1996) and Hepatitis B (Ghaffar et al. 1990), reflecting the dampening effects of *Trichuris trichiura* on antibody titers obtained from anti-malarial vaccines (Esen et al. 2012). As such, discovery of effective vaccines therefore continues to be a particular concern for communities where helminths are endemic (Wajja et al. 2017). Murine models have also displayed reduced vaccine efficacy following schistosome infection, for example, against *Mycobacterium tuberculosis* (Elias et al. 2005), reducing type 1 responses typified by IFN-γ and instead increasing type 2 responses. Schistosome infected mice had impaired clearance of vaccinia virus (Actor et al. 1994), with an increased persistence that appears mediated by suppression of CTL activity suppressive myeloid cells via secretion of soluble factors (Marshall et al. 2001). Recently, experimental murine *S. mansoni* infection inhibited effective cellular and antibody responses against novel HIV vaccines, with even the presence of schistosoma eggs alone (as a result from de-worming) able to reduce humoral vaccine responses (Dzhivhuho et al. 2018). Nevertheless, effects of anthelminthic treatment on vaccine efficacy in human cohorts remains unclear (Bruckner et al. 2015) (Bruckner et al. 2016). Although non-transplacental in nature, maternal schistosome infection during pregnancy has further documented effects upon offspring immunity in human cord blood (Ludwig et al. 2019), children (Mpairwe et al. 2011), and in transmaternal murine systems (Santos et al. 2010) (Straubinger et al. 2014). The next two sections will cover first the concept of early life priming via changes to the maternal environment in general, and then specifically in the case of maternal helminth infection, with a focus on schistosomiasis.

1.2.8 Maternal Environment during Immune Ontogenesis

Evidence is growing that the effects of early life exposures, particularly those related to maternal health during pregnancy, may present significant environmental cues during development that initiate difference through individual immune history. Maternal factors including infection, toxins, stress, activity levels and diet can impact inflammation and have been implicated in altered immune and behavioural outcomes in offspring (Bilbo et al. 2018). The delicate immunological balance at the fetomaternal interface, its vulnerability to perturbation by maternal infections, and the subsequent effects of such disruptions on immune development and responses later in life. Bacterial infections such as listeriosis remain key concerns that threaten healthy pregnancy (Charlier et al. 2017). Perinatal viral and bacterial infections have been shown to impact normal development and potentially lead to behavioural shifts in later life (Kneeland and Fatemi 2012), although the immunomodulatory effects of related transmaternal exposure, which would have been relatively commonplace throughout human history, continue to suggest a role for these processes in educating proper immune function.

Early life, including during embryogenesis and the fetal stages prior to birth, have long been considered a very important period in terms of priming responses in later life. Emerging research continues to shed light on the specific context of early life, where particular features of the immature immune system lead to tolerogenic effect, as observed in the specific tolerogenic phenotype of immature fetal dendritic cells (McGovern et al. 2017), could represent a restricted window of altered responsiveness to stimuli. In the context of parasite-derived compounds, studies where murine neonates were exposed schistosome antigens found an increased predisposition towards increased inflammatory sequelae, at least in regard to later secondary contact with schistosomes (Perrotto et al. 1974), although in utero only exposures have been found to be tolerogenic (Santos et al. 2016). While schistosome egg exposures suppressed genetically-predisposed autoimmune disease in non-obese diabetic (NOD) mouse models, the complete abrogation of disease specifically following only early age schistosome egg exposures, further supports an enhanced effect during the early life window for parasite-driven immunomodulation (Zaccone et al. 2003).

The hemochorial placenta of humans (and mice) allows contact of fetal tissue with maternal blood (Moffett and Loke 2006). This permits transfer of mature as well as haematopoietic progenitor cells, leading to varying amounts of microchimeric presence of maternal cells, which have been associated with tolerance to non-inherited maternal antigens (termed NIMA) (Dutta et al. 2009). The specific context of maternal infection through changes to inflammation may alter the rates of such cellular transfer, or, as has been shown in other maternal parasite infections, even direct the transfer of primed maternal, also during nursing, which can redirect or mobilize immune responses in the child (Darby et al. 2019).

Among other external triggers, bacterial exposure in certain early or perinatal contexts has been shown to reduce allergic susceptibility. In particular, early life contact with *Acinetobacter lwoffii* F78, a bacterium with high LPS content that is found in farming environments, was shown to lessen atopic reactivity (von Mutius 2016). *A. lwoffii* exposure also upregulates suppressor molecules in lung epithelial cells and lead to dampening of airway inflammation, with this effect also recreated by LPS exposure alone (Schuijs et al. 2015). Perinatal application of this bacterium in mouse models was further shown to lower experimental airway inflammation responses in offspring, with the increased signalling driven by maternal TLR stimulation playing a mechanistic role (Conrad, Ferstl, et al. 2009). Follow-up work identified epigenetic alterations to the T cell compartment in such perinatally-exposed offspring, where robust permissive signatures on IFNγ promoters in helper T cell populations inhibit experimentally-induced allergic airway inflammation (Brand et al. 2012; Conrad, Ferstl, et al. 2009; Brand et al. 2011). Maternal infection with *Helicobacter pylori* shows similar effects with regard to reduce allergic lung responses in offspring, with further induction of enhanced regulatory functionality in offspring T cells. The specific effect of pathogen-associated effector molecules was demonstrated through replicating this phenotype in offspring via maternal perinatal exposure to *H. pylori* immunomodulatory compound VacA (Kyburz et al. 2018).

One indirect effect of maternal infection that could alter pregnancy outcomes is through changes to cytokine levels during responses to pathogens. Indeed IFNγ levels typically rise in response to viral or bacterial exposures, and mouse systems demonstrate that direct delivery of exogenous IFNγ can skew

offspring towards lower allergic responsiveness (Lima et al. 2005). However, the pro-inflammatory effects of perinatal infection and the associated maternal immune activation (MIA), as mentioned earlier with regard to some viral and bacterial infections, can have grave short or long-term effects. The effects of MIA through viral infection have be modelled using murine perinatal exposure to TLR-agonist poly I:C, a synthetic double stranded RNA as found in some viruses. This was shown to trigger adverse effects regarding offspring behaviour as a sign of neurological impact, and mechanistically involved increased levels of pro-inflammatory cytokines IL-6 and IL-17A (Choi et al. 2016). The transmission of such effects via transfer of microbiota from poly I:C-treated mice highlight the interconnection between elements of the maternal environment during pregnancy (Lammert et al. 2018).

In utero sensing of immune stimuli can even have an effect on the fundamental structure of the lymphocyte repertoire. The presence of allogeneic antigens during development (and therefore their inclusion in the repertoire of antigens expressed in the thymus) can drive the deletion of lymphocyte clones (as discussed in (Dutta and Burlingham 2009)). Here, transmaternal exposures during early life can drive antigen-specific maternal tolerization, already demonstrated in mice using model antigen ovalbumin (OVA) (Polte, Hennig, and Hansen 2008), and for the in utero exposure-derived later tolerised acceptance of graft tissue (Billingham, Brent, and Medawar 1953). Additional similar skewing effects with regard to antigen-specific responses may also step from the differences in immature immune responses afforded during fetal life, such as demonstrated by maternal alloantigens promoting Treg cell proliferation fetal lymph nodes, mechanistically serving to spare maternal cells from clearance (Mold et al. 2008).

Aside from direct alteration of clonal diversity through deletion or expansion, further mechanisms that underlie immune changes probably involve epigenetic changes. These include stable modifications to chromatin, either directly marking the DNA molecules themselves in the case of methylation, or translational modification to histones, which through inheritance by daughter cells maintain shifts in cellular set points. These can have effects with regard to responsiveness to subsequent challenge, suppressive capacity, or altered sentinel behaviour of sensing cells such as APCs to modify their receptiveness to triggers.

Environmental interaction with the pathways governing these processes can thereby determine the regulation of gene expression and subsequent outcomes. Studies on the gut microbiome have yielded strong data on associated changes to metabolite profiles, including anti-inflammatory short-chain fatty acid (SCFA) profiles greatly implicated in gut-health-associated processes of immune tolerization (Dopkins, Nagarkatti, and Nagarkatti 2018), and serve as an important environmental cue driving immune diversity. *In utero* exposure to altered SCFA levels have been shown to have direct effects upon reducing offspring responsiveness to allergic airway inflammation, with mouse models showing this associated with additional changes to transcriptional profiles of Treg-relevant epigenetic pathways in fetal lungs (Thorburn et al. 2015). Such changes to maternal microbiome may occur more directly as a result of diet or antibiotic usage, or else in response to other changes to maternal health, such as mentioned above in relationship to MIA. As such, questions remain regarding the downstream impact of such triggering stimuli during early life on health, with the mechanisms by which such imprints are maintained into later life forming active areas of research.

1.2.9 Immunological Effects of Maternal Helminth Infection

Unlike the bacterial and viral infections detailed above, helminth infections are notable for their ability to stimulate type 2 immune responses alongside regulatory feedback loops. The relationship between helminths and allergy in infected hosts operates through the crosstalk between stimulatory and regulatory signals along similar axes that relate to inflammation and type 2 immune responses. Perinatal exposure to helminth infection, and its association with immune priming effects, represents a unique wing of the hygiene hypothesis compared to other more type 1 dominated pro-inflammatory infectious agents, offering distinct opportunities to uncover and delineate the complex processes that underlie immunoregulation and resolution of inflammation. Immunomodulatory effects of maternal schistosomiasis in humans were shown in a key study that found that the incidence of infantile eczema was lower in children of *S. mansoni*-infected mothers, with an opposing increased association with such atopic skin responses upon anti-helminth treatment of mothers (Mpairwe et al. 2011).

A further broad range of effects stemming from maternal schisostome infection indicate the likelihood that such regulatory effects comes alongside more stimulatory immune priming effects, such as those that alter maturity of the immune system. Maternal schistosome infection can sensitise human children *in utero* through altered total (polyclonal) and schistosome-specific IgE levels, alongside increased signs of maturation in B cells (King et al. 1998; Seydel et al. 2012). This may be a more general phenomenon associated with parasitic infections, as neonate cord blood cells from regions highly endemic for helminth and malarial infections show many signs of immunological maturity, including relatively less immature CD5+ B cells. This coincided with less CD27 and CD28 expression on CD4+ T cells, as indicative of downregulation through antigen-experience (Kohler et al. 2008).

Maternal infection has also been associated with enhanced antibody transfer and therefore highlights the potential range of changes to the maternal environmental induced by this infection (Ludwig et al. 2019). This can be further associated with changes to the tissues of the fetomaternal interface at the barrier between mother and fetus. Particularly, skewed expression of genes associated with placental production of steroid hormones during murine schistosome infection (Straubinger et al. 2014) and in humans (Ludwig et al. 2019) suggests metabolic changes associated with the glucocorticoid and hormonal axis that may be implicated in driving developmental changes in offspring leading to non-specific alterations in immunity.

Additional functional effects have been suggested by studies investigating the impact on effective vaccine responses in child exposure to maternal helminth infection. Cohort studies, for example, have shown that developmental exposure to maternal helminth infection, with measurable effects on offspring immune priming including altered IL-10 levels in cord blood, can reduce levels of protective IgG in response to vaccination against *Haemophilus influenzae type B* and Diphtheria (Malhotra et al. 2015; Malhotra et al. 2018). Other studies demonstrate that this effect is not always clear, for example, following Hepatitis B vaccination (Bassily et al. 1997), as is the role of any antibody-mediated effect in experimentally-induced allergy models (Straubinger et al. 2014). Other studies present findings that contradict previous results, showing no effect regarding diphtheria and tetanus, but reduced measles vaccine responses (Ondigo, Muok, et al. 2018). Overall, however, the breadth of further studies

indicating an impact of maternal helminth infection and childhood immunization responses (Ghaffar et al. 1989; Malhotra et al. 1999) demonstrates further opportunities to study transgenerational immune priming through this axis.

Experimental work performed in murine maternal infection systems have begun to clarify the processes underlying these phenomena of maternal schistosomiasis. However, the clear presence of heterogeneity and complexity of the involved immune interactions impede simplistic conclusions even in these relatively more closed experimental settings. One example of such complexity is the major impact of maternal schistosome infection phase on the resultant effects on offspring priming. Here, allergic responses in offspring reliably follow the distinct immune phase during which conception and gestation took place, with early (type 1 / IFNγ dominant) and late (regulatory) phases driving suppression of allergic asthma for offspring, with the initial patent phase (during which the type 2 immune response peaks) instead predisposing offspring towards a more pro-allergic state (Straubinger et al. 2014). IFNγ was further found to play a role in pre-patent anti-allergic priming effects, and suggested that the root mechanism in this phase-specific effect may therefore be altered maternal immune responses and cytokine profile. This was further supported by phase-dependent type 1, type 2, and regulatory cytokine responses to schistosome antigens from placental tissues (Straubinger et al. 2014). Similar suppressive effects on later allergic reactivity has also been observed using direct maternal delivery of exogeneous IFNγ alone (Lima et al. 2005) to support these conclusions. Additionally, maternal IL-10 levels during gestation have also been associated with priming effects on child IL-10 levels (Santner-Nanan et al. 2013), although the direct investigation of regulatory cytokines or IL-10 to imprint suppressed allergic responses has as-yet not been shown. Additionally, while data on whether specific individual cytokines are indeed transferred across the placenta is contentious in many cases, with evidence suggesting bi-directional transfer of IL-6, yet limited transfer of TNF (Zaretsky et al. 2004). Even without such transfer, these could act via modulation of immune processes at the decidual tissues at the fetomaternal barrier.

In addition to immune phase, the mode of maternal exposure has been found to alter offspring priming effects. The *in utero* transfer of priming effect of maternal schistosomiasis via modified gestational

environment for fetal development, for example, was found to suppress later antigen-specific responses in offspring, with an associated increased in IL-10-dominated responses to immunization (Santos et al. 2010). Conversely, nursing by a schistosome-infected mother, where antibodies and antigens are delivered to along with nutrients after birth to adult offspring, drove pro-allergic priming (Santos et al. 2016). These contrasting (pro or anti-allergic) effects were also reflected in the differential priming of co-stimulatory molecule expression (for CD40, CD80, and CD86) following immunization of adult offspring, dependent on *in utero* versus nursing-based maternal exposure (Santos et al. 2014).

Mice exposed through both routes (*in utero* and through nursing) to chronic maternal schistosomiasis were also found to display steady state alterations to the CD4+ T cell compartment, in the form of modified polarization capacity of naïve cells alongside epigenetic changes in the form of increased suppressive histone acetylation on key effector gene loci relating to allergy (Klar et al. 2017). The underlying interactions between these and *in vivo* T cell responses remain to be assessed, as does demonstration of the role that T cell priming may play within the differential effects apparent in experimental allergic asthma models, leaving clear gaps in understanding the relationship between maternal schistosomiasis and priming of offspring responses to heterologous antigens, as may underpin their modified susceptibility to inflammation.

Effects of exposure varying according to maternal immune phases during infection may represent changes to availability or load of parasite products, as well as interaction with the maternal immune response. Similarly, different time points and routes of exposure to schistosome antigens may underlie the opposing outcomes triggered by *in utero* exposure compared to via nursing. These may even involve differential transfer of maternal cells, previously shown to generate adaptive immune effects via microchimerism in other transmaternal helminth exposures (Darby et al. 2019). Components of Schistosome antigens, including molecules omega-1 and IPSE, were previously discussed to include molecules known to drive type 2 immune polarizing effects as well as directly induce regulatory changes to cell phenotypes (Haeberlein et al. 2017). Released of IPSE/alpha-1 from schistosome eggs, for example, triggered IL-4 secretion by basophils to drive modified activation of human monocytes, leading to anti-inflammatory effects (Knuhr et al. 2018). Schistosome-derived compounds have been

shown to be transported and persist within offspring, along with schistosome-specific antibodies (Attallah et al. 2006; Attallah et al. 1999; Attallah et al. 2003). In acute murine infection settings, this was further associated with modified susceptibility to subsequent infection of murine offspring (Lenzi et al. 1987; Attallah et al. 2006) (da Paz, Sequeira, and Pyrrho 2017). Similar effects were found for schistosome oligosaccharide Lacto-N-fucopentaose III (LNFPIII), otherwise known as a component found in human breast milk, which was able to effect co-cultures of dendritic cells and T cells by suppressing IFNγ production from CD4+ T cells, although CD8+ activity was not effects (Wang et al. 2010). LNFPIII also programs dendritic cells through altered CD40/CD40L to modify natural killer cell activation, and monocytes towards states of alternative activation (Zhu et al. 2012). Transfer of such molecules, during *in utero* ontogenesis, or via nursing, may offer similar training effects in a transmaternal manner, as has been described for maternal *H. pylori* infection (Kyburz et al. 2018).

In transgenerational settings, there is the further possibility of epitopes from antigenic compounds being presented within the more tolerogeneitc setting of early life. Presentation of such molecules during gestation *in utero*, or to neonates via breastmilk, may trigger similar mechanisms that induce tolerance towards maternal alloantigens or NIMA, Transfer of parasite-derived molecules to the fetus and neonates may drive adaptive responses that operate through antigen-specific mechanisms, similar to those already discussed in terms of NIMA. That is, the added presence of these antigens during early stages (e.g.: in the thymus) may affect repertoire changes, deletion of reactive clones or expansion of specific regulatory cells, or alternatively may trigger activation or modulation of other aspects of pathways involved in these responses. Maternal parasitic infection history has been shown to lead induced tolerance in children, particularly regarding the homologous pathogen itself (Dauby et al. 2012). For example, early-life exposure has been shown to yield tolerance to parasites such as malaria, suppressing of antigen-presenting cell function and modified T cell responses which can further lead to increased susceptibility to the same infection in later life (Brustoski et al. 2005). Responses to helminth infection from endemic locals has often been described as less acute that the inflammatory presentation founds in first-time infection of external visitors, and reactivity to cognate infection with schistosoma

has been suggested in mice as a further immunomodulation functional outcomes of early life exposure through maternal infection that may underlie this effect.

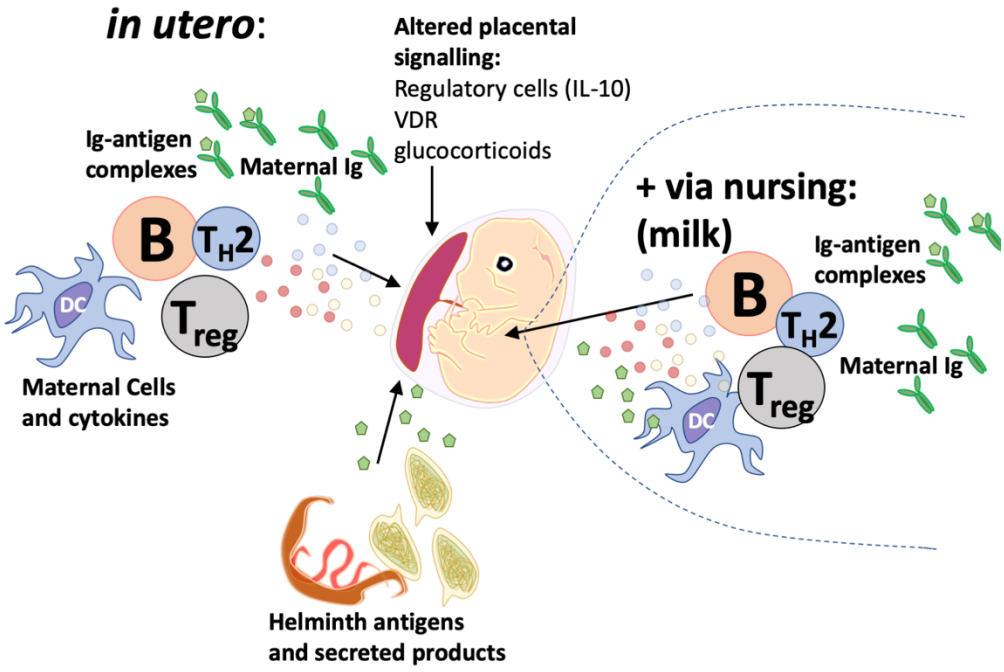


Figure 1.2-3 Mechanisms of Immunomodulation via Maternal Helminth Infection
In utero priming via maternal helminth infection may include crosstalk via: altered placental signalling pathways, including via vitamin D / VDR signalling, and changes to steroid and glucocorticoid pathways, transfer of primed cells, maternal-derived Igs, or antigen-Ig complexes, as well as cytokines, or more direct effects via transfer of parasite antigens and associated molecules, or secreted products. Similarly, maternal cells, Igs, Ig-antigen complexes, cytokines, as well as parasite-derived antigens and molecules can also be transferred during nursing via presence in mother's milk.

Parasite antigens as well as maternal parasite-specific antibodies can be transferred from mother to child, whether alone or in complex. Antibodies against molecules present in schistosome egg antigens have shown cross-reactivity to allergens including peanut (Igetei et al. 2017) and certain grass species (Igetei et al. 2018), suggesting the possibility that induction of antigen-specific regulatory responses directed at schistosomes may offer cross-reactive suppression towards antigens from other sources, in addition to truly non-specific "bystander" effects. Helminth antigens are characterised by the presence of glycans, which are absent from mammals but also found in these molecules with capacity to act as allergens, termed as cross-reactive carbohydrate determinants (CCDs) (Igetei et al. 2018). Exposure to such compounds and associated potential tolerogenic processes or immune selection in early life may thus

contributing mechanistically to the “window of opportunity”, where increased exposure to a range of such molecules at this time point may inhibit allergy formation in later life.

1.2.10 Overview and Identified Gaps

A strong case has been made for the relationship between altered maternal environment during early life and priming of immune responsiveness, as well as immune mediated outcomes. Such effects have also been demonstrated in the specific case of modulated allergic responses in children of women infected with schistosomes, as well as mouse models of schistosome infection. Here, the context-dependent demonstration of lowered incidence of atopic responses, and allergic inflammation, in association with particular modes of prenatal exposure to helminth infection direct further study into the underlying mechanisms. The relationship of these priming-induced modulations to potential additional effects, such as on vaccine efficacy, which are suggested by human cohort studies but are overall inconclusive, leave questions to be addressed.

In particular, this review has highlighted that changes to the regulation of T cell responses form a major part of maternal-driven changes that modify asthma susceptibility, as seen in the *A. lwoffii* and maternal microbiome related studies. The factors directing T cell regulation also exists in crosstalk with aspects of the immune system, including T, B, and innate cells, which are known to be affected by helminth infection, and where data already suggests, for example in the case of B cells, that maternal schistosomiasis plays a role in altered maturation. The capacity of major cell compartments of the immune system to acquire modified activity in response to maternal helminth infection therefore represents a unique opportunity for studying fetomaternal crosstalk, and the relationship between early life infection-driven priming with the behaviour of T and B cells, and the cells directing their activation, including APCs during immunisation, as well as in the context of allergy. Rather than focusing heavily on the mechanisms involved in the transfer of these effects from infected mother during the early life stage, this thesis profiles altered immune phenotype of helminth primed-offspring, within a closed experimental system to enable directly assessing the effects and consequences of cellular changes associated with modified responses to immune stimuli. In doing so, this work will direct further studies

on both the processes and features of persistent immunomoduation, as well as the induction of such effects including via early life priming through changes to maternal immune status.

1.3 Thesis Objectives and Roadmap

This thesis investigates the interaction between maternal schistosomiasis and differential immune regulation in offspring, stemming from previous research that highlighted the effects of maternal schistosomiasis, and particularly the chronic phase of maternal infection, on the development of experimentally-induced AAI. The three major objectives outlined below were addressed through continued study of murine offspring immune response and profiling of changes to cellular compartments of the immune system. This experimental work, performed using *in vivo*, *ex vivo*, and *in vitro* assays in application of this murine system of transmaternal immune priming are presented across three major chapters that comprise the results section of this thesis.

Firstly, AAI is known to involve and require T cell priming, and further studies within this murine model found steady-state alterations within the T cell on an epigenetic level. As such, the first objective was a thorough examination of outcomes relating to modified *in vivo* T cell responses following maternal schistosomiasis, particularly in the context of allergic asthma and immunization. The first chapter therefore covers a mechanistic investigation of the relationship between chronic maternal schistosomiasis and OVA-driven experimental AAI model. This was carried out across BALB/c and C57BL/6 mouse strains, evaluating previously described cellular changes to BAL composition, and importantly expanding these results to evaluate antigen-specific CD4+ T cell changes in the lung in response to AAI. Associated antigen-specific (OVA) Ig levels during AAI were assessed as a further systemic readout of allergic priming, as this previously also indicated modification after maternal priming. The presence of steady-state T cell polarisation shifts in the lung were compared to those from allergic mice, as was the transcriptional profile of key sites involved in this model, namely spleen, priming lymph node, and lungs, using RNA-seq. Earlier time points during this allergy model were also assessed, including B and T cell priming changes, alongside allergen specific antibody titres, prior to aerosol challenge to assess allergic priming alone more directly. This further included assessment of induced changes within the antigen presenting cell compartment, and establishment of local T cell

responses that could be more generalized to additional models evaluating vaccination outcomes more broadly. The induction of T cell responses was therefore assessed and compared across a range of vaccine modalities, including traditional adjuvants in common usage for pairing with protein antigens, as well delivery of antigen via a viral vector. Functional assessment of whether maternal priming impacts efficacy of vaccination was evaluated through responsiveness to further viral challenge.

The second objective was to discern whether these purported *in vivo* T cell responses stemmed directly from altered development of T cells that reduced their responsive capacity, or from factors extrinsic to the antigen-specific naïve T cells themselves, that instead modified the priming and regulation of T cell responses. The second results chapter evaluates this through a series of experiments questioning the impact of maternal schistosome-induced priming on offspring T cell biology. This begins with expansion of T cell polarization assays to build on previously published results, now assessing a wider range conditions focusing on additional T helper subsets. Previous results are also explored at earlier timepoints to give a clearer profile of developmental changes in the T cell compartment. Observations during these experiments directed further work at the potential presence of activation within the CD44+ central memory T cell compartment, including assessment of changes to proportions and transmaternal activation within this memory subset, including cytokine production capacity, whether these are indeed *de novo* primed cells produced by the offspring as opposed to transferred from the infected mothers, as well as initial experiments into their antigen-specificity. Further experiments considered altered behaviour within the highly purified subset of naïve CD4+ T cells, including functional in vivo assessment of altered capacity for polarization. This was paired with transcriptional analysis in the steady-state via RNA-seq, for both naïve CD4+ and CD8+ T cells. Whether the *in vivo* differential responsiveness to vaccination of CD8+ T cells, revealed in the first chapter, could be related to intrinsic functional changes within naïve CD8+ T cells was further assessed through additional *in vitro* assays as well as assessment of epigenetic landscape across relevant gene loci.

The third objective, growing from the data produced in exploration of the first two, was to profile steady state changes to priming across immune compartments relevant to regulation of T cell responses. This included primed T cell responses, B cell activation, and priming across antigen presenting cell

compartments. This culminated in the final aim of linking these changes to modified activation of T cell responses. As such, the third chapter begins with experiments exploring the ability of the regulatory environment, as found in later life following transmaternal priming, to modify the behavior of even exogenously-derived CD8+ T cells. This assessment served as a T cell-extrinsic counterpart to the second chapter's profiling of steady-state changes within the T cell compartment, and revisits these effects within the context of protein immunisation, protein plus adjuvant, as well as viral-vector based vaccination strategies shown to induce differential responses after transmaternal priming via helminth infection. These detail changes to CD8+ activation and proliferation that then serve as a basis for more mechanistic experiments detailing changes to immune activation after maternal priming. The focus here is on those cellular compartments involved in the differential allergic responses, and antigen-specific priming already profile, and those that regulate the activation of naive CD8+ T cells. This includes assessment of steady state activation status across B cells, DCs (including conventional DC and their subsets) and macrophages. Ultimately, functional assessment of changes within the DC compartment, in terms of their responsiveness to stimuli as well as their capacity to induce activation of naïve CD8+ T cells, serves to address the connection between altered activated set points across cell types after maternal helminth infection-induced priming, and the specific subsequent effects observed in relation to T cell responsiveness. In doing so, this chapter outlines the complexity of immune maturation changes involved within this demonstrated immunomodulatory effect, and provides specific links to the altered regulation of bystander T cell-driven inflammatory responsiveness in the form of modified DC functionality. This study therefore provides not only further evidence for the complex immune modulation that can be retained in later life from a history of maternal helminth infection during the perinatal period, which may underlie differential allergy and vaccine responses, but also the transgenerational functional memory sustained within the dendritic cell compartment.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1 Equipment

Biofuge Fresco (Small refrigerated centrifuge, radius 8.5cm)	Heraeus
Multifuge 3 S-R (Large refrigerated centrifuge, radius 19cm)	Heraeus
Cytoflex and Cytoflex S (flow-cytometers)	Beckman Coulter
Shandon Cytospin 3 (slide centrifuge)	Thermo Scientific
Sunrise (ELISA microplate reader)	Tecan
Precellys 24 (homogenizer)	Bertin Technologies
Axiovert 10 (microscope)	Zeiss
Thermocycler T3000	Biometra
Nalgene Mr.Frosty (cryopreservation container)	Sigma
Nanodrop 1000 Spectrophotometer	Thermo Scientific
Bio-Rad CFX 384 (detection thermocycler for qPCR)	Bio-Rad
Vi-Cell (cell counter and viability analyser)	Beckman Coulter
BD FACS Aria III (flow-based cell-sorter)	BD
Mo-Flo XDP (flow-based cell sorter)	Beckman Coulter

2.1.2 Reagents

Alhydrogel	Invivogen
Ammonium chloride (NH_4CL)	Roth
Bovine serum albumin (BSA)	Sigma
Brefeldin A	Sigma
CellTrace Violet (proliferation dye)	Invitrogen

Complete Freund's adjuvant (CFA)	Sigma
Collagenase D (from *Clostridium histolyticum*)	Sigma
Collagenase IV	Worthington
Cytokines (recombinant human): IL-2, TGFβ,	PeproTech
Cytokines (recombinant mouse): IL-4, IL-6, IL-12, GM-CSF	PeproTech
Deoxynucleoside triphosphate (dNTPs)	Promega
Deoxyribonuclease 1 from bovine pancreas (DNase1)	Promega
Dimethyl sulfoxide (DMSO)	Sigma
Diethyl pyrocarbonate (DEPC)-treat H_20 (molecular biology grade)	Roth
Dithiothreitol (DTT)	Roth
Dulbecco's Phosphate buffered saline PBS (cell culture-grade)	Sigma
Dulbecco's PBS (powder)	Biochrom
Eosin 1% (v/v)	Morphisto
Ethidium monoazide bromide (EMA)	Invitrogen
Ethylenediaminetetraacetic acid (EDTA)	Roth
Haematoxylin	Morphisto
Ionomycin	Sigma
Lipopolysaccharide (LPS)(isolated from *E.coli* 055:B5)	Sigma
Methanol	Roth
Narcoren	Boeringer Ingelheim
OVA (chicken egg white albumin) grade V and grade VI	Sigma
Peptides: $OVA_{323-339}$ and $OVA_{257-264}$ (SIINFEKL	peptides&elephants
Phorbol 12-myristate 13-acetate (PMA)	Sigma
Potassium hydroxide (KOH)	Merck
Propidium Iodide (PI)	Biolegend
RNAlater	Ambiont
Roti Histofix 4% (v/v)	Roth

3,3', 5,5'-Tetramethylbenzidine (TMB) substrate	eBioscience
Tris(hydroxymethyl)aminomethane (TRIS)	Roth
Trypan blue solution 0.4% (v/v)	Sigma
Tween 20	Sigma

2.1.3 Culture Media and Supplements

Fetal Calf serum (FCS)	PAA
β-Mercaptoethanol for cell culture	Gibco
Non-essential amino acids (100x)	PAA
Penicillin/Streptomycin (100x)	PAA
RPMI 1640 (with L-Glutamine)	Sigma
Sodium Pyruvate (100nM)	PAA
Zombie Aqua and NIR (fixable live/death dyes)	Biolegend

2.1.4 Buffers

ACT buffer (erythrocyte lysis)	17 mM 160 mM pH 7.2	TRIS NH_4CL
Complete RPMI medium	1x 10% (v/v) 1% (v/v) 1% (v/v) 1% (v/v) 0.1% (v/v)	RPMI 1640 FCS Penicillin/Streptomycin Non-essential AAs Sodium pyruvate β-Mercaptoethanol
Lung Digest Solution	1x	PBS

	1mg/ml 0.5mg/ml 2%	Collagenase D (Roche) DNase1 (Roche) FCS
DC Digest Buffer		Collagenase IV (Worthington) DNase1 (Roche)
FACS Buffer (FACS bf)	1x 2% (v/v)	PBS FCS
MACS Buffer	1x 2mM 0.5% (v/v)	PBS EDTA FCS
ELISA washing buffer	1x 0.05% (v/v)	PBS (from powder) Tween 20
ELISA stop solution	2 M	H_2SO_4

2.1.5 Anti-Mouse Facs Antibodies

Target	Fluorochrome	Clone	Dilution	Provider
CD4	APC	GK1.5	1:200-1:400	eBioscience
CD4	PE-Cy7	RM4.5	1:200-1:400	eBioscience
CD4	BV421	GK1.5	1:200-1:400	Biolegend
CD4	eFluor 506	RM4.5	1:200-1:400	eBioscience
CD8	PerCP-Cy5.5	53-67	1:200	eBioscience
CD8	APC	53-67	1:200	Biolegend

CD8	APC H7	53-67	1:200	BD
CD8	FITC	53-67	1:200	eBioscience
CD8	AF594	53-67	1:200	Biolegend
B220	FITC	RA3-6B2	1:200-1:400	BD
B220	PE	RA3-6B2	1:200-1:400	BD
B220	APC H7	RA3-6B2	1:200-1:400	Biolegend
CD44	PE-Cy7	IM7	1:300	eBioscience
CD44	PE	IM7	1:300	eBioscience
CD44	FITC	IM7	1:300	BD
CD62L	FITC	MEL-14	1:200	BD
CD62L	PE	MEL-14	1:200	BD
CD62L	eFluor450	MEL-14	1:200	eBioscience
CD45.1	APC	A20	1:200	Biolegend
CD45.1	FITC	A20	1:200-1:400	Biolegend
CD45.1	BV650	A20	1:200-1:400	Biolegend
CD45.1	AF700	A20	1:200-1:400	Biolegend
CD45.2	FITC	104	1:200-1:400	Biolegend
CD3	PerCP	145-2C11	1:200	Biolegend
CD3	FITC	17A2	1:200	eBioscience
CD19	PE	eBio1D3	1:200-1:400	eBioscience

CD69	FITC	H1.2F3	1:100	eBioscience
IL-4	APC	11B11	1:200	Biolegend
IL-4	FITC	11B11	1:200	Biolegend
IL-4	PE	11B11	1:200	Biolegend
IL-13	eFluor450	eBio13A	1:200	eBioscience
IL-13	PE	eBio13A	1:200	eBioscience
IL-17	FITC	eBio13A	1:200	eBioscience
IL-17	eFluor450	eBio17B7	1:200	eBioscience
IFNγ	PE-Cy7	XMG1.2	1:200	eBioscience
IFNγ	APC	XMG1.2	1:200	eBioscience
IFNγ	PE	XMG1.2	1:200	eBioscience
IL-10	AF488	JES5-16E3	1:200	Biolegend
IL-10	PE	JES6-5H4	1:200	eBioscience
IL-2	APC	JES6-5H4	1:200	eBioscience
TNF	APC	MP6-XT22	1:200	eBioscience
GATA3	PE	16E10A23	1:200	Biolegend
T-bet	APC	4BIO	1:200	Biolegend
FoxP3	eFluor450	FJK-16S	1:200	eBioscience
FoxP3	FITC	FJK-16S	1:200	eBioscience
FoxP3	PE	FJK-16S	1:200	eBioscience

RORγt	APC	B2D	1:200	eBioscience
PD-1	APC	RMPH-30	1:200	Biolegend
SiglecF	PE	E50-2440	1:200	BD
XCR1	BV650	ZET	1:200	Biolegend
CD11b	BV510	M1/70	1:200	Biolegend
CD11b	PE	M1/70	1:200	BD
CD11c	PE-Cy7	N418	1:200	Biolegend
MHC II (I-A/I-E)	eFluor450	114.15.2	1:200	eBioscience
F4/80	PE	BM8	1:200	eBioscience
CD64	PerCP-Cy5.5	4-5/7.1	1:200	Biolegend
CD24	AF700	M1/69	1:200	Biolegend
MHC I (H2-Kd)	APC H7	SF1-1.1	1:200	Biolegend
CD80	FITC	10A1	1:200	Biolegend
CD86	APC H7	GL-1	1:200	Biolegend
CD40	PE	44986	1:200	Biolegend
CD103	PE	M290	1:200	BD
CD25	PE	PC61.5	1:200	eBioscience

2.1.6 Antibodies For ELISA, Standards, and Culture

Name	clone:	conjugate:	dilution:	provider:
Anti-mouse IgE	RME-1	biotin	1:400	Biolegend
Anti-mouse IgG1	RMG1-1	biotin	1:400	Biolegend
Anti-mouse IgG2a	RMG2A-62	biotin	1:400	Biolegend
Mouse Anti-OVA IgE	2C6	none	Standard curve	Bio-Rad
Mouse Anti-OVA IgG1	TOSG1C6	none	Standard curve	Biolegend
Mouse Anti-OVA IgG2a	TOSGAA1	none	Standard curve	Biolegend
Anti-mouse CD3	145-2C11	none	See culture protocol	eBioscience
Anti-mouse CD28	37.51	none	As above	eBioscience
Anti-mouse IL-4	11B11	none	As above	Biolegend
Anti-mouse IFNγ	R4-6A2	none	As above	Biolegend
Anti-mouse CD16/32 (Tru Stain FcX Fc-block)	93	none	1:1000	Biolegend

2.1.7 Primers and Custom Probe for S.mansoni Stool PCR

Forward Primer	5’ CAACCGTTCTATGAAAATCGTTGT 3’
Reverse Primer	5’ CCACGCTCTCGCAAATAATCT 3’
Dual labelled probe (with 5’ and 3’ modifications)	5’ [6FAM]TCCGAAACCACTGGATTTTTATGAT[BHQ1]3’

2.1.8 Kits and Systems

Cytofix/Cytoperm (fixative and wash buffer concentrate)	BD
Diff-Quik staining kit	Medion Diagnostics

DNeasy stool DNA isolation kit	Qiagen
GenElute Mammalian Total RNA Miniprep Kit	Sigma
GoTaq DNA polymerase and reaction buffer	Promega
FoxP3 staining buffer kit (fixative and wash buffer concentrates)	eBioscience
Light Cycler 480 Master Mix and Universal Probes	Roche
Mouse MACS isolation kits (CD4CD62L, CD11c, Pan T-cell)	Miltenyi Biotec
Mouse Ready-Set-Go ELISA kits (IL-4, IL-5, IL-6, IL-12, TNF, IFNγ)	eBioscience
Mouse Duo-Set ELISA kit (IL-10)	R&D
Mouse T cell activation/expansion kit (with anti-CD3 and anti-CD28)	Miltenyi Biotec

2.1.9 Software

FlowJo 10	Treestar
GraphPad Prism 8	GraphPad Software
Magellan	Tecan
Nanodrop1000 V 3.7.0	Kisker
Bio-Rad CFX Manager	Bio-Rad
R Studio	RStudio PBC

2.2 METHODS

2.2.1 Mousekeeping, *Schistosoma mansoni* Lifecycle and Infection

Animal experiments were performed under national and European Union guidelines 2010/63, and in accordance with specific approval by regional governmental authorities of Upper Bavaria (license AZ ROB-55.2-2532.Vet_02-17-145, as well as earlier licenses 12-67 and 14-115). BALB/c and C57BL/6J mice were purchased from Envigo (Germany). CD45.1+/+ Rag1-/- OT-I TCR-transgenic mice as well as OT-2 transgenic mice on a C57BL/6 background, were maintained at our in-house breeding facility. All mice were fed standard chow and housed under specific pathogen-free conditions at the Institute of Medical Microbiology, Immunology, and Hygiene (MIH), Munich, Germany.

Biomphalaria glabrata snails, infected with the Brazilian strain of *Schistosoma mansoni*, were placed in distilled water under warm light. Released cercariae were counted microscopically, and 150-250 were injected subcutaneously into 3-week-old male NMRI or Swiss Webster mice. Following 8 weeks development towards patent-infection (assessed through visual inspection of liver and intestines following sacrifice via cervical dislocation), miracidiae were isolated from intestines and further used to infect additional snails.

2.2.2 Model of Maternal Chronic Schistosomiasis

For experimental infection, 6-8-week-old female C57BL/6 or BALB/c mice were infected via subcutaneous injection in the flank with 180 or 90 *S. mansoni* cercariae, obtained as per 2.2.1. After 16 weeks of infection, these were mated with naïve male mice of the appropriate strain. For congenic labelling of mice, CD45.1/1 male mice were mated with infect and non-infected C57BL/6 females. Following weaning of offspring at 21-days of age, infected dams were euthanised with sodium-pentobarbital (Narcoren, Boeringer Ingelheim) at a dose of 5ml/kg (800mg/kg) bodyweight, with normal dosage of 50µl for a 20g mouse (to enable worm observation in the portal vasculature). These were then definitively assessed for degree of infection via determining *S. mansoni* egg numbers per liver as described previously (Straubinger et al. 2014). Briefly, the entire liver was removed, weight, and a small

piece (0.05-0.1g) weighed out, then dissolved (shaking at 37°C for 2h) in 5ml of 5% (w/v) KOH solution. The resultant digest was centrifuged (480g x 5min). Excess 4.5ml liquid was removed, leaving 500µl. This was resuspended and 10µl was inspected microscopically for number of eggs, performed in triplicate, with the mean egg number per 10µl then used to extrapolate total number of eggs in the piece (as within the total 500µl digest) and then in the total liver, according to entire weight. Uninfected age-matched female mice, originating from the same batch served as control, housed for the same time period as chronic infection, usually in a neighbouring cage of the same row and rack.

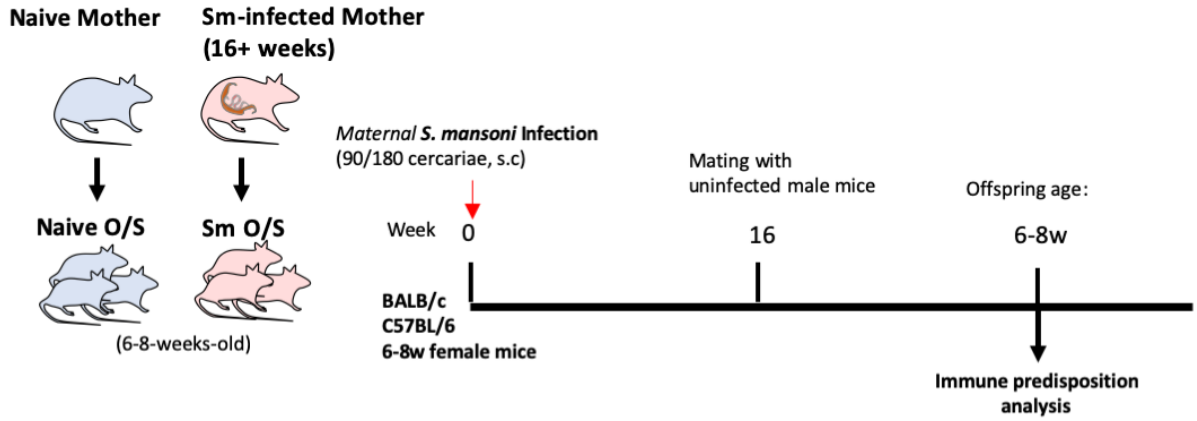


Figure 2-1 Model of Perinatal Exposure to Schistosomiasis through Maternal Infection

For an infection-free model of transmaternal schistosomiasis exposure, dams were injected twice with 5,000 schistosome eggs (as purified from the livers of hamsters or mice, as per SEA preparation) in 200µl of PBS i.p, on day 0 and 7, before mating with male mice on day 14.

Initial experiments used qPCR-based determination of schistosome DNA in stool as a diagnostic measure of maternal infection. Briefly, mice were separated for 48h and stool collected, from which DNA was extracted using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. qPCR was then performed using the following reaction mix and settings:

reagent:	[conc]	
LightCycler 480 Master Mix	10µl	
Forward Primer 10µM	1µl	Run in a CFX384 Thermocycler, Bio-Rad:

Reverse Primer 10µM	1µl	Hot Start: 5min, 95°C
Custom Probe (1:16 pre-dilution)	1µl	Amplification: 10s, 95°C
DEPC H_20	5µl	30s, 58°C (38x)
Sample DNA	2µl	Cooling: 15s, 45°C

dH_20 alone or stool from uninfected mice was used to determine a negative cut-off, however false-positive results, potentially caused by cross-contamination through coprophagy, lessened the utility of this method. As an alternative, measurement of SEA-IgG1 and SEA-IgE in serum was performed with in-house ELISA as adapted from (Hoffmann, Cheever, and Wynn 2000). Briefly, 1µg of SEA in 50µl PBS was used to coat each well of a 96-well microtitre plate overnight (NUNC). After washing 3x with PBST, these were blocked for 2h at RT with 3% (w/v) in PBS. After a further 3x washing, 100µl of serum diluted in BSA blocking buffer was probed for 2h at RT, washed again, then probed with 100µl detection antibody (biotinylated anti-mouse IgE or IgG1) diluted at 1:400 for 2h at RT. After washing, detection antibody was conjugated to 50µl Avidin-HRP peroxidase diluted 1:1000 for 30min at RT (in the dark), washed, then 50µl undiluted TMB substrate (eBioscience) was added per well and allowed to develop in the dark, before stopping with 50µl of 2 N H_2SO_4. Again, false positives as animals injected with cercariae acquired substantial antibody titres, irrespective of subsequent clearance. However, this did prove to be effective using a method of grading of infection using a cut off value (determined on a case-by-case basis through visual assessment of livers for mice with the lowest antibody titres, ascending until substantial levels of infection were reach as determined by visual inspection of livers.

For later experiments, high infection rates and relative inaccuracy of tests precluded the need for additional diagnostic testing, although liver palpation through abdominal skin served to confirm well-defined liver fibrosis typical of chronic infection.

For more detailed assessment comparing infection between Sm offspring and naïve offspring, granuloma area was calculated from formalin fixed (4%) paraffin-embedded cut sections (4µM) of liver and intestine samples after standard H & E staining and microscopic analysis as previously described

(Layland, Wagner, and da Costa 2005) using up to 40 granuloma per mouse to calculate an average area μm^2 per mouse.

2.2.3 Inflammation and Immunisation Models

OVA-AAI was induced in 6-8-week-old C57BL/6 mice as previously described (Conrad, Yildirim, et al. 2009) (Ritter et al. 2014) according to scheme in Fig. 3.1. On days 0, 7, and 21, 10µg ovalbumin (grade VI, Sigma) suspended in 200µl sterile PBS was administered per mouse via subcutaneous injection in the flank (control animals received only PBS). On days 28, 29, and 30, mice were challenged with 1% w/v nebulised ovalbumin (grade V, Sigma) in PBS, for 20min per day. 48h following the third challenge, mice were euthanised with Narcoren as described above. Adjuvant-free OVA sensitization ("**OVA-s.c**") was performed similarly to OVA-AAI, with mice analysed one day after the final injection on day 22. **OVA-TR** variation on OVA-s.c immunization using Texas Red-labelled fluoresecent OVA (Thermofischer), with 10µg/200µl given s.c in the flank as per unlabelled OVA. **OVA-ALUM** alum-adjuvanted immunization was performed via intraperitoneal (i.p) injection of 10µg OVA precipitated in a total volume of 200µl of 1:1 PBS and aluminum hydroxide gel (Alhydrogel®, Invivogen). **CFA-OVA** immunization with complete Freund's adjuvant (CFA)(Sigma) was also performed 1:1 with 10µg OVA in PBS, injecting 100µl total final volume s.c. Modified Vaccinia virus Ankara (MVA) expressing OVA (**MVA-OVA**) was produced as previously described (Thiele et al. 2015), and 10^8 IU (infectious units) was delivered i.p in 200µl PBS per mouse.

2.2.4 Protein/Adjuvant and Viral-vector-based Hepatitis B Immunization and Viral Challenge

The murine model of HBV infection used a "prime-boost" plus challenge system as modified from (Kosinska et al. 2019). Briefly, a priming immunization with Hepatitis B surface antigen (HBsAg) (5µg expressed endotoxin-free in yeast) was adjuvanted with 10µg cyclic di-adenylate monophosphate (ci-di-AMP, Invivogen) and administered i.p on day 0, followed on day 14 by viral-vector-based boost with Hepatitis B core antigen- (HBcAg) and HBsAg -expressing recombinant MVA (**MVA-HBV**), with 10^7 IU given i.p in 200µl PBS, or PBS alone to non-boosted groups. Acute viral challenge was administered

on day 28 with an adenoviral vector-based delivery of HBV genomes (**Ad-HBV**) produced as previously described (Loffredo-Verde et al. 2019), with 10^8 IU delivered i.v two weeks prior to end analysis. To assess viral copies as a measure of viral load or persistence, DNA isolated from liver using DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions and was assessed via real time quantitative PCR using SYBR Green I Master Mix (Roche) for HBV genome copy numbers (as used in (Loffredo-Verde et al. 2019)) in collaboration with the Institute for Virology, TUM.

2.2.5 Dissection and Organ Preparation

After euthanasia using cervical dislocation or Narcoren, mice were sprayed with 70% EtOH and removed organs were placed in ice-cold sterile PBS. For RNA analysis, small samples of organs were cut and placed immediately in Eppendorf tubes the flash frozen in liquid nitrogen, then stored at -80°C. Blood was collected either directly from the heart, or via buccal bleeding, transferred into treated collection tube then centrifuged at 13,000 RPM (9500g) x 5min at RT, and serum (supernatant) collected and stored at -20°C for further use. OVA-specific antibody titres in sera were measured by in-house ELISA as previously (Straubinger et al. 2014), using similar protocol to SEA-specific ELISA, as above, coating plates instead with 1µg/ml OVA (grade VI) in 100µl PBS per well O/N.

Bronchoalveolar lavage (BAL) cells were obtained and enumerated as previously described (Straubinger et al. 2014) for microscopic analysis of eosinophil content. After sacrificing mouse, chest was opened up to the mouth, cutting along the sternum, cutting away excess rib cage, and careful not to rupture vessels near shoulders. Cutting away revealed the trachea, and after sliding closed scissors underneath trachea to isolate it, trachea was gently lifted with scissors to isolate it, while opening the scissors slightly to release the trachea somewhat from the underlying tissues., and uppermost part of trachea was simultaneously clamped. 1ml of ice-cold sterile PBS was inserted directly longitudinally into trachea with a 1ml syringe using a 23-gauge Sterican needle and used to inflate the lungs, plunging up and down a few times, before final aspiration of all fluid, which was ejected into a 1.5ml Eppendorf tube, weighed, and centrifuged (2000 RPM [380g] x 5min at 4°C). Supernatants were retained at -20°C for further assessment, and cell pellet resuspended in 200µl PBS, with 20µl aliquot diluted 1/10 in PBS for counting

using trypan-blue-based cell counting and viability assessment using a Vi-Cell XR (Beckmann Coulter). 150µl of remaining cell suspension (subject to standardisation based on cell count) was then centrifuged onto glass slides using Shandon Cytospin 3 Centrifuge (Thermo Scientific) using 400 RPM x 7min eith medium acceleration. After removal from cassettes and overnight drying, slides with Diff-Quick staining kit (Medion Diagnostics, Langen, Germany), with slides dipped 5 times in fixative solution (containing Fast Green in methanol), then 5 x 1 second in Stain Solution I (containing eosin Y, with sodium azide preservative), then 5 x 1 second in Stain Solution II (contains thiazine dye). Slides were rinsed with dH_20, then again allowed to air-dry overnight. Cells were then assessed microscopically to determine numbers of macrophages, eosinophils, neutrophils, and lymphocytes, in 100 counted cells, which was further extrapolated using BAL total cell count.

PBS-perfused lungs (10ml PBS delivered directly into tip of heart after cutting inferior vena cava, until lungs turn white) were minced (with sterile small scissors, directly in Eppendorf tubes until mostly homogenised and no large pieces remained, roughly 2min, in 500µl RMPI) prior to 30min shaking at 37°C enzymatic digestion by adding another 500µl per sample of 2x Lung Digest Solution, to allow extraction of lung infiltrating lymphocytes. For assessment of DCs and other APCs, spleens and lymph nodes were subjected to similar mincing before digest using for 20min shaking at 37°C.

Single cell suspensions were made by passing spleen, lymph node, or digested lung samples through 70µm strainers in PBS, centrifugation (480g x 5min at 4°C), supernatants discarded and then erythrocytes-depleted using ACT buffer (5min at RT) before a further centrifugation (ACT for spleen and lung samples only), supernatants again removed and then resuspended in 1ml of complete RPMI for Vi-Cell-based counting as before. For *ex vivo* stimulations, cell suspensions were standardised to 5x10^6/ml (to give 5x10^5 cells per 100µl aliquot), and then stimulated with the following conditions, at 200µl final volume for bulk determination of cytokine release via ELISA-based detection of secreted cytokines in supernatants, or 250µl final volume (after addition of BFA) for intracellular cytokine staining (ICS) analysis via FACS. DC-enrichment was performed via positive selection using anti-mouse CD11c UltraBeads, as was pan-T-cell pre-enrichment or CD4CD62L+ enrichment, all using MACS kits with LS or MS columns (Miltenyi Biotec) according to manufacturer's instructions.

2.2.6 Flow Cytometry and Cell Sorting

Staining for flow-cytometry was generally performed in 96-well plates. Dead cells were excluded by staining with 50-100µl ethidium monoazide bromide (EMA) (Invitrogen), diluted at 1:1000 in FACS bf and stained for 20min under light on ice, or Zombie Aqua or NIR (Biolegend), diluted at 1:1000 in PBS, stained in the dark at 4°C for 20min. To wash, this was then diluted up to 200µl with FACS bf and centrifuged (1350 RPM [387g] for 4min at 4°C, before proceeding to staining. Alternatively (and in the case of flow-sorting) dead cells were excluded using final suspension after staining in 1:1000 propidium iodide (PI) (Biolegend). After washing, surface staining of cell was performed in FACS bf for 20min at 4°C in the dark. At this point, for flow-sorting, cells were filtered using 40µM filters into 5ml FACS tubes, and desired populations sorted directly into FCS. After sorting, these were centrifuged and re-suspended in FCS with 10% DMSO (Sigma) for cryopreservation using a freezing container ("Mr. Frosty") (Nalgene/Sigma) for gradual freezing at -80°C, or re-suspended in RPMI medium or FACS bf for further use.

For ICS, cells were then washed again, and concurrently fixed / permeabilised for 30min at 4°C in the dark using 50-100µl of Cytofix/Cytoperm (BD) solution and subsequent washing and intracellular staining steps performed using Fix/Perm wash buffer (BD). For intranuclear staining of transcription factors, FoxP3 staining buffer kit set (eBioscience) was used, with fixation performed for 20min at RT in the dark. In each case, after washing, subsequent intracellular/nuclear staining was performed in the respective buffer for 30min at 4°C in the dark, before final washing and resuspension in 100-200µl FACS bf for acquisition. If required, and additional fixation step in 2% paraformaldehyde (Roti Histofix, Roth) was performed for 20min at 4°C, then washed and left until analysis.

Data was acquired using a Cytoflex S (Beckman Coulter) and analyzed in FlowJo 10 (TreeStar).

2.2.7 T Cell Adoptive Transfer

For experiments where immunization was paired with adoptive T cell transfer, CD8+CD44^{lo} cells were flow sorted from the erythrocyte-depleted single-cell suspensions of spleens and lymph nodes of OT-I

donor mice (or similar CD4+ T cells from organs of OT-2 donor mice). 1-2x10^5 naïve OT-I/2 cells were administered i.p or intravenously (i.v) to recipient mice one day prior to subsequent immunization, with prior staining with CellTrace Violet (Invitrogen) for proliferation analysis.

2.2.8 Cell Stimulation and Co-Culture

Ex vivo stimulation of cell suspensions was for 5h with OVA (grade VI, 100µg/ml), OVA immunodominant peptides including $OVA_{323-339}$ and $OVA_{257-264}$ (SIINFEKL) (1µg/ml, peptides&elephants), or phorbol 12-myristate 13-acetate (PMA, 50ng/ml, Sigma) and ionomycin (1µg/ml, Sigma), all in the presence of Brefeldin A (BFA, 10µg/ml, Sigma)(in some cases, protein/peptide stimulation was overnight, and the following day proceeded with an additional 4-6h culture with BFA). For 48-72h bulk assessment of secreted cytokines in supernatant, cell suspensions were treated with OVA or SEA (20µg/ml), or anti-T cell receptor (TCR) stimulation via anti-CD3/CD28 (1µg/ml each). Unstimulated controls standardised to similar volumes using medium alone. For HBV experiments, splenocyte cell suspensions were re-stimulated for 5h with HBcAg (10µg/ml) or TCR stimulus for 48h.

Sorted naïve CD8+ T cells were co-cultured (in a ratio of 2:1) with bone-marrow derived dendritic cells (BMDCs) that had been prepared over 7 days as previously described (Bhattacharjee et al. 2019) from the bone marrow of C57BL/6J donor mice with 20ng/ml recombinant GM-CSF (Miltenyi Biotec). Along with the addition of anti-CD3 (clone: 145-2C11, eBioscience) at the time of co-culture to overcome antigen specificity, the BMDC had been either pre-pulsed with OVA protein (100µg/ml), or with BMDCs pre-infected with MVA-OVA (90min with 2.5x10^5 IU). At 72h supernatant was sampled for analysis of cytokine secretion via ELISA, and followed by 6h additional stimulation in the presence of BFA followed by ICS staining. Additional experiments evaluated the suppressive effects of B cells on this process included the addition of flow-sorted (B220+ Lymphocytes) B cell at a ratio of 1:1 with OT-1/2 cells. Additional experiments used such BMDCs to evaluated the antigen-specific response of CellTrace stained CD4+CD44+ flow-sorted T cell from Sm offspring and naïve offspring, and were in this case pulsed with 20mg/ml SEA. DC-enriched splenocytes were cultured for 72h with or without

2ng/ml LPS (isolated from *E.coli* 055:B5, Sigma Aldrich), or loaded with 1µg/ml $OVA_{257-264}$ SIINFEKL and cultured 1:1 with flow-sorted naïve OT-I CD8+ T cells, pre-stained with CellTrace, as above.

2.2.9 T Cell Differentiation Assays

To analyse the capacity to differentiate into T helper subsets during activation of naïve CD4+ T cells, 100,000 MACS (CD4+CD62L+) or flow-sorted (CD4+CD62L+ $CD44^{lo}$) isolated cells were plated per well of a 96-well cell flat-bottom culture plate, that had been pre-coated with 50µl of 5µg/ml of anti-mouse CD3 in 50mM TRIS for 2h at 37°C (CD3-coating buffer aspirated just before adding CD4+ T cells). 100µl of one of the following polarisation master mixes was then added (containing combinations of cytokines and antibodies):

T_H type:	reagent:	[final conc]	T_H type:	reagent:	[final conc]
T_H0:	a-IL-4	10µg/ml	T_H17:	rm-IL-6	20ng/ml
	a-IFNg	5µg/ml		a-IL-4	10µg/ml
	rh-IL-2	10ng/ml		a-IFNg	5µg/ml
	a-CD28	1µg/ml		rh-TGFb	0.5ng/ml
				a-CD28	1µg/ml
T_H1:	rh-IL-2	10ng/ml			
	IL-12	20ng/ml	Treg:	rh-IL-2	20ng/ml
	a-IL-4	10µg/ml		a-IL-4	10µg/ml
	a-CD28	1µg/ml		a-CD28	0.5µg/ml
				a-IFNg	5µg/ml
T_H2:	rh-IL-2	10ng/ml		rh-TGFb	2ng/ml
	rm-IL-4	20ng/ml			
	a-IFNg	5µg/ml			
	a-CD28	1µg/ml			

T_H1 and T_H2 cells were cultured over 6 days, with cells divided into 2 wells and topped up to 200µl with complete RPMI on day 3. T_H17 and Treg polarisations assessed at 72h. T_H0 cells were cultured alongside

other subtypes as a control. Induced parameters in polarised cells were assessed via flow-cytometry, transcription factors via intranuclear staining immediately upon harvest, or cytokine production after 4h additional culture with PMA/ionomycin and BFA, as described above.

2.2.10 Transcriptomic Analysis Via RNA-Seq

Total RNA from snap frozen organs derived from steady state or OVA-AAI BALB/c mice (igLN, spleens, or lungs), or from cryopreserved sorted naïve CD4+ or CD8+ ($CD44^{lo}CD62L+$) T cells, was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and diluted to 5mg/ml, prior to transcriptomic analysis performed through TranslaTUM-based core facility housed within laboratory group of Prof. Roland Rad, where library preparation for 30-sequencing of poly(A)-RNA was performed as described previously (Parekh et al. 2016) (Bhattacharjee et al. 2019). Briefly, barcoded cDNA was generated per sample with a unique molecular identifier (UMI) plus adaptors using a Maxima RT polymerase (ThermoFisher). cDNA 5' ends were extended with a template switch oligo (TSO) before full length cDNA amplification using primers directed at the TSO-site and adaptor, followed by tagmentation using Nextera XT kit (Illumina). 30 end-fragments were then amplified using primers with Illumina P5 and P7 overhangs, and the library was sequenced on a NextSeq 500 (Illumina) with 75 cycles for the cDNA in read1 and 16 cycles for the barcodes and UMIs in read2. Data processing was performed as described in (Macosko et al. 2015), where sequence reads were demultiplexed and mapped according to sample barcode and unique molecular identifier filtering, generating a differential gene expression (DGE) matrix of counts mapped to a specific gene per sample. Differential gene expression analysis between samples from Sm offspring and controls was performed using R package DESeq2 (version 1.16.1). An adjusted p-value of less than 0.1 was used as a cut-off for differentially expressed genes. Overrepresentation of highly expressed genes reduced effective read depth particularly for OVA-AAI samples from whole organs, obscuring detection of immune-related genes and pathways, particularly in lung and spleen samples.

2.2.11 Chromatin Immunoprecipitation

Potential differences in the epigenetic landscape of naïve T cells from Sm offspring and naïve offspring were analysed comparing the relative enrichment of promoter regions for T helper relevant genes with permissive histone marks. Using chromatin immunoprecipitation (ChIP), this was initially performed for CD4+CD62L+ flow-sorted naïve T cells from 3-week-old offspring to complement similar analysis of 6-8-week-old offspring previously conducted, and additionally for sorted CD8+ T cell samples (CD62L+CD44lo, from 6-8-week-old offspring), both as modified from previous studies (Harb et al. 2015) and in collaboration with members of the laboratory of Prof. Holgar Garn at Philips University of Marburg. Briefly, following cryopreservation, chromatin in CD4+ or CD8+ T cells were crosslinked using paraformaldehyde, and then were lysed and chromatin then sheared using a Bioruptor® Pico (Diagenode). Sheared chromatin was precleared with Protein-A/G-coated sepharose beads (GE Healthcare Life Sciences) and immunoprecipitated for modified histone residues via anti-H3K4me3 polyclonal rabbit antibody (Diagenode), anti-H3ac polyclonal rabbit antibody (Merck), and anti-H4ac polyclonal rabbit antibody (Active Motif). Immunoprecipitated chromatin was de-crosslinked, then DNA purified from denatured histones using Microchip Diapure columns (Diagenode) was assessed via quantitative PCR using SYBR Green (Qiagen) for enrichment of effector CD8-related gene (including *IFNG*). Target DNA sequences of the desired primers were downloaded from *UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly* and primers designed using online tool *Primer-BLAST*. Relative enrichment was calculated as: % recovery $=100* 2^{[(Ct(input) - 3{,}32) - Ct(IP)]}$, and further normalized to isotype control (IgG), and positive control gene *RPL32* as previously (Klar et al. 2017).

2.2.12 Statistical Methods

Statistical tests were performed using GraphPad Prism 8.0.2 for Windows (GraphPad Software, San Diego California USA). Data was subjected to D'agostino and Pearson omnibus normality test. Significant outliers were identified using Grubbs' test. Where specified, normalization was calculated using standard or z-values, where *Z = (x - mean of original experiment cohort) / standard deviation of original sample cohort*, which allowed re-scaling of data between repeat experiments, with normalized

value calculated as: *Z*(repeat experiment standard deviation) + mean of repeat experiment cohort*, to minimize artefacts when pooling repeat experiments. Differences between sample sets with a normal distribution were analyzed using an unpaired, two-tailed Student's t-test, while non-parametric data was assessed using an unpaired, two-tailed Mann-Whitney U-test, where p-values ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1 MATERNAL SCHISTOSOMIASIS MODIFIES ALLERGIC T CELL RESPONSES

3.1.1 Chronic Maternal Schistosomiasis Modulates OVA-AAI across Mouse Strains

Previous results from our group demonstrated that maternal infection with parasitic helminth *S. mansoni* had infection-phase-specific modulatory effects upon allergic airway parameters in a murine model using BALB/c mice, including lowering lung inflammation, and lowering splenic IL-5 responses to re-stimulation with OVA during acute and chronic maternal infection. Less leucocyte infiltration into the bronchoalveolar spaces was observed, as assessed by lavage (BAL), including a greatly reduced proportion of eosinophilic infiltration. Serum OVA-specific antibody titres also appeared affected by maternal pre-priming (Straubinger et al. 2014), although further analysis shows inconsistencies regarding Ig-responses indicated potential complexities in this model compelling further analysis. The reproducibility of this previously shown phenotype was tested in repeat experiments with additional cohorts of offspring murine BALB/c dams with chronic (16+ week) schistosomiasis (referred to here as "Sm offspring"). Additionally, similar experiments were carried out in C57BL/6 mice, assessing mouse strain specificity and further permitting mechanistic experiments using C57BL/6 and on the C57BL/6 (including CD45.1+ mice, and OT-I and OT-II mice with transgenic TCRs).

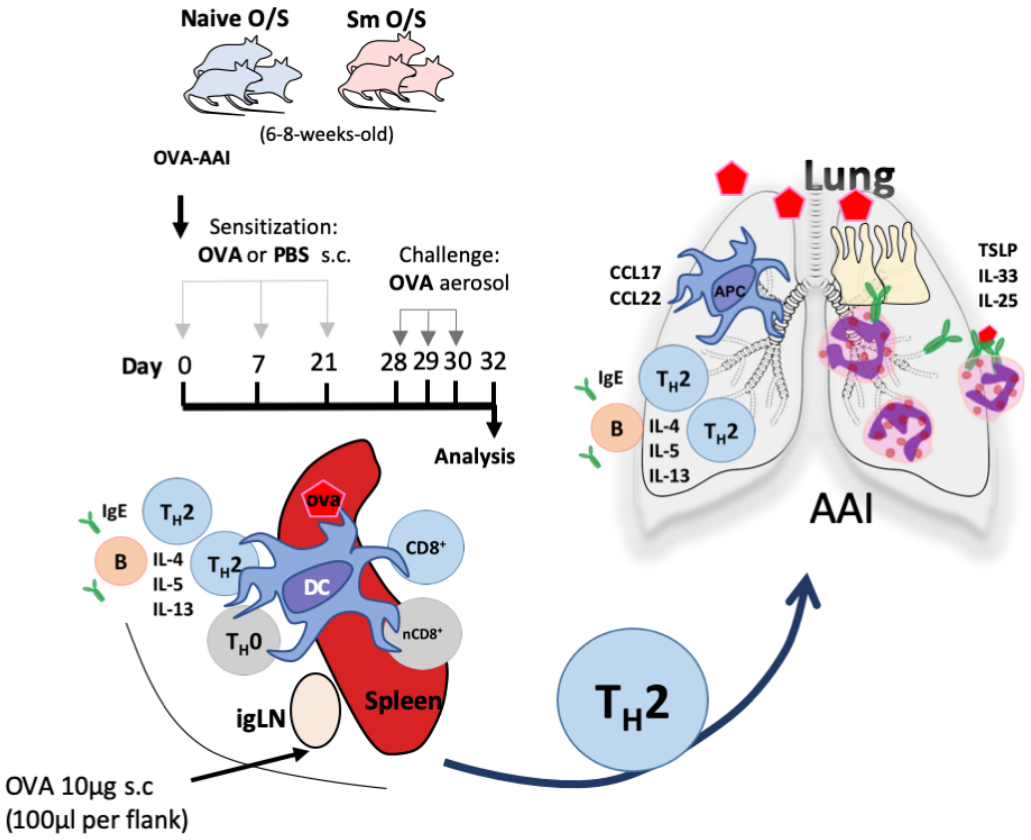


Figure 3.1 Murine Model for Transmaternal Modulation of OVA-AAI through Chronic Schistosomiasis
Transgenerational exposure to chronic maternal schistosomiasis performed by mating C57BL/6 or BALB/c female mice 16 weeks after infection, or uninfected controls. Allergic sensitization is performed through sequential subcutaneous immunisations with model allergen ovalbumin (OVA) in 6-8-week-old female offspring exposed to chronic maternal infection ("Sm offspring"), or offspring of age-matched female mice from the same cohort, expanding OVA-specific T and B cells, as well as serum OVA-specific antibody titres. Experimental allergic airway inflammation (AAI) is subsequently induced by repeated aerosol exposure to OVA, and characterised by local type 2 inflammation, including OVA-responsive T helper cells producing IL-4, IL-5, and IL-13, and local eosinophilia, with goblet cell hyperplasia.

Here, the aluminium hydroxide adjuvant used in the previous study was removed to simplify the complex immune interactions involved (Fig 3.1 B). OVA dissolved in PBS, administered subcutaneously with no adjuvant, has previously been shown to equally effective at pre-sensitising mice to a subsequent aerosol challenge with OVA to yield substantial AAI (Conrad, Yildirim, et al. 2009). The particular effectiveness of this procedure likely concurrent induction of epithelial damage signals to polarise and activate APCs, together with the presence of foreign antigen (Lambrecht and Hammad 2015). As depicted in 3.1, experimental AAI models are largely accepted to require the induction of

antigen-specific CD4+ helper T cells (Das et al. 2006) primed through successive sensitizations, drawn into the lung via local allergen challenge where they co-ordinate local inflammatory responses. As in other allergic conditions, otherwise harmless molecules coupled with damage responses lead to alarmin release (including TSLP, IL-33, and IL-25), as well as chemokines for type 2 cells including CCL17 and CCL22. More pro-allergenic compounds are those with an ability to induce such damage responses, such as through protease activity or other triggers of endogenous danger signals (Kool et al. 2008), and initiate (disproportionate) type 2 tissue healing responses, which are expanded in chronic exposures. Type 2 cytokine responses contribute, over time, to tissue remodelling and collagen deposition in a fibrotic response, evident in the shorted term through goblet cell hyperplasia and mucous overproduction, as well as (through increased IL-5) recruitment and activation of eosinophils.

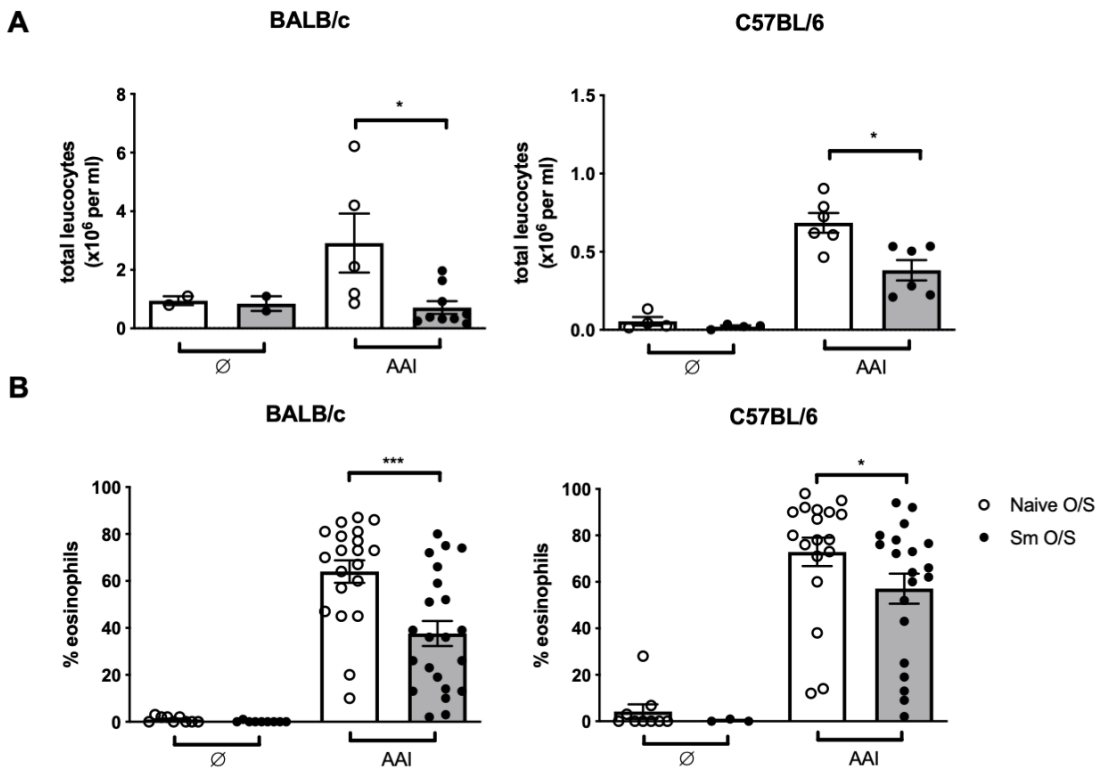


Figure 3.2 Transmaternal Schistosomiasis Effects on BAL Eosinophilia during OVA-AAI.
(A) Enumeration of absolute leucocytes in extracted bronchoalveolar lavage (BAL) fluid following AAI in BALB/c and C57BL/6 mice. (B) Percentage eosinophils in BAL. Total leucocytes each from a representative experiment, with eosinophil percentages showing pooled data from 3 independent experiments per mouse strain, each experiment with 6-10 offspring from ≥ 3 *Sm* mothers. After normality testing, differences in eosinophilia compared using Mann-Whitney U-test.

Microscopic analysis of eosinophilia in BAL fluid provides a robust indicator of OVA-AAI induction, as for untreated mice this is usually not present. Total numbers of leucocyte cells in BAL is also increased in AAI (with lymphocytes and neutrophils, as well as total macrophages contributing), to a lesser degree in Sm offspring of both BALB/c and C57BL/6 mouse strains (Fig. 3.2A). Representation of eosinophilia as a percentage of total BAL cells allows pooling of experiments, removing some variability of AAI-response magnitude between experiments, as well as technical variability in retrieval of absolute BAL cell number. Across experiments, clear and statistically significant reductions in final BAL eosinophilia is evidence in both BALB/c and C57BL/6 Sm offspring (Fig. 3.2B), recapitulating the reduced eosinophilic alveolar infiltration during AAI previously observed in BALB/c offspring of dams in the regulatory phase of schistosomiasis (Straubinger et al. 2014).

3.1.2 Transmaternal Effects on Lung CD4+ T Cell Responses during OVA-AAI

Splenic antigen-induced IL-5 production was shown to be lower in Sm offspring with AAI in our previous study, indicating a potential reduction in antigen-specific T cell responses during this model. This finding, and other immunisation-based immune challenge modulations derived from transmaternal exposure to schistosomiasis (Santos et al. 2010) (Santos et al. 2014) (Santos et al. 2016), suggest effects upon antigen-specific priming in the T cell compartment as-yet not addressed. To expand upon our previous experiments to determine the involvement of altered CD4+ responses in the AAI model, cell suspensions from digested, perfused lungs of mice, with and without AAI, were re-stimulated with OVA in the presence of BFA to assess their responsiveness in terms of cytokine production. Perfusion prior to dissociation was used to remove circulating cells and instead focus analysis on cells infiltrating the lung tissue itself.

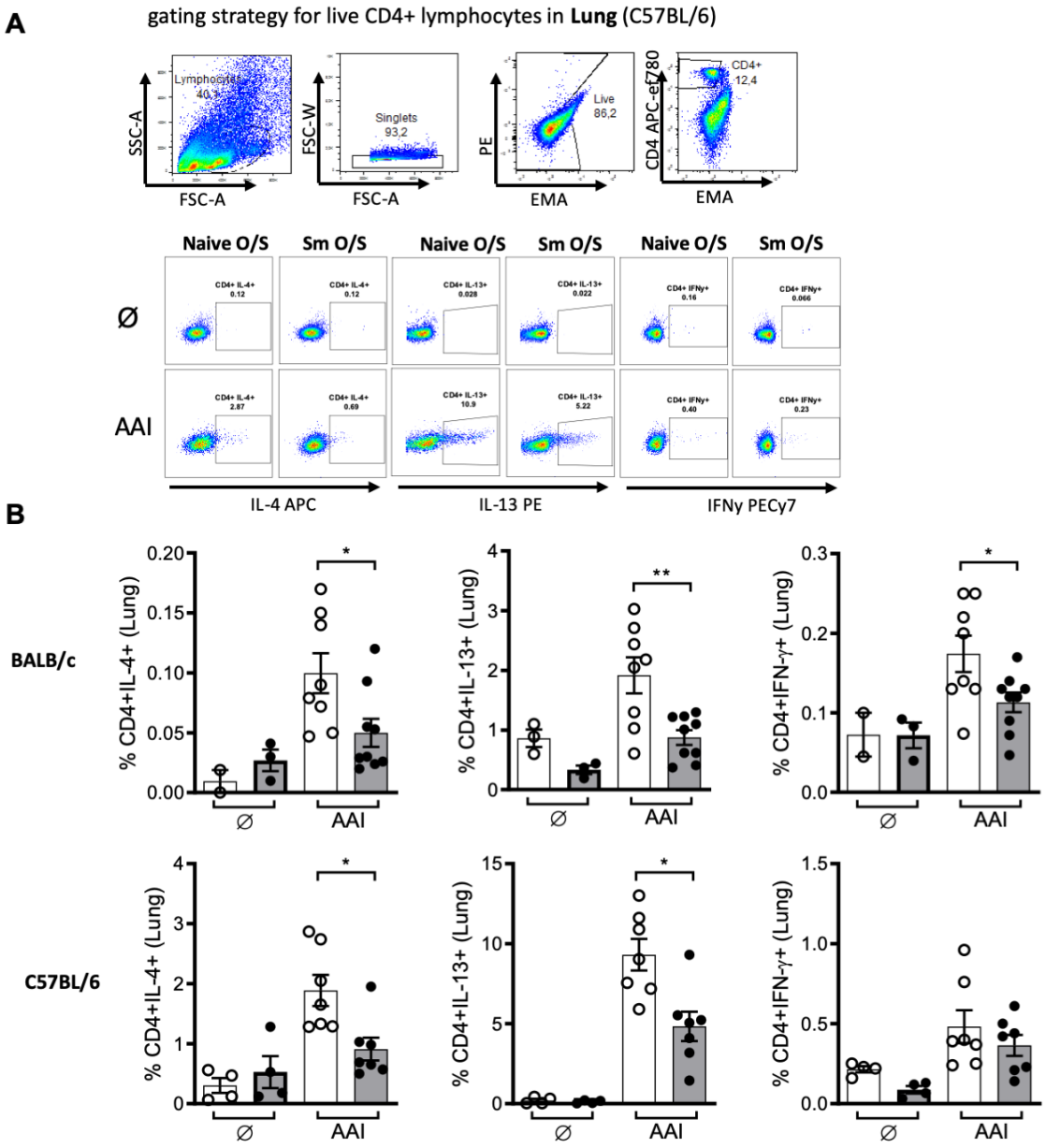


Figure 3.3 Antigen-Induced CD4+ T Cell Cytokine Responses in Lung during OVA-AAI
(A) Representative flow cytometry plots showing gating strategy for identifying live CD4+ lymphocytes, and proportions cytokine-positive cells (B) Proportions of CD4+ T cells positive for intracellular cytokines IL-4, IL-13, and IFNγ as examined via flow cytometry in cell suspensions from digested, perfused lungs following re-stimulation for 5h with ovalbumin and BFA prior to staining. C57BL/6 data AAI data shows 7 female mice per group, with Sm offspring derived from 3 individual infected mothers, representative from one of two independent experiments. BALB/c FACS shows data for 8-9 OVA-AAI offspring per group (each dot representing the stimulated lung cells of a single offspring mouse), with Sm O/S derived from 4 mothers, and C57BL/6 FACS data has 7 offspring per group representative of 2 independent experiments, with Sm O/S derived from 3 mothers.

After identification of CD4+ cells in the lung suspensions, intracellular cytokine staining revealed that those cells infiltrating the lungs of Sm offspring contained fewer CD4+ helper T cells positive for type 2 cytokines IL-4 and IL-13 compared to naïve mice. This was statistically significant for both BALB/c and C57BL/6 mouse models. Less CD4+ T cells in the lung were positive for IFNγ in BALB/c Sm offspring compared to naïve counterparts, with a non-statistically significant similar trend observable in the C57BL/6 groups (Fig. 3.3B).

These results support the hypothesis that dampening of inflammation in Sm offspring is associated with modifications to T cell functionality. In the type 2 prone inflammatory environment of the allergic lung, this includes altered cytokine production in an antigen-specific manner, of both type 2 (IL-4 and IL-13 measured), and type 1 (IFNγ) cytokines. These differences could result from lower recruitment of these T cells to the lung niche during challenge-based inflammation, or may result from differences in initial priming of antigen-specific T cells during the sensitisation phase, potentially via reduced expansion leading to lower frequencies of activated memory cells, or their reduced reactivity.

3.1.3 Maternal Schistosomiasis Skews Allergen-specific Antibody Responses

Alongside innate and adaptive cellular responses to allergen, the immune cascade in AAI also can involve allergen-specific immunoglobulins, although some studies show that while these can play a role in this process, they may not be required to develop inflammation. Specifically, antibodies of the IgG1 and IgE subclasses derived from class-switching of responsive B cells in the presence of type 2 help from T cells. These contribute to allergen recognition by binding receptors on mast cells and basophils, with allergen-mediated cross-linking promoting their effector functions in a type 1 hypersensitivity response.

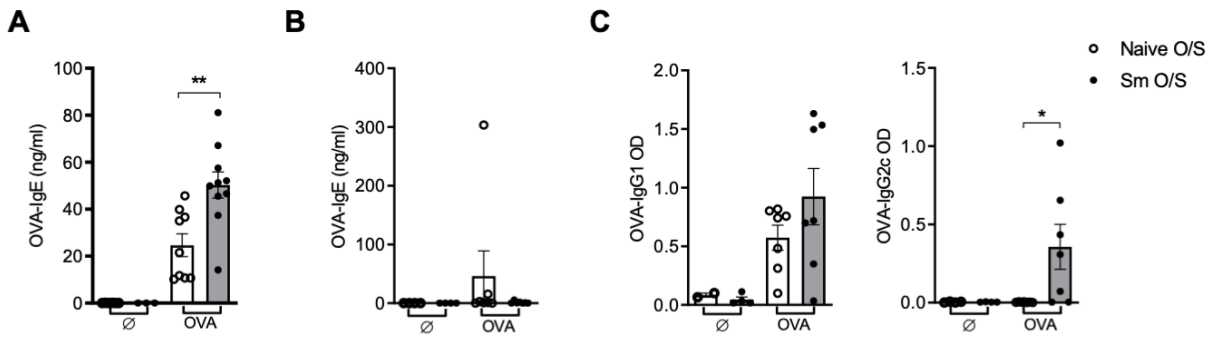


Figure 3.4 Serum Levels of OVA-Ig during OVA-AAI
Comparison of OVA-specific IgE levels in serum (as measured by ELISA) following OVA-AAI induction in both **(A)** BALB/c and **(B)** C57BL/6 Sm offspring versus controls, with **(C)** showing OVA-specific IgG1 IgG2A levels in the latter. Showing experimental groups as per Fig. 3.3.

Both BALB/c and C57BL/6 Sm offspring show clearly reduced lung inflammation and eosinophilia in this AAI model. In measuring OVA-specific antibodies by ELISA, further analysis of BALB/c Sm offspring reveals enhanced serum levels of ovalbumin-specific IgE antibodies, otherwise also taken as a parameter of allergic responsiveness (Fig. 3.4A). C57BL/6 appear to produce, overall, lower levels of OVA-IgE, regardless of maternal exposure (Fig. 3.4B), but further analysis of other OVA-specific antibody subclasses, IgG1 and IgG2c, reveal similar trends towards higher titres indicating increased antigen-specific humoral responsiveness in Sm offspring. This could be further indicative of systemic polarisation effects that are not reflected in the lower cytokine responses so far observed in the lungs of Sm offspring after AAI-induction.

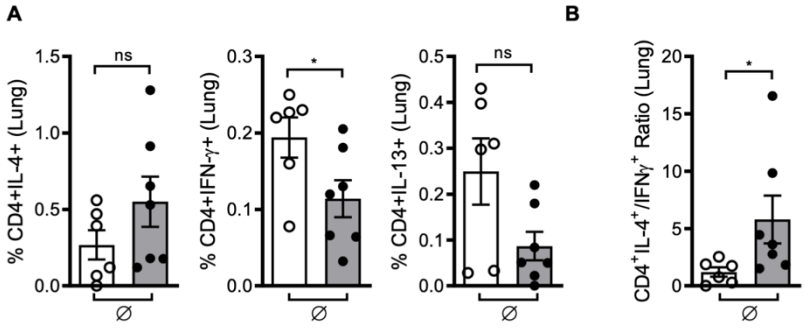


Figure 3.5 Steady State Lung CD4+ Polarisation
(A) Steady state proportions of IL-4+, IFNγ+, and IL-13+ CD4+ T cells in lung cell suspensions after similar *ex vivo* ICS conditions as per Fig. 3.2, with **(B)** showing the ratio of IL-4+/IFNγ+ cells within this compartment. Data is normalised and pooled from independent BALB/c and C57BL/6 experiments depicted in Fig. 3.2.

Across BALB/c and C57BL/6 offspring lung CD4+ T cells, in contrast to the overall reduction in OVA-stimulated cytokine responsiveness in T cells from Sm offspring, there is polarising effect which appears congruent with the increased antibody responses. Here, trends to higher proportions of IL-4+ CD4+ T cells and lower IL-13+, with significantly lower IFNγ+ CD4+ T cells (Fig. 3.4A), leading to a skewed IL-4/IFNγ+ CD4+ T cell ratio (Fig. 3.4B). Higher tendencies towards baseline IL-4 production, as well as lower pro-inflammatory cytokines such as IFNγ, may indicate steady-state activation of pathways that would support humoral activation, including IL-4-mediated class-switching, and yet coincide with priming effects that otherwise, or via these same mechanisms, lower inflammation as observed in the lungs during AAI.

As such, these findings suggest that developmental Sm-exposure causes a potential shift in immune axis induced in this allergy model, between T cell-associated inflammatory responses induced by allergen-exposure directed to the lung, and immunoglobulin levels in circulation induced by repeated sensitising immunisations. Whether this reflects a competitive regulation between inflammation-driving T cells and antibody-producing B cells (as observed for schistosome antigen-induced regulatory B cells (van der Vlugt et al. 2017), or the cells induced to ensure their functionality (e.g.: IL-4 producing helper T cells) remains to be seen. The indications of immune imprinting effect on sensitisation, as well as inflammation-associated T cell functionality alongside differential antibody titres, support the inclusion of less supervised approaches including RNA-seq, and further exploration of more global effects on T cell priming in the following sections.

3.1.2 Modified Transcriptional Profile of Priming Lymph Node during Allergy Model

Experimental OVA-AAI is induced over multiple immunological niches. First in the priming inguinal lymph node (igLN, found close to the site of subcutaneous injection on the flank) and systemic priming that involves circulation of and reactivation of induced memory cells (found, for example, in other secondary lymphoid organs, such as the spleen which is commonly used and readily accessible in mice), and ultimately in the lung during localized challenge with aerosolized allergen. Transcriptional analysis was performed on all three of these tissue types (igLN, spleen, and lung) in Sm offspring and naïve

counterparts, in both untreated mice representing the steady state, as well as from tissue at end-analysis of OVA-AAI (from the BALB/c experiment in Fig. 3.3 demonstrating lung intracellular cytokine analysis). RNA-seq was performed to uncover potential clues as to pathways that might be differential activated in Sm offspring during OVA-AAI compared to naïve mice, and to observe these potential differences at sites representative of systemic immune responses and priming, as well as locally at the site of inflammation.

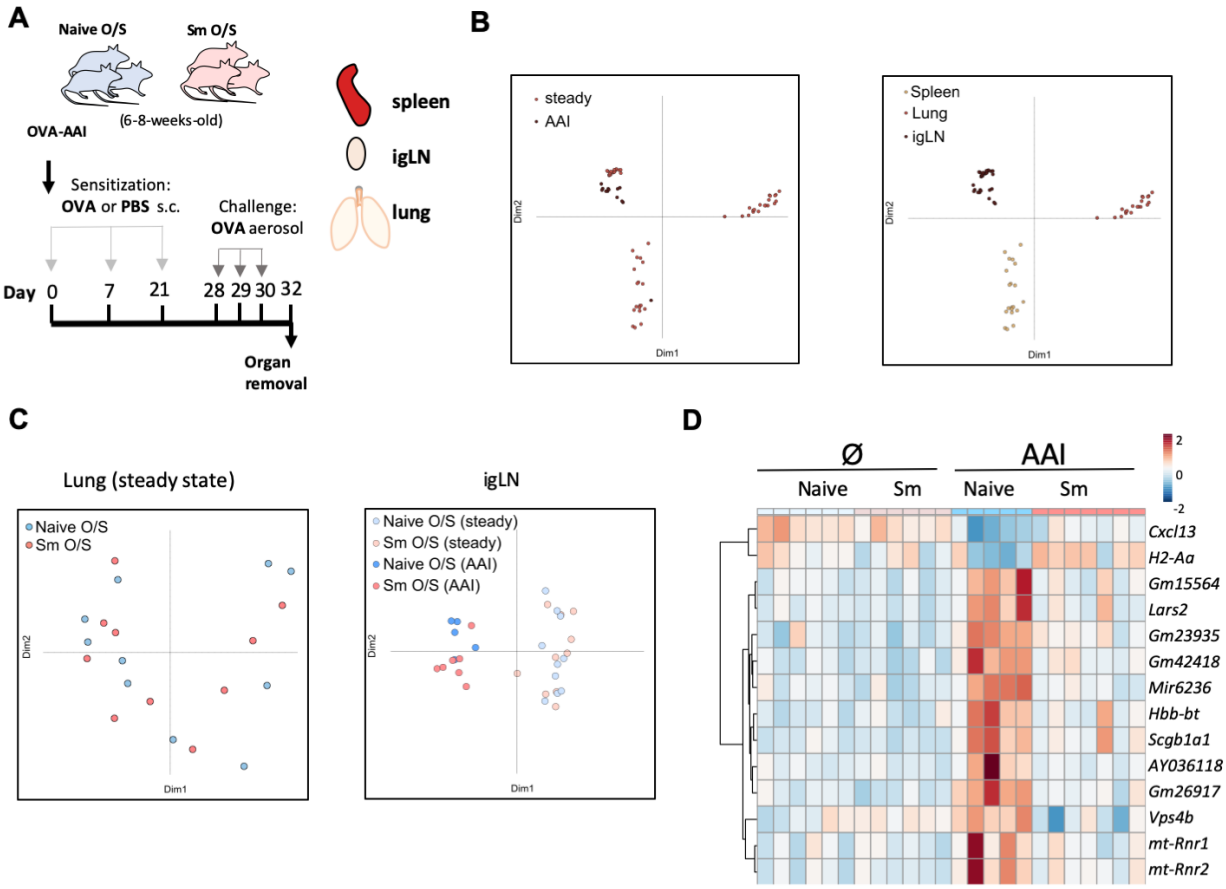


Figure 3.6 Organ Sampling and Bulk RNA-seq.
(A) Scheme of maternal priming followed by OVA-AAI induction, with parallel organ sampling during end-analysis for igLN, Spleen, and Lung tissues for transcriptomic assessment using RNA-seq, including sample extraction, library prep, sequencing, mapping of sequence reads, and DGE analysis using DEseq2 in R. (B) MDS plots with dimensionality reduction, with clustering of samples assessment against state (steady-state or OVA-AAI) or maternal status (naïve or Sm infection), after exclusion of samples with insufficient effective sequencing depth. (C) *Left:* Identification of Naïve and Sm offspring-derived samples in lung for cluster analysis in the steady state, *Right:* Comparison of unsupervised clustering of igLN samples according to Naïve and Sm offspring in steady state and during OVA-AAI. (D) Heatmap of genes in differentially express in igLN between Naïve

mice and Sm offspring during OVA-AAI, ordered by adjusted p-value. RNA-seq data obtained from 96 samples: 32 samples per organ, including 10 steady state samples per group and 5-7 per OVA-AAI group, with 3-4 mothers per group.

Quality control of samples based on differentially expressed genes across the experiment was initially performed to determine whether there was an over-representation of the 10 highest expressed genes, indicative of effective low sequencing depth by skewing representation towards those highly abundant transcripts. Unlike the cell suspensions in Fig. 3.3, which allowed specific gating on target populations through flow-cytometry to discover clear T cell responses, the whole tissue sampled for bulk RNA-seq, particular lungs and spleens during AAI, were highly enriched for structural and signalling genes involved in lung inflammation, as well as involved in haemoglobin pathways. This resulted in no detection, for example, of IL-13 mRNA sequence reads, despite encoding a protein known to be clearly induced during AAI in the lung, and was clearly a product of activated CD4+ in these same lung samples (as measured *ex vivo* by ICS). As such, only the OVA-AAI-derived samples for the igLN achieve sufficient depth to provide meaningful analysis. Initial assessment of the total cohort of samples reveals clear discrete clustering into 3 groups. Cross-referencing sample ID reveals that these clusters relate to organ type – indicating that the clearest difference in transcriptional profile is between different tissues, with further clear separation between igLN samples from mice in steady state or after AAI (Fig. 3.6B).

In the steady state lung samples reveal no difference in MDS plot-based clustering based on maternal status (Fig. 3.6C, *left*). The igLN group is clearly made up of 2 main sub-clusters, which when viewed relative to state reflect the differences between the steady state and AAI. When viewed in closer detail, the AAI cluster further appears to form subgroups when viewed according to maternal infection status (Fig. 3.6C, *right*). This distance of this clustering represents genes that are differentially expressed to a statistically significant level in a group-wise manner between Sm offspring and matched naïve mice. These are represented ordered according to adjusted p-value in the heatmap (Fig. 3.5 D). The majority of genes appear to be upregulated in naïve mice with OVA-AAI, yet to a lesser degree in Sm offspring, in line with the overall AAI hyporesponsiveness of Sm offspring, already observed in terms of eosinophilia and lung CD4+ T cell activity. These include ribosomal RNA (*mt-rnr1* and *mt-rnr2*), hemoglobin (*Hbb-bt*), inflammatory signaling molecules (*Scgb1a1* and *Vps4b*), plus long non-coding

and micro RNAs alongside RNAs for predicted genes or pseudogenes (*Gm15564*, *Mir6236*, *Lars2*, *Gm23935*, *AY036118*, *Gm26917*), some of which have been associated with inflammasome activation (Zhang et al. 2019). The two most differentially expressed genes, however, display a different pattern. The most differentially expressed transcript between offspring groups was CXCL13, a chemokine ligand for the receptor CCR5, found highly expressed on B cells and also on follicular helper CD4+ T cells (Tfh) (Poholek et al. 2010). This dropped significantly during AAI compared to in the steady state, although less so in the samples from Sm offspring. Similarly, the second highest differentially expressed gene is H2-Aa, the gene encoding MHCII, found on all professional antigen presenting cells and in high amounts on B cells. Given that other APCs generally make up a small proportion of the total numbers of cells in a lymph node, while B cells are a major lymph node population, changes to the proportion of H2-Aa mRNA in transcripts could indicate altered movement of B cells into or out of the lymph node. Coupled with data finding increased rather than decreased OVA-specific antibody titres in conjunction with reduced T cell-associated inflammatory responses, this could denote an interaction between T and B cell responses that is modified by priming effects of exposure to maternal helminth infection.

3.1.3.1 Maternal Schistosomiasis Modulates T and B Cell Responses to Allergic Sensitization in Offspring

Lung CD4+ T cell responses during OVA-AAI were modified by prior exposure during development to maternal schistosomiasis. Antibody titres and transcriptional findings suggested potential alterations to B cell responses, and in conjunction with changes to T cell cytokine production indicate that differences in antigen-specific priming could underlie modulated AAI response. These may even occur on a more systemic level, in light of the antibody-related data. Given the complex milieu of allergic asthma, a simplified version of this model would help clarify whether there is a more generalizable effect of this maternal infection on priming of offspring T cell responses. As such, the primary activation of T cells during the allergic priming that occurs with subcutaneous immunization was examined, including CD4+ and CD8+ T cell responses during ovalbumin sensitization alone, without subsequent airway challenge.

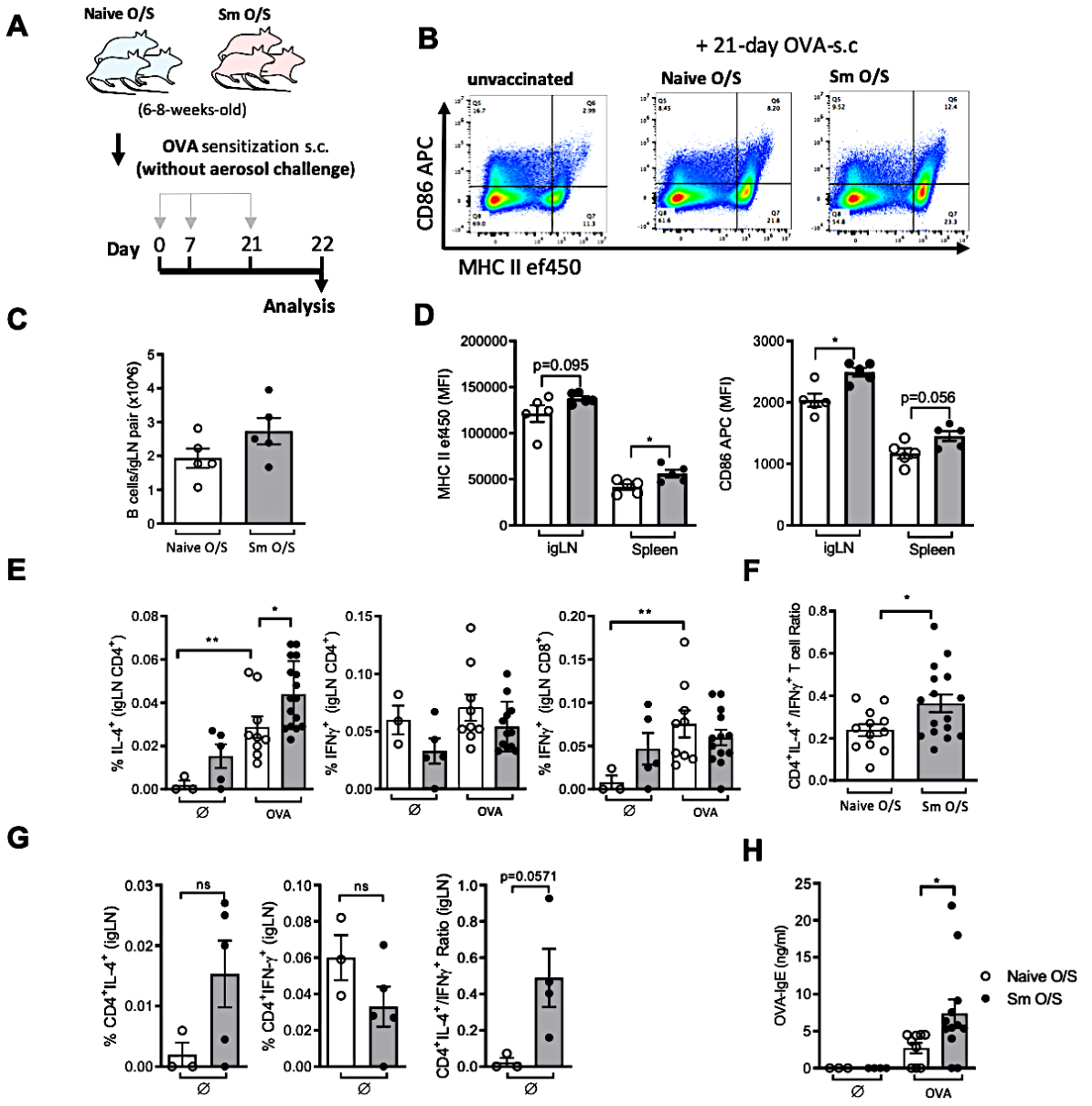


Figure 3.7 Transmaternal Modulation of Allergic Sensitization to Subcutaneous OVA.
(A) Scheme for OVA sensitization, as per OVA-AAI, without lung challenge component. **(B)** Representative plots of CD86 and MHCII expression in splenocytes in non-immunised mice, versus immunised offspring cohorts. **(C)** Comparison of B cell numbers in spleen and igLN during steady state and sensitization as measured by flow cytometric analysis of B220+ lymphocytes. **(D)** Activation state of B cells assessed via mean fluorescence of markers MHCII and CD86, in spleen and igLN. **(E)** Proportions of CD4+ or CD8+ T cells positive for intracellular cytokines IL-4, or IFNγ via flow cytometry in igLN and spleen suspensions re-stimulated O/N with ovalbumin or OVA peptide and 5h with BFA prior to staining. **(F)** Comparison of IL-4+/IFNγ+ T cell ratio in igLN between immunised Sm offspring and naïve counterparts. **(H)** Demonstration of steady-state CD4+ IL-4/IFNγ ratio in igLN of non-immunised offspring cohorts. **(H)** Serum OVA-specific IgE levels following sensitization protocol, as determined via ELISA. B cell data in (C) and (D) shows 5 female mice per group, (E) and (H) from

an experiment using 9-12 offspring per immunized group, (for Sm O/S, mothers n = 5). (F) pools z-normalized data from 2 independent experiments, n = 14-17 per group, and (G) represents data from unimmunized controls in E.

As suggested by RNA-seq transcriptions of igLN during AAI, Sm offspring display increased proportions of MHCII+ cells in the igLN during priming with OVA (representative plot in Fig. 3.7B), largely composed of B cells, and indications of increased B cell sin igLN (Fig. 3.7C). The B cells in the IgLN as well as the spleen additionally display higher MHCII expression, and similar patterns for co-stimulatory molecule CD86 (Fig. 3.7D).

The increased B cell activity was associated with a shift in T cell cytokines in the igLN during sensitization. Following this 21-day sensitization-only protocol, *ex vivo* re-stimulation of igLN cell suspensions gave increased CD4+IL-4+ T cells overall following OVA-s.c, which was significantly increased in the Sm offspring (Fig. 3.7E). This is not entirely surprising given the IL-4/B-cell axis, but is an initial clue that T and B cell responses are not only both modulated in Sm offspring, but that this modulation may be linked. OVA-responsive CD4+IFNy+ cells do not clearly increase here, although there is a trend towards less IFNγ+ CD4+ T cell in Sm offspring, regardless of immunization status (Fig. 3.7E, *middle*). Unlike CD4+ T cells, there was a clear increase in OVA-responsive CD8+ IFNγ+ in immunised naïve offspring, with this increase less clear in Sm offspring (Fig. 3.7E, *right*).

These findings also result in a shift in ratio of IL-4/IFNγ producing T cells in igLN in Sm offspring compared to naïve responses (Fig. 3.7F). In fact, calculation of this ratio within CD4+ T cells for steady state mice yields a similar shift (Fig. 3.7G), indicating similar IL-4-skewing and potential antagonism of IFNγ production, even prior to immunisation. These indications of an enhanced B cell response in the Sm offspring (increased numbers and activation markers), alongside increased IL-4+ T cells (needed to class-switching) were further associated with the functional outcome of increased Ig titres for OVA-specific antibodies (Fig. 3.7H).

Together, these results indicate that the lower AAI-responsiveness previously observed in Sm offspring could be further associated with an overall lower predisposition towards mounting antigen-specific pro-inflammatory T cell responses in response to allergic sensitization. Here, a higher predisposition to induce IL-4 producing CD4+ cells, in conjunction with enhanced B cell responses, appear coupled to

steady-state reduction in CD4+ IFNγ producing cells, and reduced capacity to expand IFNγ producing CD4+ as well as cytotoxic CD8+ T cells in response to antigen, that may prove to have functional outcomes during immunisations outside of the context of allergic sensitization, such as prophylactic vaccination.

3.1.3.2 Local T cell Responsiveness to Adjuvant-Free Subcutaneous OVA Immunisation

The data so far demonstrate an effect of maternal schistosome infection upon igLN transcriptional profile during OVA-AAI, and T as well as B cell responses, and development of OVA-specific Ig-titres, when the subcutaneous OVA immunisation alone is used to induce allergic sensitisation. In both settings this is used without additional adjuvant. To further evaluate the hypothesis that altered T cell priming in the igLN is involved in sensitization to OVA, experiments were conducted to confirm the involvement of the igLN in the s.c-based OVA directed into the flank. This was initially observed using OVA fluorescently labelled with Texas Red (OVA-TR), and delivered via multiple routes, namely: s.c, i.p, and i.p with the addition of alum, followed by flow cytometric analysis of TR+ cells from spleen, igLN, and the mediastinal lymph node (medLN, previously shown to be a preferential site for draining and activation on CD4+ DO11.10 T cells after i.p injection with OVA-ALUM (Kool et al. 2008).

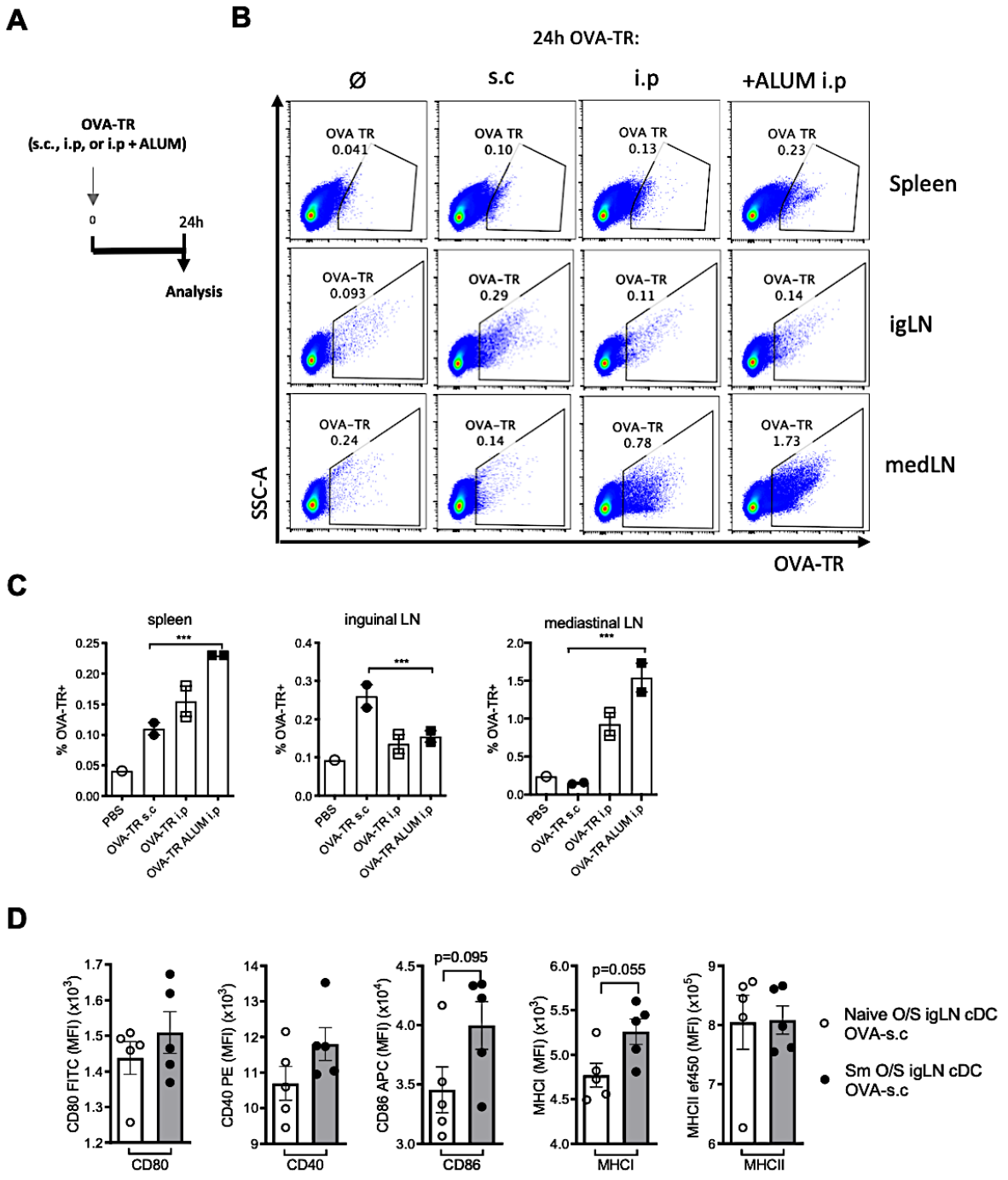


Figure 3.8 Subcutaneous OVA into Flank Preferentially Primes igLN
(A) Scheme of OVA-TR immunisation via different routes of s.c, i.p, and i.p with the additional of alum as adjuvant. **(B)** % of live, OVA-TR+ cells in spleen, igLN, and medLN after immunisation **(C)** Comparative enumeration of OVA-TR+ cells across organs with n=2 mice per immunisation modality. **(D)** Comparison of co-stimulation molecules expression (CD80, CD40, CD86, MHCI, MHCII, all assessed via MFI using flow-cytometry) on igLN conventional CD11c^{hi} dendritic cells from 21-day OVA-s.c. immunised offspring cohort BALB/c mice, experimental groups as per Fig. 3.7 (C) and (D).

First, the use of fluorescently labelled OVA injected i.p, particularly with the addition of alum lead to increased OVA-TR+ in spleen and medLN, in line with previous findings of a role for these sites. OVA-TR-s.c. injection in the flank instead showed localised accumulation of OVA-TR+ cells preferentially in the igLN (Fig. 3.8C). This data confirms that the sensitisation protocol used to induce OVA-AAI via subcutaneous injection of adjuvant-free OVA into the flank induces initially draining of antigen (or migration of antigen-loaded DCs) into the igLN for presentation. The localisation of this effect is further confirmed by the enhanced retrieval of OVA-TR+ in the igLN following s.c injection compared to i.p injections. As indicated by previous studies, i.p injection led to OVA-TR+ cells in the spleen and medLN, increasing with the additional of alum (Fig. 3.8C).

The local effects on igLN APCs in the OVA-s.c model, suggested as particularly relevant by the localised presence of antigen-loaded APCs, was further evaluated in OVA-s.c. immunised offspring cohorts, otherwise treated as per Fig. 3.7. Here, the altered expression of co-stimulatory molecules on B cells from immunised Sm offspring was further reflected within the $CD11c^{hi}$ cDCs of immunised Sm offspring relative to immunised naïve mice. The clearest trends are towards higher expression of CD86 and MHCI (Fig. 3.8D).

The localisation of T cell activation following from adjuvant-free OVA delivered s.c. in the flank was then clarified using adoptive transfer (i.p) of CD45.1+ naive (CD44lo) OT-I or OT-2 cells, pre-stained with CellTrace proliferation dye.

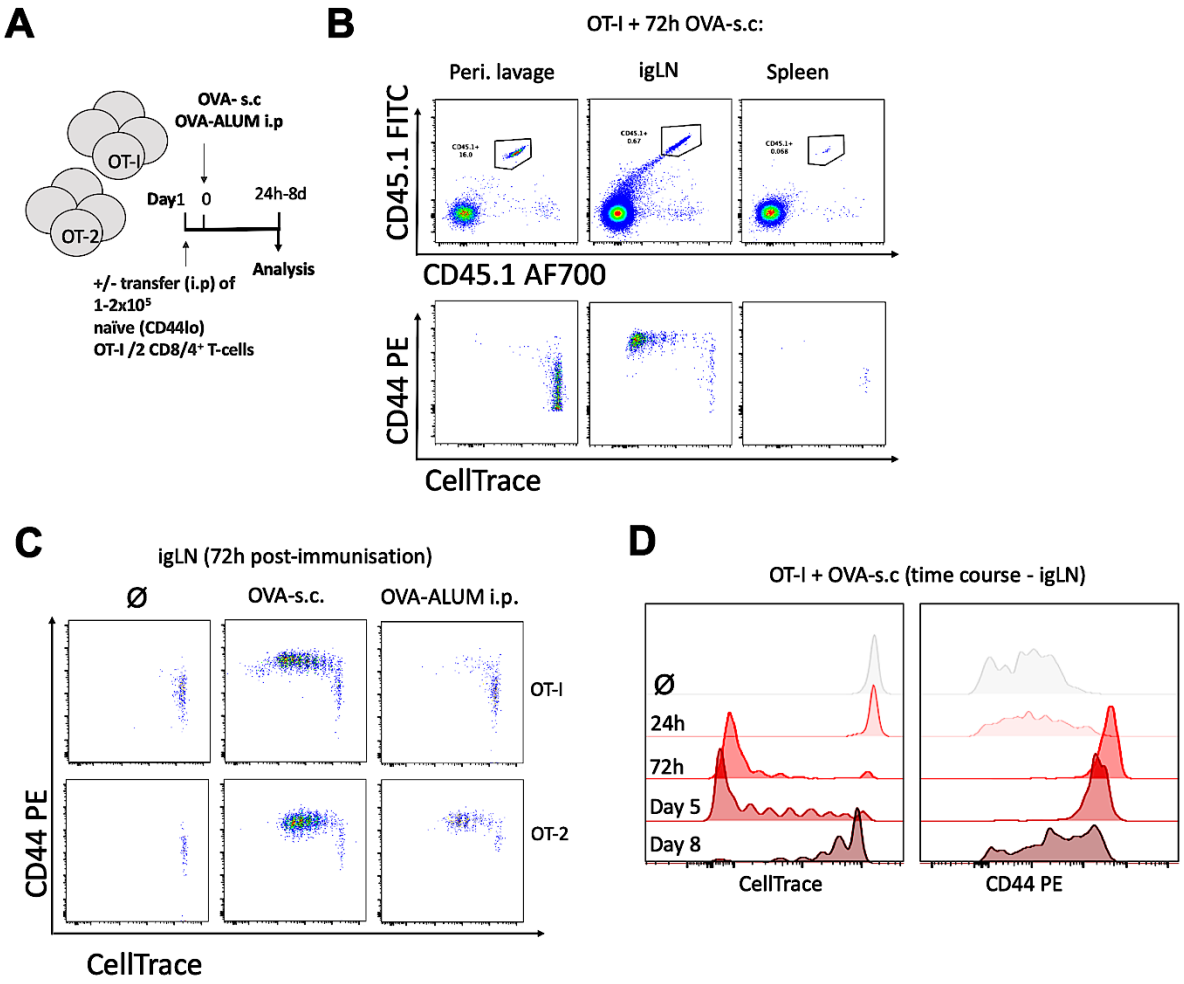


Figure 3.9 OVA-s.c. Drives Local CD4 and CD8 T cell Activation in igLN
(A) Scheme for adoptive transfer of naïve (CD44lo) CD45.1+ OT-I or OT-2 cells followed by OVA-s.c. immunisation, as compared to PBS or OVA-ALUM i.p. (B) Representative FACS plots from OT-I cells retrieved from distinct locations (peritoneal lavage, igLN , or spleen) 72h after OVA-s.c., *upper*: identification of CD45.1+ OT-I cells, *lower*: activation status visualised through CD44 upregulation and CellTrace dilution (pre-gated on CD45.1+ cells). **(C)** Comparison of activation status (CD44+ and CellTrace dilution) of CD45.1+ OT-I and OT-2 cells retrieved from igLN after OVA-s.c, PBS injection, or OVA-ALUM i.p. **(D)** Time course of CellTrace and CD44+ profiles from OT-I from igLN over 8 days following OVA-s.c. immunisation.

The i.p delivery of transferred OT-I cells led to a significant population in the peritoneal cavity, present in peritoneal lavage fluid as a major population (here 16%) of retrieved CD8+ cells there (Fig. 3.9B, *top left*). 72h following OVA-s.c., these largely maintained retained a CD44lo naïve phenotype, with an undivided, bright CellTrace peak (Fig. 3.9B, *bottom left*). 72h after OVA-s.c. immunisation, active division of transferred OT-I and upregulation of CD44 was detected in the igLN (and in igLN higher proportions of cells were retrieved compared to spleen)(Fig. 3.9B, *central panels*), while the fewer cells

retrieved from the spleen at this time point largely retained a naïve phenotype. (Fig. 3.9B, *right panels*). Further analysis of the igLN found that OVA-s.c. activation occurred in OT-I and OT-2 transferred cells. OVA-s.c. drove higher accumulation and proliferation in igLN than if i.p immunization was used, even i.p with alum as an adjuvant, with particular specificity for local igLN CD8+ proliferation (Fig. 3.9C), confirming this as a preferential site for local antigen-specific CD4 and CD8 responses in this system (with previous studies showing that instead the mediastinal lymph nodes would be the more direct draining sites of i.p immunization (Kool et al. 2008)). Further, a time-course of OT-I activation found peak division and CD44 upregulation between days 3-5 following injection (Fig. 3.9D). The returning bright CellTrace signal by day 8 signifies a loss of activating OVA signal, and probable replenishment from the naïve reservoir retained in the peritoneal cavity (as observation in the lavage fluid in Fig. 3.9B). Consequently, OVA-s.c. (as used earlier in AAI and allergic-sensitisation) triggers local activation of CD4+ and CD8+ T cells in the igLN. The modified T cells responses of Sm offspring following this route of priming, with and without challenge, therefore warrant further examination of T cell responses to ascertain how translatable this effect is more generally to regulation of T cell responses outside of an allergy setting.

3.1.5 Early Schistosomiasis Exposure Primes Modified Type 1 CD4+ And CD8+ T Cell Responses to Vaccination

In the previous section, the altered CD4+ T cell responses from Sm offspring during experimentally-induced AAI were found to be associated with shifts in both T and B cell response during immunization-based priming alone, with an altered IL-4/IFNy responsiveness ratio. Further, the OVA-s.c immunisation modality was observed to trigger local activation of CD4+ and CD8+ T cells, indicating potential involvement of these processes of T cell priming in the mechanisms of modified inflammation in Sm offspring. By extension, exposure to chronic maternal schistosomiasis may therefore yield differential T cell responses to other vaccination modalities (as has been theorized for helminth-endemic populations, reviewed in (Lacorcia and Prazeres da Costa 2018)), that compromise vaccine immunogenicity (particularly in the case of lower CD8+ memory T cell expansion and overall IFNγ

responses could result in reduced protective immunity against pathogens where such responses are required, for example in antiviral responses.

To test this hypothesis, additional vaccine strategies were used to further clarify this T cell phenotype. The first more traditional adjuvant-based approach was ovalbumin administered with aluminium hydroxide (scheme in Fig. 3.10A), a common adjuvant included in many licensed vaccines, including for Hepatitis B (GlaxoSmithKline 2019). Alongside strong humoral responses and T_H2 polarisation, alum has been shown to boost antigen-specific activation of T cells in parallel with innate immune cells (McKee et al. 2009), and to induce significant IFNγ production in CD8+ but not CD4+ T cells (Serre et al. 2010). Similar outcomes regarding T cell activation between alum usage and adjuvant-free subcutaneous sensitization with OVA is not surprising considering that both protocols are used in sensitization for later allergic asthma models based on aerosol challenge. Similarly, the finding that Sm offspring displayed lowered AAI response when alum was used as an adjuvant during the sensitization phase (Straubinger et al. 2014) invites speculation that their T cell responses to alum may be similarly altered. Complete Freund's Adjuvant (CFA) is commonly used to prime mixed predominantly type 1 immune responses from CD4+ and CD8+ T cells using dried and inactivated mycobacteria products to enhance the immunogenicity of protein antigens in a mineral oil emulsion. CFA has been previously shown to yield lowered cytokine responses in male mice exposed to maternal schistosomiasis, with differing outcomes dependent on nursing versus gestational effect (Santos et al. 2010). In contrast to the protein antigen +/- adjuvant methods so far described, additional mice were immunized with Modified Vaccinia Ankara virus (MVA) expressing ovalbumin (MVA-OVA) as a viral vector-based vaccine (as used in [25]) (also according to scheme in Fig. 3.10A). Such live viral vector-based modalities have more recently been clinically employed in an attempt to yield improved vaccine efficacy in low-responding populations [26]. For example, adenoviral vector-based immunization strategies are amongst those in development against highly virulent and difficult to control infectious agents such as Ebola virus (as reviewed in [27]).

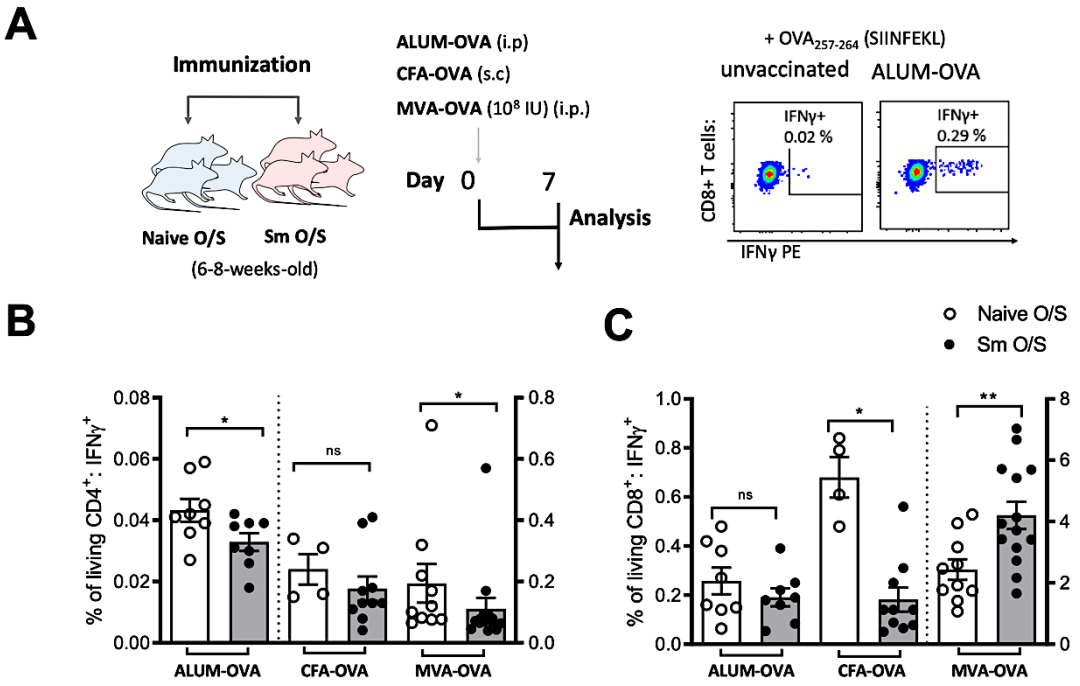


Figure 3.10 Vaccination Modality-Dependant Transmaternal Effects: OVA-ALUM, OVA-CFA and MVA-OVA
(A) Offspring cohorts were immunized with either: ALUM-OVA (i.p), CFA-OVA (s.c), or MVA-OVA (i.p), *right:* representative plot for CD8+ IFNγ+ cells following subsequent *ex vivo* stimulation, depicting impact after OVA-ALUM. (B) Proportions of IFNγ+CD4+ T cells after 5h re-stimulation with $OVA_{323-339}$ peptide + BFA (of splenocytes for i.p vaccinations, igLN for s.c). (C) IFNγ+ proportion in CD8+ T cells stimulated with $OVA_{257-264}$ peptide (SIINFEKL). ALUM-OVA: 8 female mice per group, (Sm mothers: n = 4). CFA-OVA and MVA-OVA: 4-9 mice per group, each representative of 2 independent experiments (Sm mothers n = 2-3 per experiment).

7-days following intraperitoneal immunization, peptide re-stimulation of splenocytes revealed that ALUM-OVA did not drive a detectable increase in antigen-stimulated IFNγ production by CD4+ splenocytes (as previously described for this model in (Serre et al. 2010)). However, as previously found in the adjuvant-free immunization (Fig. 3.7), immunized Sm offspring showed lower proportions of IFNγ+ CD4+ T cells compared to naïve controls (Fig. 3.10B). This could indicate immunization induced-effects in Sm offspring that were able to suppress numbers of IFNγ+ cells to lower than the baseline of unimmunized control mice, such as type 2 or regulatory responses), but also could reflect potential steady state suppressive effects on IFNγ producing CD4+ T cells following maternal schistosomiasis. ALUM-OVA appeared to induce a reduced proportion of IFNγ+ CD8+ T cells in Sm offspring of responsive to peptide re-stimulation, observed in both mediastinal lymph node (previously shown to be a key draining site of intraperitoneal immunisations(Kool et al. 2008)) and splenocyte cell

suspensions (Fig. 3.10C), although despite this trend observed across organs, in both cases this difference was not found to be statistically significant. 7-days following subcutaneous immunization with CFA, CD4+ and CD8+ T cell responses were evaluated using peptide re-stimulation, with higher proportions of antigen-responsive IFNγ+ cells in both CD4+ and CD8+ compartments. Variability within the numbers of induced CD4+ IFNγ producing cells from Sm offspring obscures any statistically significant difference (although visually indicated). There is however a clear reduction in IFNγ+ CD8+ cells Sm offspring compared to controls (Fig. 3.10C).

Consistent with the results obtained for earlier allergic models as well as alum and CFA-adjuvants, CD4+ cells from Sm offspring appeared largely unresponsive to MVA-OVA-based induction of IFNγ+ production when compared to those from vaccinated naïve controls (Fig. 3.10B). However, MVA-OVA was able to induce clearly higher proportions of IFNγ+ CD8+ cells than previously observed. Further, in contrast to the previously used vaccination protocols, MVA-OVA led to an even larger increase in numbers of IFNγ+ CD8+ T cells following *ex vivo* peptide re-stimulation of splenocytes from Sm offspring compared those from vaccinated control mice (Fig. 3.10C). Beyond offering further support that immune reactivity is altered by prior exposure to maternal schistosomiasis, these findings suggest that such modified T cell responses are amenable to altered antigen delivery strategies, the functional implications of which may be highly relevant for individuals with lower vaccine responses and varied history of immune priming.

3.1.6 Early Schistosomiasis Exposure Alters Anti-Viral HBV Responses but is Amenable to Optimized Vaccination Modalities

Immunizing Sm offspring with an MVA vector was sufficient to overcome the suppressive effects regarding CD8+ T cell priming using the model antigen OVA. The functional outcome of the ineffective CD8+ T cell priming with traditional adjuvants following exposure to maternal schistosome infection, however, was further tested by immunizing and challenging Sm offspring with a murine model of Hepatitis B virus (HBV), the clearance of which decisively requires effective CD8+ T cell responses. As mice are not naturally susceptible to HBV, this involved using an established mouse model of HBV

by employing an adenovirus-mediated genome transfer of replication competent HBV genomes [29; 30] (Ad-HBV), which has recently been used to demonstrate the efficacy of a "prime-boost" vaccination system using both immunization (first "priming" with Hepatitis B surface antigen (HBsAg) protein with adjuvant, then "boosting" with MVA-based viral-vector HBV antigen delivery (MVA-HBV)), prior to challenge with Ad-HBV (as modified from (Kosinska et al. 2019)).

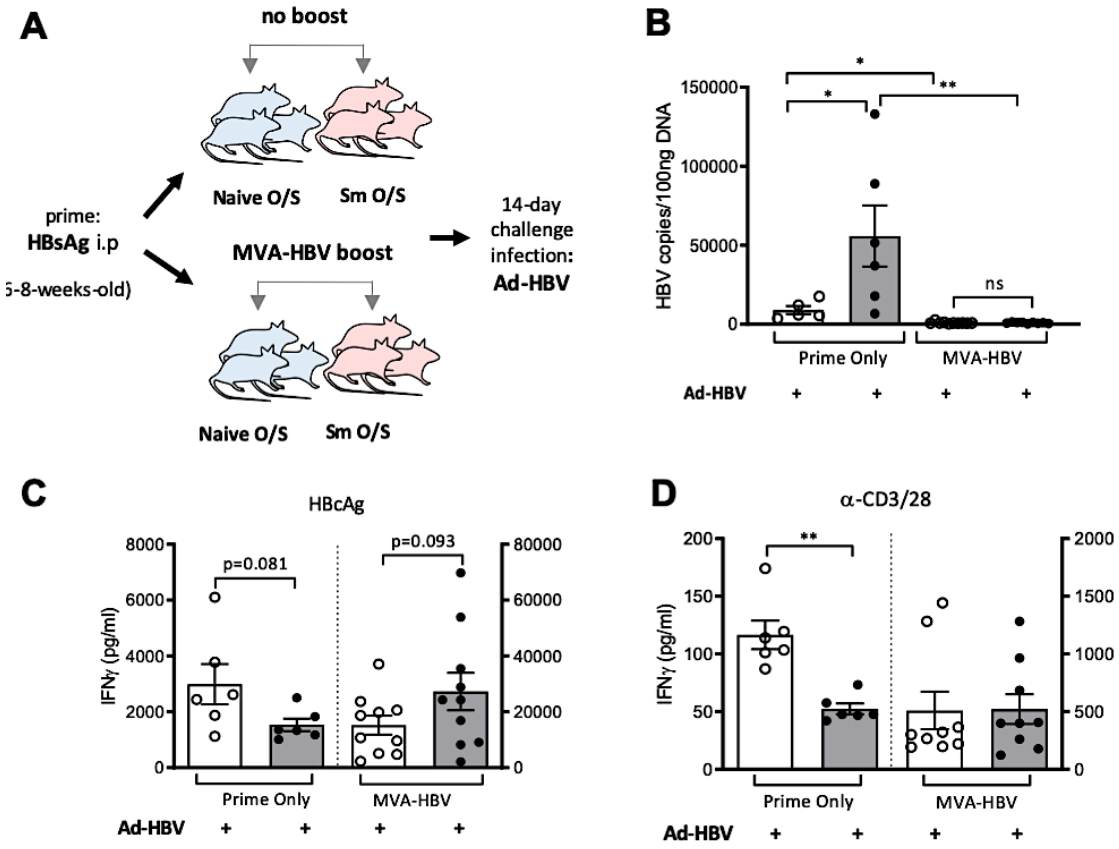


Figure 3.11 MVA-based Vaccination Rescues Suppressed Anti-Viral HBV Responses in Sm Offspring
(A) Experimental scheme for murine HBV model, where 6-8-week-old C57BL/6 Sm offspring and age- and sex-matched naïve controls were subjected to vaccination with HBsAg plus adjuvant (i.p) on day 0, followed by an additional boost with MVA-HBV or only PBS as a control on day 14, prior to challenge with Ad-HBV on day 28. **(B)** Copies of HBV DNA per 100g of liver DNA as determined by PCR. **(C)** Levels of secreted IFNγ as determined by ELISA in splenocyte cell culture supernatants after re-stimulation with HBcAg (5h) or **(D)** anti-TCR stimulus (48h). Data is pooled from two independent experiments each with 4-6 mice per group receiving MVA-HBV boost, and 3 per group receiving Priming alone.

When only immunized with protein plus adjuvant prior to viral challenge, Sm offspring indeed appeared to display weakened antiviral immunity. This included higher amounts of HBV viral DNA (Fig.3.11C), indicating susceptibility or reduced viral clearance. Replicating the reversal effect as seen with the model

antigen OVA, MVA-HBV-based boosting prior to viral challenge was able to override the immunosuppressive effect on apparent antiviral, responses induced by previous exposure to maternal helminth infection, greatly reducing the number of viral DNA copies compared those Sm offspring who only received a prime-based vaccination (Fig. 3.11B). These phenotypes regarding viral control were further associated with impaired T cell responses in Sm offspring receiving prime-only immunisation, included reduced responsiveness to anti-HBV core antigen (HBcAg, the immunodominant antigen for CD8+ T cell responses) as indicated by lowered IFNγ secretion in response to HBcAg (Fig. 3.11C), as well as lowered IFNγ responses to TCR-based re-stimulation of the total T cell compartment (Fig. 3.11D). Coinciding with the MVA-HBV boost providing Sm offspring with normalised clearance of virus as observed via DNA, Sm offspring displayed corrected IFNγ secretion in response to HBcAg or TCR *ex vivo* re-stimulation, meeting or exceeding those responses from vaccinated and challenged offspring from naïve mice (Fig. 3.11C + D). The application of an MVA-based viral vector therefore appears to not only effectively primed and expanded virus specific CD8+ cytotoxic T cells in otherwise hyporesponsive Sm offspring, but has also led to the functional effect of normalizing apparent viral clearance to levels comparable to those observed in control mice.

3.2 DEVELOPMENTAL ORIGINS OF MODIFIED T CELL RESPONSES

3.2.1 Early Schistosomiasis Exposure Modifies Epigenetic and Functional Signature in CD4+CD62L+

The findings so far presented have described how suppressed allergic inflammation responses by Sm offspring include modified T cell cytokine reactivity. Even with the removal of inflammatory lung challenge, Sm offspring have alternatively skewed priming responses to immunization with protein antigen, which further translates into modified vaccination outcomes that are generally skewed towards lower IFNy production, although application of the MVA viral vaccine vector was able to overcome this hyporesponsive effect with regard to cytotoxic CD8+ T cell responsiveness. A key functional outcome of such reduced capacity to mount type 1 responses following traditional immunization was weakened

viral clearance in an HBV model, where normalization of CD8+ responsiveness with an MVA-based vaccine had the further functional outcome of normalized viral clearance.

Prior to direct measurement of CD4 and CD8 T cells during allergy and immunization, a study from our group assessed the epigenetic signature on CD4+CD62L+ T cells from Sm o/s and naïve controls as may relate to allergy. That study found different patterns of differentiation of these cells, in terms of cytokine production when subjected to *in vitro* polarization assays (more induced IFNy+ production, less induced IL-4+ production), to be linked to the degree of histone acetylation in the promoter regions of genes encoding associated cytokines (Klar et al. 2017). Further investigation into these effects, however, including control activation under neutral conditions as a control, as well as the steady-state polarisation observed so far in this thesis, problematize the concept of clear epigenetic skewing of the naïve T cell compartment to give rise to the modified T cell responses so far observed using *in vivo* allergy, inflammation, and vaccination models. This secondary investigation into epigenetics effects within this cellular compartment derived from exposure to maternal schistosomiasis began with an additional complimentary polarization studies using CD4+CD62L+ T cells from 6-8-week-old offspring.

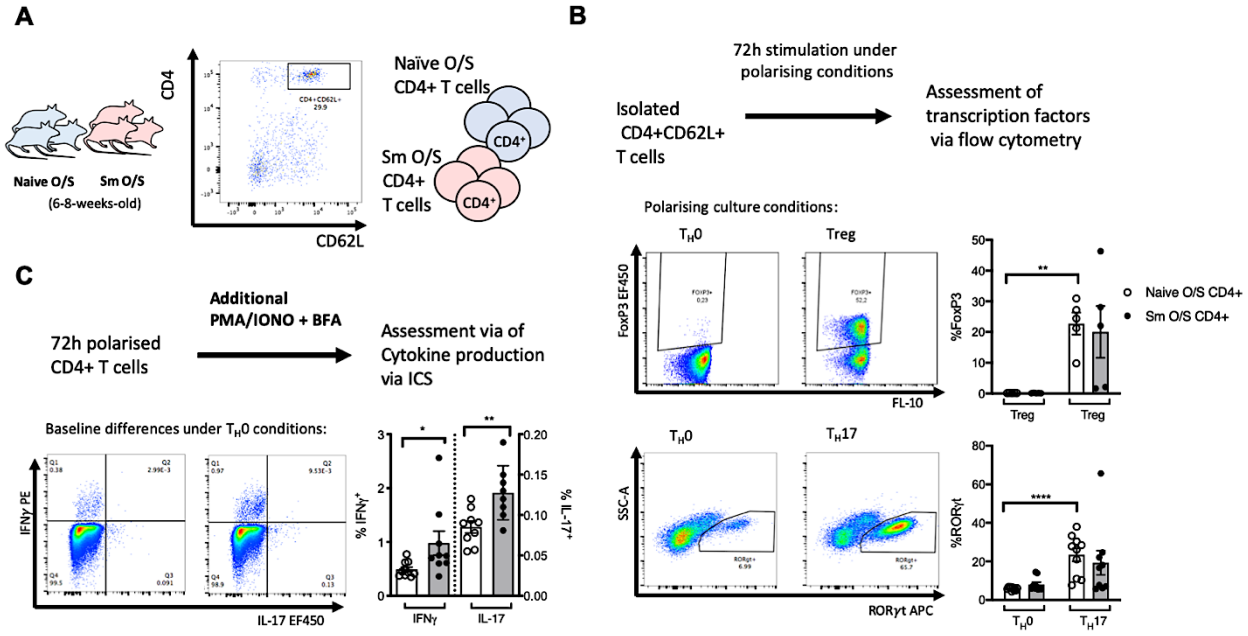


Figure 3.12 Maternal Schistosomiasis Alters Functional Capacity of CD4+CD62L+

(A) Scheme for isolation of CD4+CD62L+ "naïve" T cells from 6-8-week-old Sm offspring and naïve counterparts, which were then activated and polarized in vitro into T helper subtypes (namely Th17 or regulatory T cells) using antibody and cytokine cocktails. (B) Comparison of transcription factor FoxP3 and RORγt under respective conditions. (C) Comparison of cytokine production (IFNγ and IL-17) from T_H0 cells, activated under neutral conditions.

While Treg-polarising conditions induced a substantial increase in FoxP3+ cells (Fig. 3.12B, *middle row*), no clear trend was apparent between naïve and Sm offspring-derived cells in the initial pilot experiment. This was also true for RORγt+ cells, which were induced under T_H17 conditions (Fig. 3.12B, *lower row*), and was unaffected by maternal status of offspring. Results of polarisation *in vitro* are problematized by baseline differences within the T_H0 condition, where already higher levels of baseline IFNy and IL-17 indicate a degree of steady-activation of cytokine-competent cells (Fig. 3.12C).

These additional polarisation assays were accompanying by repeat ChIP assays on cells from 3-week-old offspring to track the development of changes observed in adult mice.

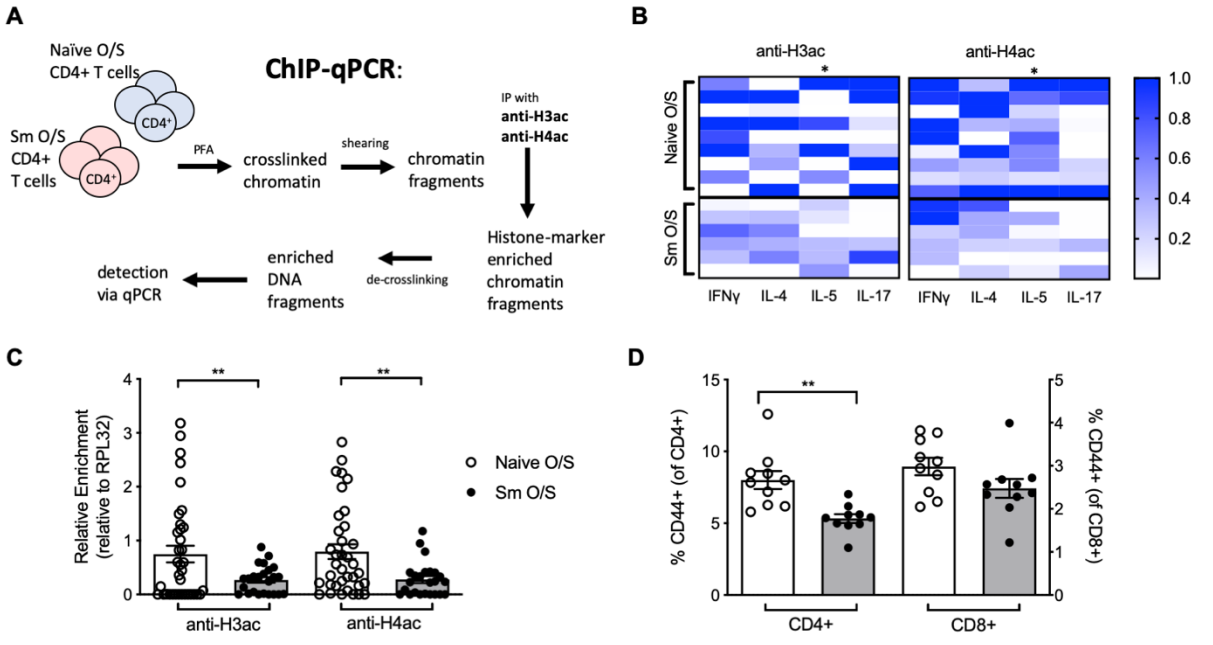


Figure 3.13 Maternal Schistosomiasis alters Epigenetic Landscape in CD4+CD62L+
(A) Scheme of ChIP-based epigenetic analysis of histone acetylation of CD4+CD62L+ T cells compartment in 3-week-old Sm offspring and naïve controls. (B) Heatmaps of enrichment at cytokine loci using anti-H3ac and anti-H4ac ChIP targeting acetylated sites as permissive modifications. (C) Comparison of pooled enrichment across all measured cytokine loci (IFNγ, IL-4, IL-5, and IL-17) for both H3ac- and H4ac-directed ChIP. (D) Comparison of CD44 proportion within CD4+ and CD8+ compartments for 8-week-old naïve and Sm offspring.

The degree of histone acetylation at the promoter sites of key cytokines associated with T helper subsets were compared in the CD4+CD62L+ T cells via ChIP (scheme in Fig.3.13A) from Sm offspring and matched naïve mice. Across IL-4, IL-5, IL-17, and IFNγ promoter loci, there is a strong trend towards lower amounts of acetylation (i.e.: a less permissive epigenetic state) for all of these cytokine promoters, and significantly lower on the IL-5 locus, on both H3 and H4 histones (Fig. 3.13B. The IL-5 promoter showed statistical significantly less acetylated histone-associated IL-5 in the samples from Sm offspring. The findings of lowered histone acetylation at all of these sites in Sm offspring samples, clarified through pooling data across loci (as in Fig. 3.13C), indicate a potential global process (or potential artefact) within these samples that skews the histone acetylation profile. The data of clear increases of cytokine production in the polarization assays, and increased in IL-4+ polarised CD4+ in steady-state igLN and lung relative to usual ratios, despite epigenetic data indicating the opposite trend, making conclusions difficult to draw from this data.

This bulk analysis of chromatin, however, cannot distinguish differences between in changes to the status of otherwise homogenous cell populations (i.e: naïve T cells with different degrees of permissiveness regarding their cytokine genes) or altered compositions of heterogenous cell populations (i.e, changes to the relative proportion of cells such as already differentiated, activated or antigen-experienced cells, such as memory T cells, which each would bear different epigenetic signatures). It is known that the CD62L+ compartment contains CD44+ memory cells, and that the classic members of the central memory (Tcm) subset fit within this category. One possibility then, is that rather than displaying the true epigenetic status overall of the naïve T cells, these histone-associated promoter measurements describe the relative amounts of more activated cells, such as CD44+ Tcm, within the CD62L+ compartment between groups. Here, there potential explanation for the observed difference, as assessment of the basic composition of the T cell compartments in Sm offspring reveals that overall they possess proportionally less CD44+ T cells, with clearer reduction in CD4+CD44+ in Sm offspring (Fig. 3.13D). It is therefore likely that the overall lower levels of permissive epigenetic marks could instead represent changes to the proportion of memory cells, and not an imprinting of epigenetic restriction upon the chromatin of a homogenous pool of naïve T cells.

These potential confounding factors therefore identify the need to assess potential differences in within the CD44+ memory compartment of Sm offspring. Instead of bulk CD4+ analysis, this would also require more narrow assessment of naïve T cell modifications to the relatively more homogenous CD62L+CD44lo subset of naïve T cells.

3.2.2 T Cell Modifications driven by Maternal Schistosomiasis Restricted within CD44+ Compartment

Previous analysis of CD4+CD62L+ T cells from Sm offspring revealed difference in cytokine production, most prominently increased IL-4 production. This did not match the reduced epigenetic status across the bulk of these cells at the *IL4* promoter. To clarify whether modifications to the priming of naïve or memory T cells could be linked to differences in in vivo inflammation and T cell activation models, this phenomenon was further examined, including through culture of CD44+ and CD44- sub-compartments.

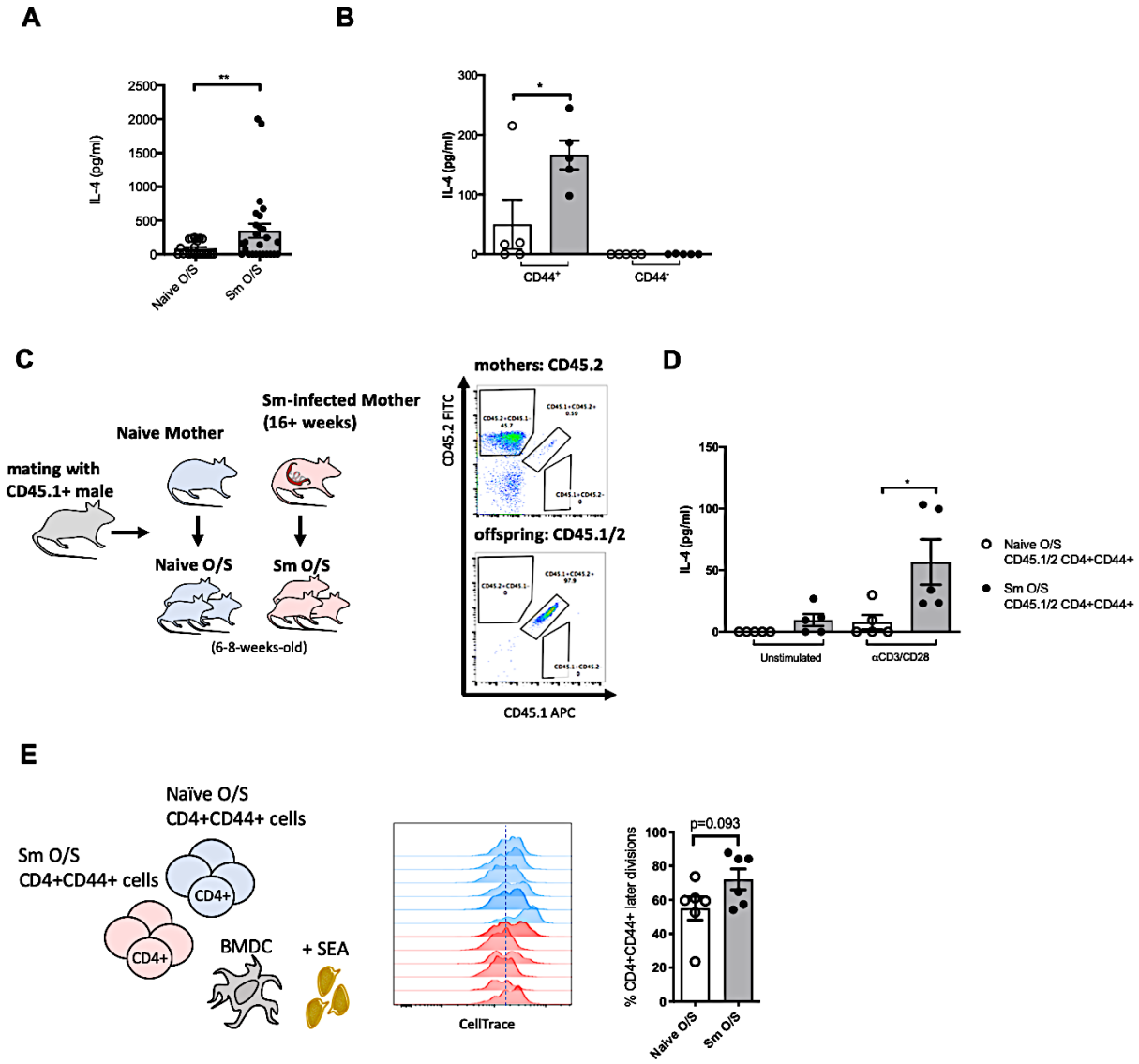


Figure 3.14 Maternal Priming of CD44+ Compartment
(A) Offspring CD4+CD62L+ isolated cells were cultured under T_H0 neutral conditions, with IL-4 levels in supernatants measured via ELISA after 6-days. (B) Levels of IL-4 in supernatants were compared after pre-sorting of CD44+ versus CD44- fractions of CD4+CD62L+ cells. (C) Scheme for mating CD45.1+ males with WT (CD45.2+) naïve or chronic Sm-infected females, resulting in CD45.1+ congenic labelling of offspring-derived cells, with offspring-derived CD4+CD44+ T cell additional sorted as CD45.1+/.2+ cells. (D) IL-4 levels in supernatants of offspring-derived cells after 48h stimulation with anti-CD3/CD28. (E) CellTrace proliferation dye-labelled CD4+CD44+ T cells from offspring cohorts were co-cultured with BMDCs that had been pre-pulsed with SEA (20µg/ml, O/N) for 72h, right: relative proportion of CD4+CD44+ in later CellTrace divisions. (A) depicts 3 independent experiments, (B) shows a representative experiment, (D) contains data pooled from 2 independent experiments, with mothers n=3, with (E) showing one of two representative experiments, again with mother n=3.

Further T_H0 neutral culture was performed alongside T cell polarization assays. Here, the T_H0-cultured CD4+CD62L+ T cells from Sm offspring clearly had an increased production of IL-4 (Fig. 3.14A). CD44+ and CD44- subpopulations were further flow sorted and subjected to the same culture conditions, revealing that this IL-4 production was restricted to the CD4+CD62L+CD44+ T cells from the Sm offspring (Fig. 3.14B), indicative of an altered Tcm compartment. Weaker but similar tends were observed for IFNγ, IL-10, and IL-17, denoting a more generalized activation of this compartment in Sm offspring, although the clearly distinct differences in IL-4 levels remained the most notable.

The identification of these cells in adult offspring mice, as differentially activated cellular populations primed in response to early life exposure to an altered maternal environment, could prove important for deciphering how maternal immune priming relates to the end-phenotype of modified inflammatory responses to heterologous antigens. One potentially key question regarding these cells is whether they are transferred cells of maternal origin (with such microchimeric cells reported to be active in other maternal helminth infection models and provide additional, helminth-specific cellular immunity (Darby et al. 2019)), or offspring-derived cells activated *de novo* in response to exposure to maternal infection. To address this wild-type (CD45.2) C57BL/6 chronic infected or matched naïve female mice were mated with CD45.1+ male mice. This marked the offspring-derived cells as CD45.1+CD45.2+, and permitted the distinction of offspring cells from maternal cells (which are instead CD45.2+ only) (Fig.3.14C, *central panels*). A similar approach has been used in fetal and neonatal mice to observe chimeric transfer of maternal cells (Solano et al. 2014). In adult 6-8-week-old mice, no clear maternal cellular population was retrieved through such flow cytometric analysis of splenocytes. Instead, CD45.1+CD45.2+CD4+CD44+ T cells from Sm offspring and naïve counterparts were flow sorted, and cultured for 48h, with and without TCR-stimulus. Consistent with previous assays, ELISA-based assessment of cytokines in the supernatant revealed notable amounts of IL-4 production from these offspring-derived memory CD4+ T cells, compared to the minimal amounts detected in those cells from naive mice (Fig. 3.14D). This further signifies offspring-derived cellular populations primed through maternal exposures, whether through direct antigenic exposure with schistosome antigens, as have been

shown to transfer to offspring *in utero* and through nursing, or through the altered cytokine milieu during development.

A further key question is whether these cells would show altered responses to schistosome antigens, and therefore demonstrate a degree of antigen-specificity. To test this, BMDCs were pre-pulsed with SEA overnight, follow by 72h co-culture with proliferation dye-labelled CD4+CD44+ T cells from Sm offspring and naïve mice. Likely due to the mix of antigens and other compounds present in SEA, including those that stimulate and suppress immune processes and TLR signalling (Kaisar et al. 2018; Bhattacharjee et al. 2019), responses to the BMDC-SEA stimulus were observed across CD44+ from both offspring groups. Proliferation curve analysis revealed a trend towards increased division of Sm-offspring-derived cells (Fig. 3.14E, *right*), with further studies required to study the potential specificities and activities of these cells. For example, optimisation would be required to more precisely assess cytokine secretion, and focused experiments with increased cell yield to effectively control non-specific activation differences (e.g.: with TCR stimulus), BMDC activation and cell viability during assay, and specific T cell sub-sets (e.g.: with sufficient cell numbers for ICS analysis).

3.2.3 Subsequent Cognate Infection of Exposed Offspring with Schistosomiasis

A more functional approach to assessing immunomodulation to cognate schistosome antigens is cognate infection of pre-exposed offspring themselves directly with schistosomes. In previous studies exploring such effects, "infected mother" mice were mated at 3-weeks (early T_H1 immune phase), and had subsequent reduced parasite load with apparent more aggressive clearance of worms (Attallah et al. 2006). Alternatively, mothers with 9-weeks infection (middle of T_H2 immune phase) lead to increased transfer of antibodies and associated reduction in necrosis in conjunction with granuloma formation (Lenzi et al. 1987). It would be reasonable to expect different outcomes in a chronic (16+ weeks) maternal infection setting, especially considering the heterogenous effects of maternal infection phase. The hypothesis here is that alongside priming of enhanced regulatory features regarding bystander antigens, chronic Sm offspring may show reduced anti-schistosome responses during infection, potentially associated with reduced clearance or increased parasitic load, as a form of primed tolerance.

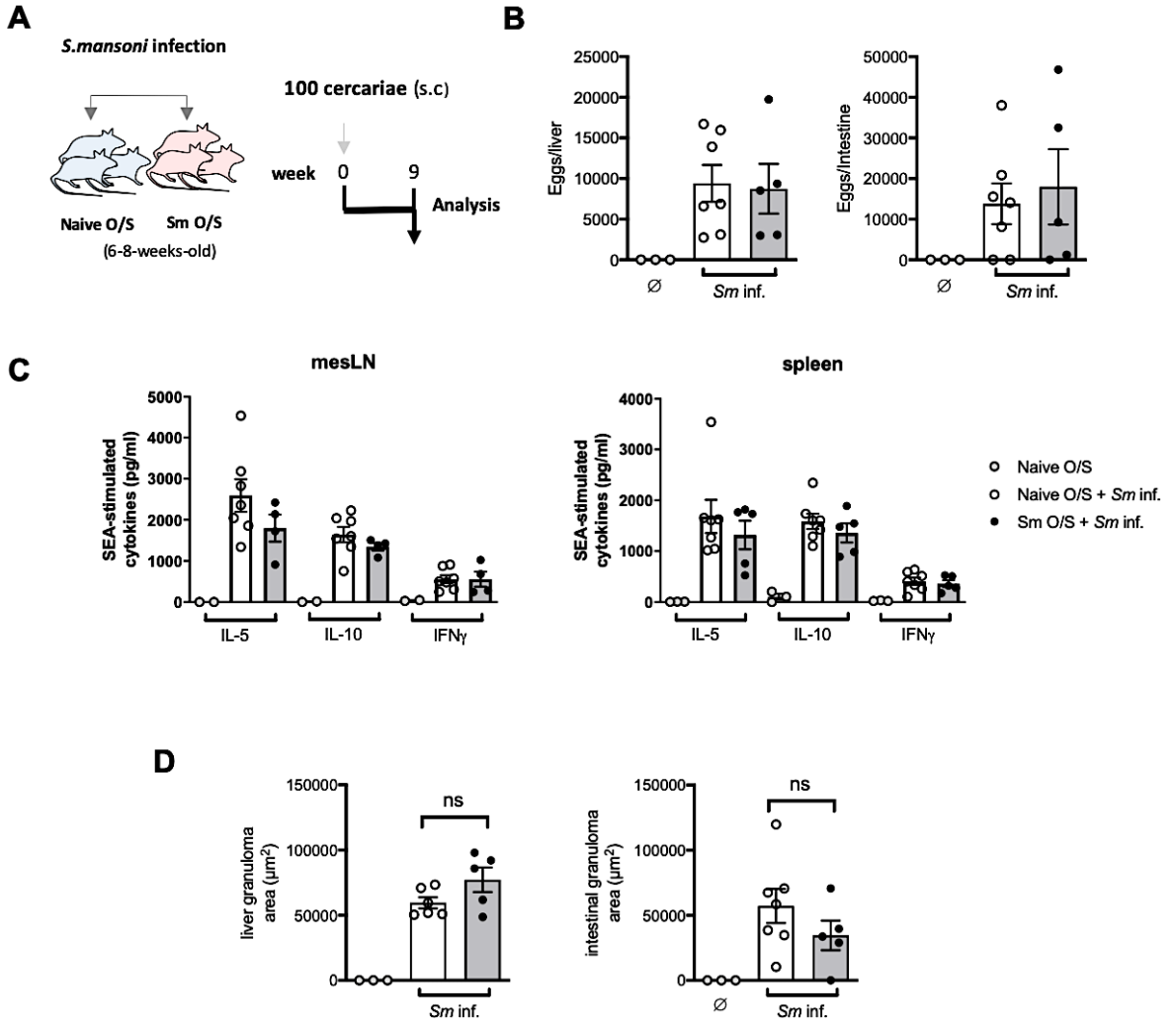


Figure 3.15 Cognate Schistosome Infection of Early Exposed Offspring
(A) Scheme for *S. mansoni* infection of Sm or naïve offspring via injection with 100 cercariae (s.c) analysed 9 weeks later. (B) Comparison of total egg counts per liver or intestine between offspring cohorts after 9-week infection, extrapolated from samples digested in KOH and microscopic enumeration. (C) Levels of IL-5, IL-10, and IFNγ in supernatants of cell suspensions from mesenteric lymph nodes (mesLN) or spleens stimulated *ex vivo* for 72h with SEA (20µg/ml) were determined via ELISA. (D) Total average granuloma area (from up to 40 measured granulomas, per mouse per tissue type) for liver and intestine via microscopic assessment.

Infection of offspring cohorts was analysed after 9-weeks to assess the effects upon the peak type-2 immune reaction dominated parameters typical of schistosomiasis (scheme depicted in Fig.3.15A). Egg deposition into the liver or intestines did not appear affected by prior maternal exposure (Fig. 3.15B). Antigen-specific cytokine responses did not strikingly differ in SEA-restimulated cell suspensions from mesenteric LN or spleens, although there were indications of trends towards lower levels of IL-5 and IL-10, which were clearly induced during this T_H2-dominated phase of infection, which although not

significant, were mirrored across both organs (Fig. 3.15C). This indicates similar lowered cytokine responsiveness, but would require further experimental work for clarification. Similarly, size of granulomas (formed around deposited eggs through T_H2-dependant processes and involving granulocytes including eosinophils [predominantly in liver granulomas] as well as alternatively activated macrophages and the deposition of collagen, reviewed in (Schwartz and Fallon 2018)) were not clearly different between offspring cohorts, with a non-significant trend towards increased liver granuloma size and smaller intestinal granuloma size in Sm offspring (Fig. 3.15D). Overall, the minor hints of differences in cytokine production could reflect similar modulations as evident in antigen-specific AAI hyporesponsiveness, but across parameters there was no major effect on cognate infection, at least under these conditions, in Sm offspring from chronic infected mothers.

3.2.4 Priming Effects in Purified Naïve (CD62L+CD44-) CD4+ and CD8+T Cell Compartments

The previous in vitro assays of "naïve" CD4+ T cell functional differences between Sm offspring and naïve mice used a CD4+CD62L+ population that contained CD44+ T cells. This yielded differential cytokine responses during *in vitro* assays, with increased cytokine production associated with multiple activated T helper subsets but largely and most consistently IL-4 indicative of type 2 helper T cells, that were incompatible with the epigenetic information from the ChIP data (as partially shown in (Klar et al. 2017)). As discussed, this could result from difference in the ratio and activation level of the CD44+ subpopulation.

In order to further clarify whether primed intrinsic differences in more "truly" naïve cell population drive the *in vivo* differences in T cell responses observed in allergy and immunization models, further analysis focused on CD62L+CD44lo subpopulation was performed. This removal of potential experienced cells allowed more direct assessment of the naïve cells that would recognize and react to heterologous antigens, such as to OVA-reactive T cells during the immunization or AAI models. For CD4+ T cells, this involved repeat *in vitro* activation and polarization assays (depicted in Fig. 3.16A), now excluding the CD44+ sub-compartment.

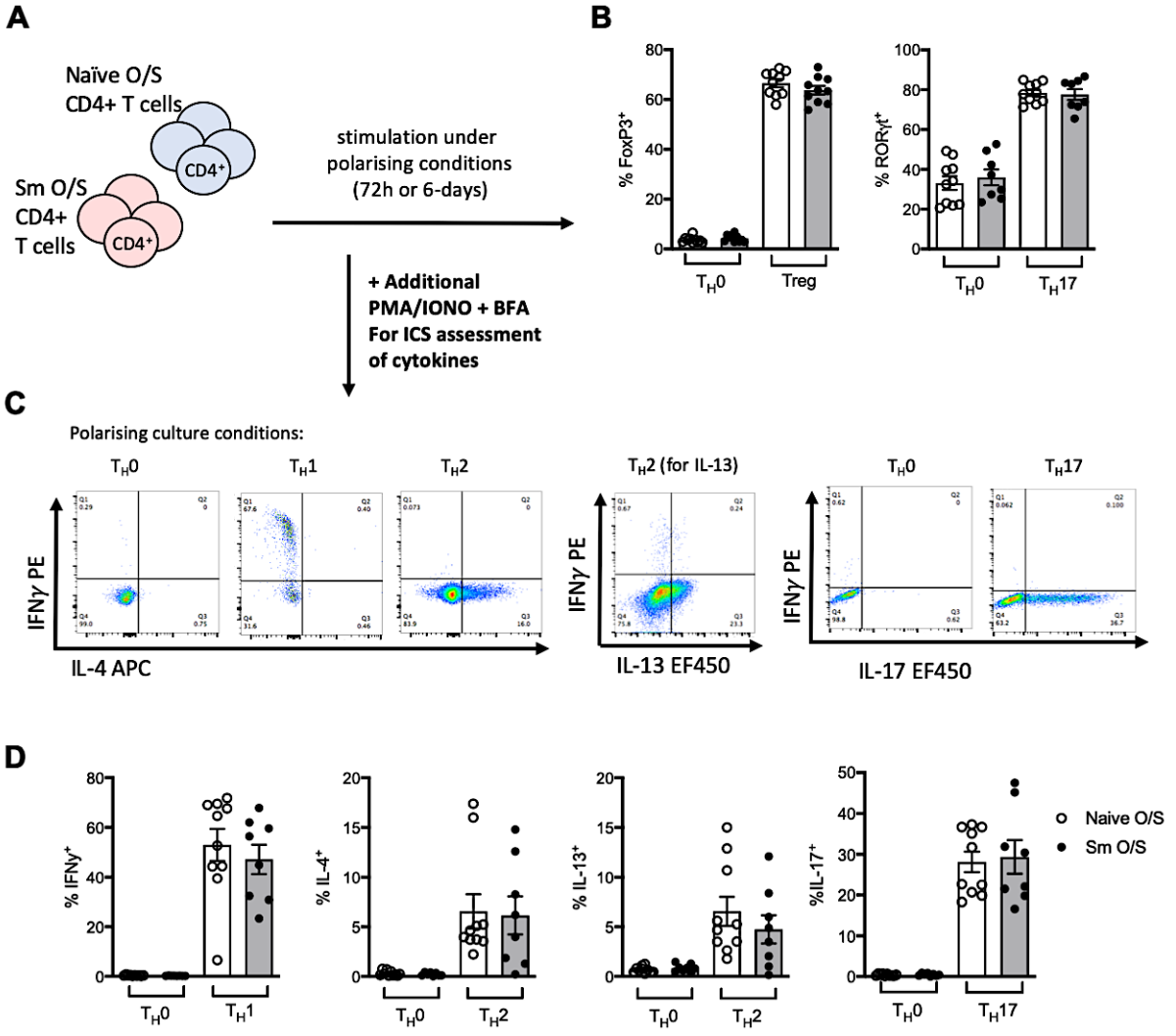


Figure 3.16 Functional Changes to CD44- T Cells
(**A)** Scheme for isolation of CD62L+CD44lo "naïve" CD4+ T cells from splenocytes of 6-8-week-old BALB/c Sm offspring and age- and sex-matched naïve controls in the steady state. **(B)** CD4+ T cells were activated and polarized *in vitro* into T helper subtypes (namely T_H1, T_H2, T_H17, or regulatory T cells) using antibody and cytokine cocktails, with transcription factors (FoxP3 and RORγt) measured directly via flow-cytometry, **(C)** showing representative flow cytometry plots for cytokine staining. **(D)** Production of polarization-relevant cytokines (IFNγ, IL-4, IL-13, IL-17) measured via ICS. Pooled data from 3 independent experiments using mixed gender BALB/c offspring cohorts.

As with prior experiments with total CD4+CD62L+ in Fig. 3.12, expression of FoxP3 (Treg) and RORγt (TH17) transcription factors were not different between offspring cells based on maternal priming (Fig. 3.16B). Similarly, and in contrast to the cultures where some CD44+ were present, there was no clear trend of increased polarization in any of the T helper sub-types assayed (representative plots in Fig. 3.16C), with similar levels of IL-4 and IL-13 (T_H2 polarisation), IFNy (T_H1 polarisation) or Il-17 (T_H17 polarisation) (Fig. 3.16D).

In tandem, aliquots of flow-sorted CD4+ and CD8+ CD62L+CD44lo T cells were cryopreserved and later subjected to transcriptomic analysis, with the rationale that priming may be reflected in differential expression of transcription factors, metabolic enzymes, or co-receptors associated with the likelihood to generate activated cells of a particular subtype, or housekeeping genes including those involved in maintaining the epigenetic status of the cell. One indicator of altered epigenetic mechanisms in T cell differences driving their responses, on a transcriptional level to equate with histone acetylation changes, would be alterations to epigenetic machinery: e.g.: transcriptional changes in histone acetyl transferases (HATs) and other modifying enzymes.

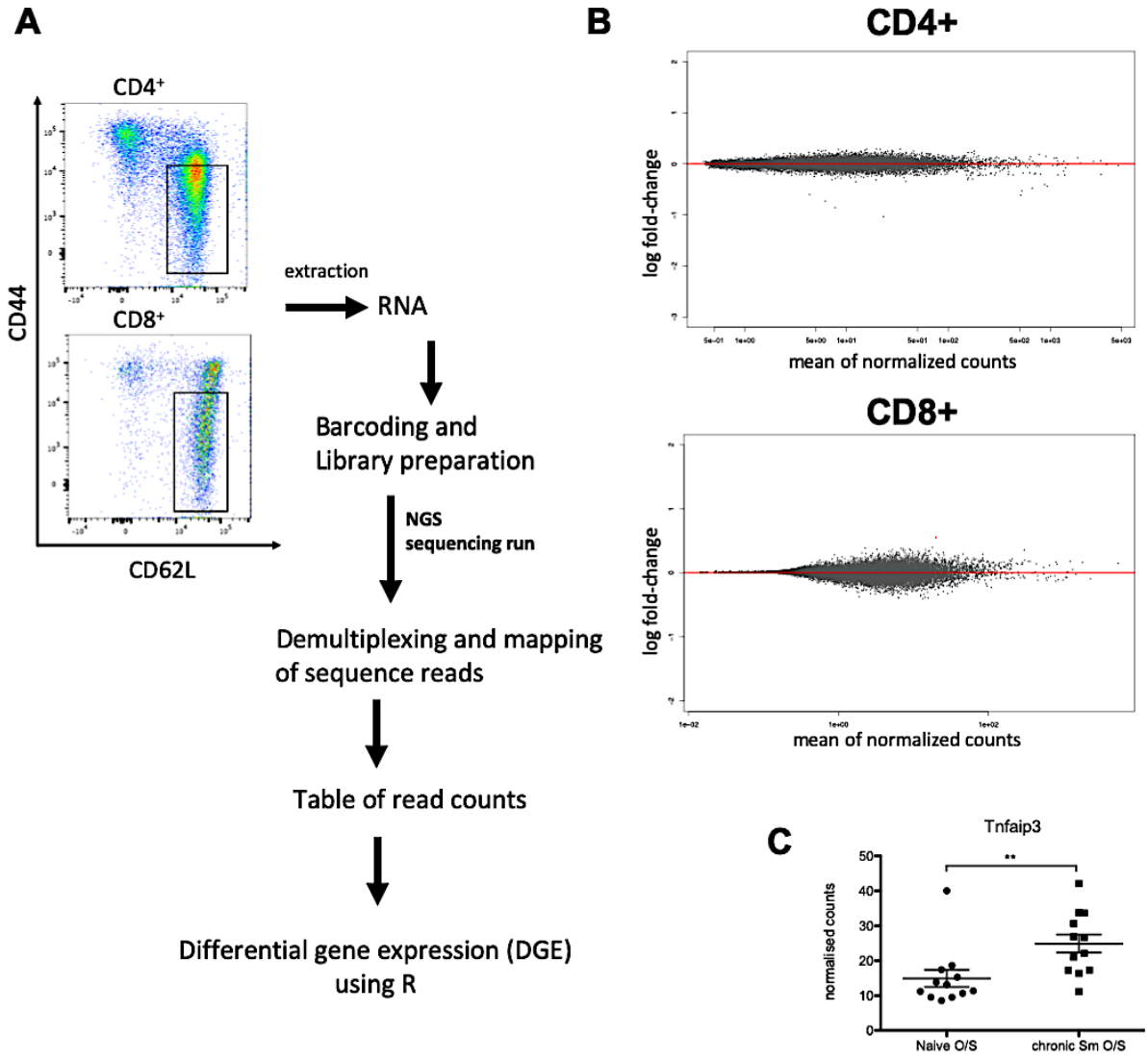


Figure 3.17 Transcriptional Profile in CD4 and CD8 CD44^{lo} T cells in Sm Offspring
(A) Scheme of transcriptomic analysis using bulk RNA extracted from flow-sorted of naïve T cells **(B)** MA plots comparing differential gene expression between cells from Sm and control offspring, generated using R package DESeq2 following RNA

extraction, library preparation, and Illumina NGS sequencing. (C) Differential sequence-read counts of *Tnfaip3* in CD8+ T cells RNA. RNA-seq performed comparing samples from 12 naïve offspring to 12 Sm offspring (mothers: $n \geq 3$ per group).

Transcriptomic analysis via RNA-seq of CD4+ samples similarly yielded no genes that reached a statistically significant adjusted p-value for differential expression between the groups (with similar MA plots, as in Fig. 3.17B). These results indicate together high levels of similarity between Sm offspring and naïve mice within this relatively pure, naïve CD4+ T cell compartment, giving no evidence to support that differences to heterologous antigen-specific CD4+ T cell responses *in vivo* arise from intrinsic differences in priming in naïve T cells themselves. As with naïve CD4+ T cells, transcriptomic profiles for the CD8+ T cells from Sm offspring and naïve controls also show a high level of similarity. The one exception was higher transcripts for *Tnfaip3*, a gene known to regulate CD8+ activity (Giordano et al. 2014), being found in the naïve CD8+ cells from Sm offspring compared to lower levels in naïve controls, indicating that these cells might show minor differences in the steady state. Following from these (albeit minor) differences, chromatin immunoprecipitation (ChiP)-based analysis was also performed on these sorted naïve CD8+ T cells for a range of CD8+ T cell-relevant gene loci.

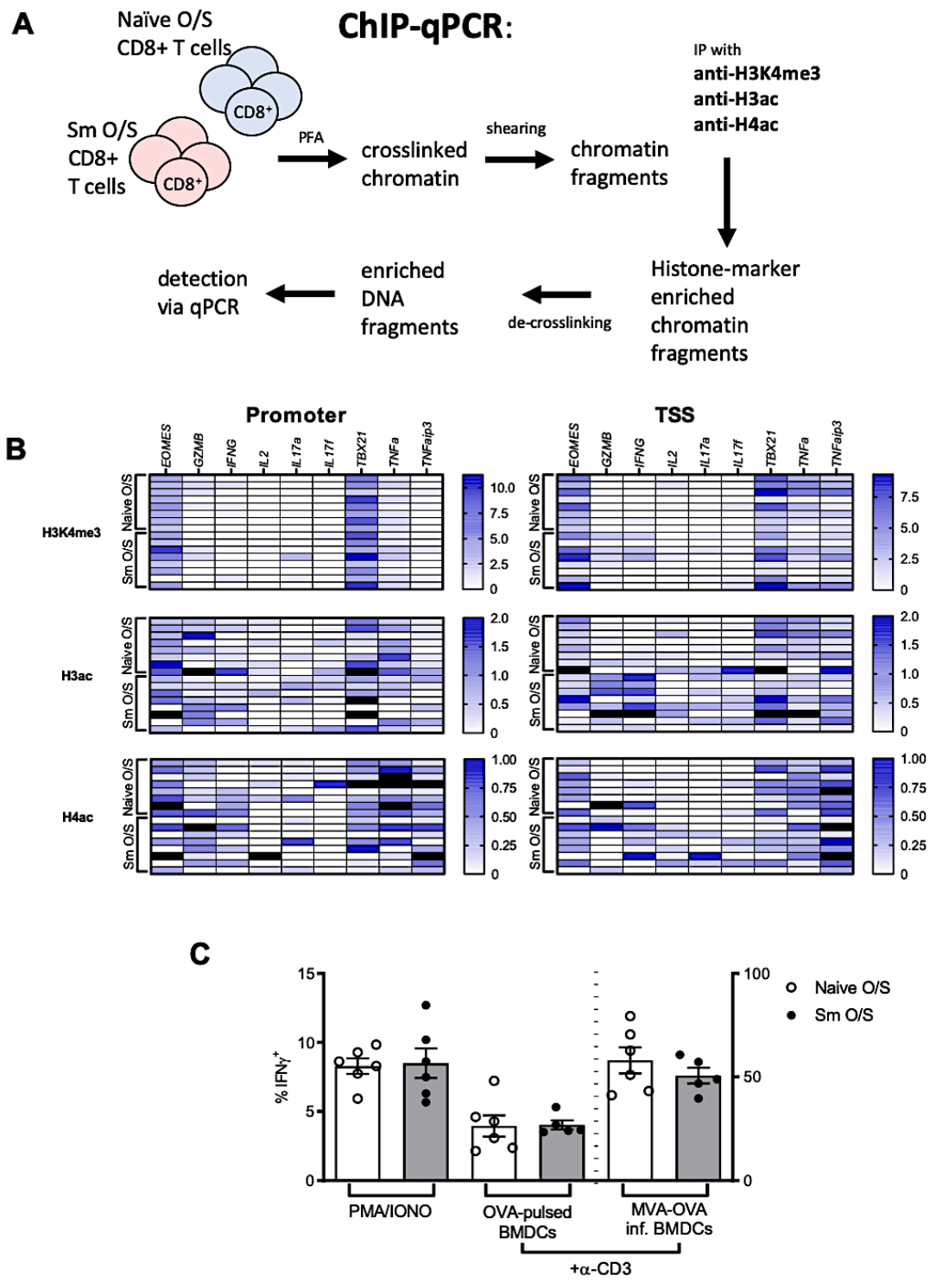


Figure 3.18 Epigenetic and Functional Analysis of Naive CD8+ T cells
(A) Workflow for chromatin immunoprecipitation (ChIP) assay of sorted CD8+ T cell samples targeting histone methylation via anti-H3K4me3 antibody, or histone acetylation via anti-H3ac and anti-H4ac antibodies, followed by quantitative PCR, assessing differential enrichment at the **(B)** promoter and TSS region of the gene loci. **(C)** IFNγ production assessed by flow cytometry of similarly sorted naïve CD8+ T cells from Sm offspring and naïve controls following: stimulation with PMA/Ionomycin for 5h in the presence of BFA, or 3-day co-culture with anti-CD3 + bone-marrow derived Dendritic Cells

(BMDCs), which were either pre-pulsed with OVA protein, or with BMDCs pre-infected with MVA-OVA followed by 6h additional stimulation in the presence of BFA, plus ICS staining. ChIP performed on 8 samples per group (mothers: n ≥ 3 per group). Culture experiment shown was conducted with offspring: n = 4-5, mothers: n ≥ 3 per group.

Potential epigenetic changes to the promoters of a panel of CD8-related effector genes were evaluated, which were: *EOMES*, *GZMB*, *IFNG*, *IL2*, *IL17a*, *IL17f*, *TBX21*, *Tnfaip3*), yet this too revealed high homology between Sm and naïve offspring, with suggestion of enriched permissive markers, for example on H3ac TSS for *IFNG* and *GZMB* for Sm offspring-derived T cells, further reducing the likelihood that direct intrinsic changes to these cells would be responsible for modulated suppressed T cell functionality *in vivo* immunization models.

As functional analysis, *ex vivo* stimulation of these sorted naïve CD8+ T cells with PMA/Ionomycin revealed equal numbers of IFNγ+ cells (Fig. 3.18C). Similar results were obtained through co-culture experiments, with naïve bone-marrow-derived dendritic cell (BMDCs) pre-pulsed with OVA alone, or pre-infected with MVA-OVA to mimic the stimuli from the *in vivo* vaccinations). The addition of anti-CD3-based provided T Cell Receptor (TCR) stimulus. As was expected from the *in vivo* experiments, MVA-OVA infection of DCs indeed induced higher proportions of IFNγ+ CD8+ T cells following co-culture compared to OVA-pulsing. However, the differential *in vivo* response from Sm offspring was not observed, with both stimulations yielding equal proportions of IFNγ+ cells as well as similar levels of secreted IFNγ as compared to control offspring (Fig. 3.18C). As such, the naive CD8+ T cell compartment of Sm offspring revealed no clear apparent differences that reflected their differential *in vivo* immunization responses, nor that would support the hypothesis that their divergent responses derived from induced changes to CD8+ T cell development during ontogenesis in response to maternal schistosomiasis.

As such, assessment of the CD62L+CD44^{lo} compartment of naïve cells gives no convincing evidence to support the hypothesis that the *in vivo* T cell phenotype observed in Sm offspring derives from developmental priming differences in the pool of naïve, antigen-inexperienced T cells, which would include those reacting to OVA epitopes.

3.3. EARLY PRIMING OF T CELL EXTRINSIC REGULATORY NETWORKS

3.3.1 Immunization Response of Exogeneous T Cells in Transmaternally-Primed Recipients

3.3.1.1 Effects on subcutaneous Allergic Sensitization

Despite the clearly modified induction of T cell responses, most notably CD4+ polarisation shifts and CD8+ inflammatory T cell responses during the various immunization models, no functional differences were found in isolated naïve (CD44lo) CD4+ or CD8+ T cells from Sm offspring, when compared to those from naïve mice. Additionally, no major clear transcriptional changes were found, nor epigenetic shifts in the case of CD8+ T cells. Aside from developmental changes to naive T cells, an alternative hypothesis is that the divergent immunization-induced CD8+ responses of Sm offspring arise from altered regulatory responses, that modify the activation or function of antigen-specific CD8+ T cells. The following experiments explore this first by using Sm or naïve offspring as recipient mice for *in vivo* transfer of exogenously-derived antigen- (OVA)-specific CD8+ T cells that had not been exposed to maternal schistosomiasis during development. This allows segregation of the influence of an altered regulatory environment from naïve T cell intrinsic changes, and tracking of antigen-specific T cell responses more directly. CD44-low naïve CD8+ transgenic OT-I cells were flow sorted from mice with congenic CD45.1 expression. These were then transferred into wild type (CD45.2+) Sm offspring and control mice as recipients (via i.p), prior to *in vivo* activation via subcutaneous immunization with ovalbumin protein alone (Fig. 3.19A), as used earlier in experiments examining endogenous responses, and as initially used to sensitize mice prior to AAI induction. CD45.1+ staining allowed discrimination of these antigen-specific transferred OT-I cells from endogenous populations (Fig. 3.19A, *right*) for subsequent analysis.

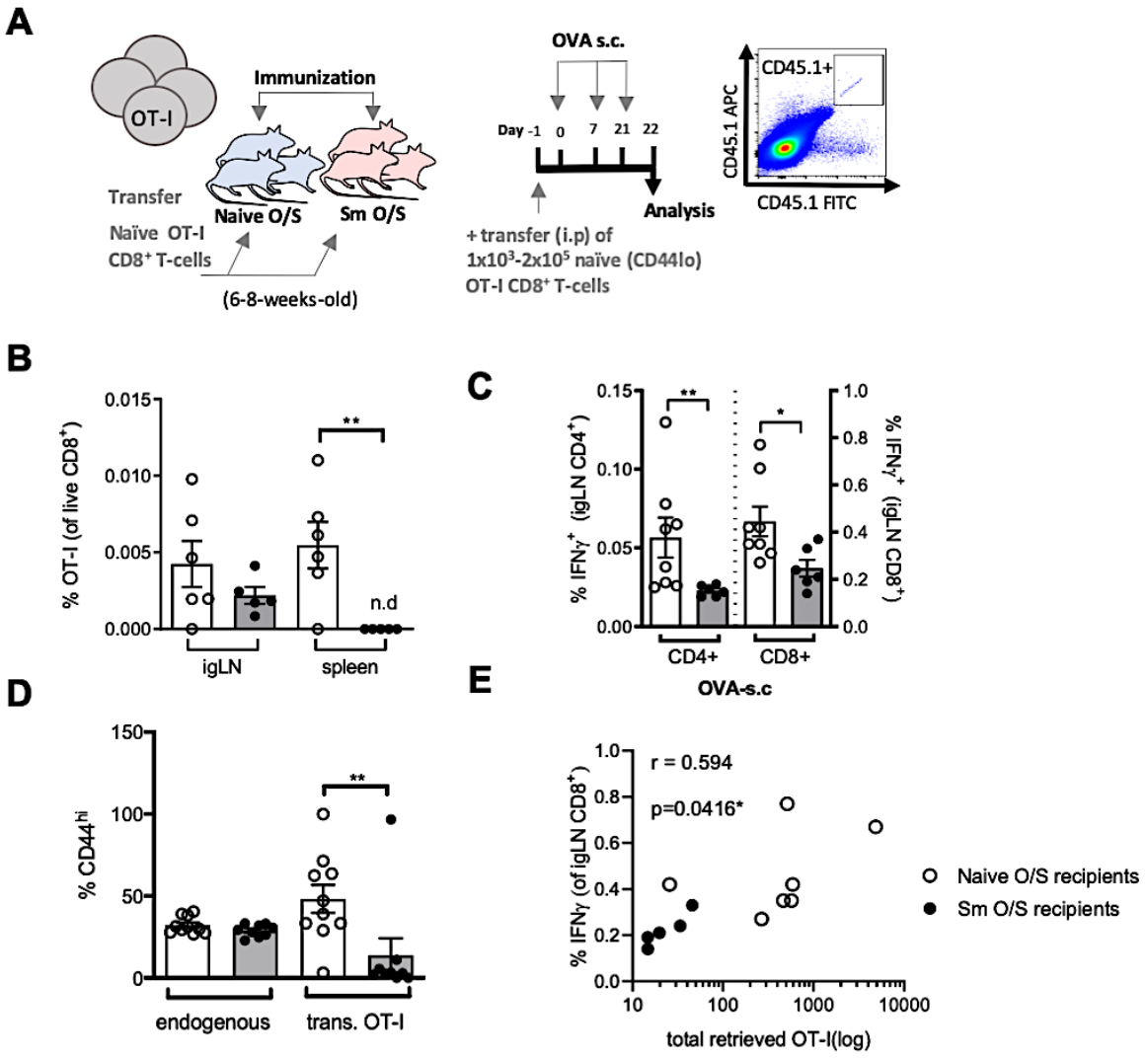


Figure 3.19 Behaviour of Exogenous T Cells within Sm O/S Recipients
(A) Experimental scheme for adoptive transfer of 1-2x10^5 flow-sorted $CD45.1^+CD8^+CD44^{lo}$ naïve OT-I cells into recipient C57BL/6 Sm or naïve offspring mice, followed by immunization with ovalbumin (s.c.) using the same allergic sensitization scheme over 21 days as per Fig. 3.7, with representative dot plot for discriminating CD45.1+ donor cells from CD45.1-CD45.2+ endogenous cells. **(B)** Overall retrieval of OT-I T cells from Sm offspring versus naïve control recipients (proportion of CD8+ T cells in igLN and spleen), using low OT-I number (10^3 cells) transfer **(C)** Assessment of altered IFNγ production from *ex vivo* OVA re-stimulated draining lymph node (igLN) cell suspensions following 21-day immunization model, as determined by ICS. **(D)** Proportion of CD8+ T cells with high expression of CD44, from both endogenous (CD45.1-) and transferred (CD45.1+) subpopulations (in spleen), as determined by surface staining and flow cytometry, 24h after the final immunization (using high cell number transfer of 2 x 10^5 OT-I). Adoptive transfer experiment in (A-E) using 6-8 immunised offspring per group, except (D) conducted with 9-10 immunized offspring per group after higher number OT-I transfer, each time using Sm offspring derived from two separate infected mothers.

CD8+CD45.1+ OT-I cells were first transferred (as per scheme Fig. 3.19A) at levels just above the physiologically appropriate level (which would be around 100 epitope-specific cells in a mouse) of 10^3 cells per recipient. After 21-day OVA-s.c. immunization, this resulted in a weak trend towards lower CD8+ retrieval from igLN of Sm offspring recipients, but more clearly, no transferred OT-I were retrieved from the spleens of Sm offspring, unlike from naïve recipients (Fig. 3.19B), providing initial evidence of modified expansion of CD8+ T cell responses. Low numbers of retrieved OT-I inhibited further direct phenotyping, such as of the previously observed phenotype of lower immunisation-induced pro-inflammatory cytokines. However, across total cells, Sm offspring here again showed lower IFNγ responsiveness to *ex vivo* OVA-re-stimulation in igLN cell suspensions, in both CD4+ and CD8+ T cells (Fig. 3.19C).

CD44 expression in the endogenous CD8+ T cell population in the spleen revealed a minor decrease in Sm offspring compared to naïve controls, supporting previous findings of altered proportions of endogenous memory cell populations (as observed in Fig. 3.13D). Adoptive transfer of higher (highly supraphysiological) numbers of OT-I (2×10^5) permitted further analysis of phenotype of transferred cells. Here the transferred CD45.1+ OT-I cells retrieved from the spleen of Sm offspring contained much lower proportions of cells with high expression of CD44 (Fig 3.19D). This result signifies a clear difference in the accumulation of antigen-specific CD8+ T cells with an activated or memory phenotype regarding CD44 expression following immunization in Sm offspring, and suggests inhibited development of an activated memory population in systemic circulation. In contrast to T cell developmental changes, these findings support the hypothesis that altered regulation of T cell responses is involved in driving the differential CD8+ T cell vaccination responses, and corroborates the previous failure to demonstrate major functional or intrinsic differences in endogenous CD8+ T cells alone. A further link between this functional outcome (of lower circulating memory populations) of altered activation capacity and the observed drop in IFNγ levels, is that retrieved numbers of OT-I in this system showed a correlation with OVA-stimulated IFNγ levels detected via ICS in the LN (Fig. 3.19E), further linking these two phenomena.

3.3.1.2 Effects on Alum versus MVA-Vector Immunization

Adoptive transfer of exogenous OT-I cells into Sm offspring showed alteration to the function of those cells following immunization, supporting the concept that regulation of T cell responses is a key driving factor in the modified phenotype observed in Sm offspring, as opposed to developmental imprinting within the naïve T cell compartments. The regulation of CD8+ T cell activation, however, involves complex interactions of factors. These include initial activation by TCR-recognition of MHC-I-peptide complexes, co-stimulation, including by professional antigen presenting cells, as well as additional signals provided by CD4+ T cell help, and antagonistic effects from cells with suppressive capacity, including Tregs and Bregs. Tracking the dynamics of these processes, as occur in expansion through activation, may also be complicated, particularly over the multi-week allergic-sensitisation-based immunisation OVA-s.c scheme used here for T cell activation, by the peritoneal cavity acting as a reservoir of naive transferred cells after i.p transfer.

To eliminate the potential dynamics that may be operating over the 21-day immunization period, and to focus on whether the initial activation of antigen-specific CD8+ T cells is modified (that is, at the initiation of the T cell responses), a 48h model of T cell activation was chosen to evaluate the proliferation and acquisition of activation marker CD44 during OVA immunization. OVA-ALUM was chosen to give effective activation in a short time period (although still with a modality with demonstrated alternate effects in Sm offspring, both in immunization alone and AAI). OVA-ALUM was assayed alongside MVA-OVA, the antigen-delivery vector that gave the opposing effect of apparent heightened CD8+ T cell activation despite maternal helminth priming, and which normalised the anti-viral responses of Sm offspring to HBV. Prior to transfer, OT-I cells were stained with CellTrace Violet proliferation dye to track divisions in response to immunization.

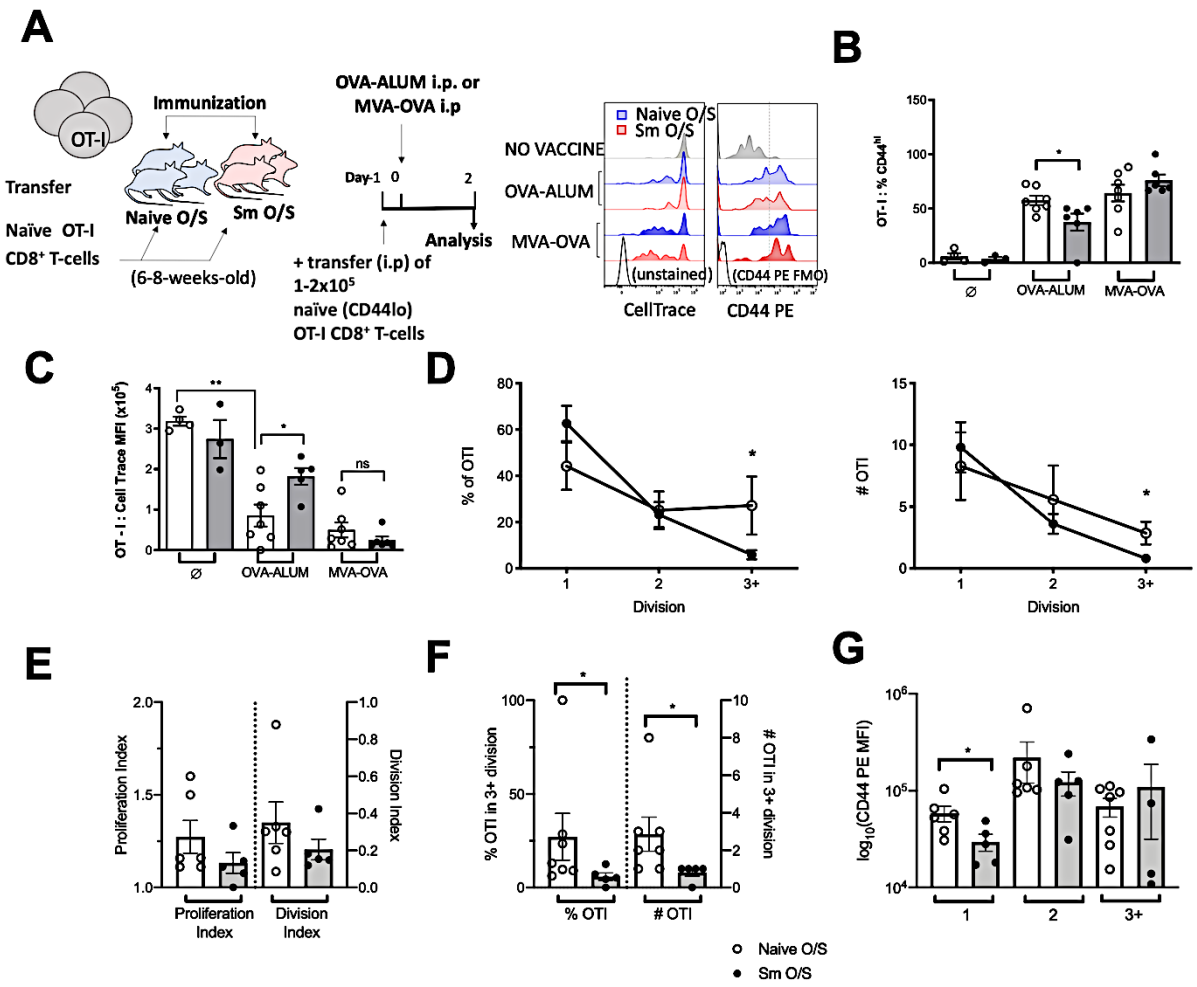


Figure 3.20 Vaccine Modality-dependant Behaviour of Exogenous CD8+ T Cells within Sm O/S Recipients
(A) Experimental scheme for adoptive transfer of 1-2x10^5 flow-sorted CD45.1$^+$CD8$^+$CD44^{lo} naïve OT-I cells into recipient C57BL/6 Sm or naïve offspring mice (i.p), followed by immunization with OVA-ALUM or MVA-OVA, as described earlier (both i.p), or no additional immunization. **(B)** Proportion of transferred CD45.1+ OT-I with high expression of CD44 in spleen, as determined by surface staining and flow cytometry, 48h after immunization. **(C)** Analysis of cell proliferation using flow cytometric analysis of Cell Trace division peaks.

Consistent with the findings obtained using the 21-day OVA-s.c scheme, this shorter OVA-ALUM protocol (in Fig. 3.20A) drove differential CD44 acquisition for transferred OT-I dependent on recipient maternal infection history, with lower proportion of CD44^{hi} in Sm offspring recipients already at this early 48h timepoint. OT-I cells from mice having undergone MVA-OVA immunization remaining similar regardless of maternal infection status, supporting a role for the MVA vector in overcoming the inhibitory effects of maternal schistosomiasis with regard to CD8+ T cell activation (Fig.3.20B). Through the additional analysis of CellTrace dye dilution peaks (representing cell divisions), those OT-I CD8+ T cells further appear not only to exhibit lower levels of the activation marker CD44, but also

less cell division. This was apparent as less overall dilution of the proliferation dye (as in OVA-ALUM groups in Fig. 3.20C), and less divisions after OVA-ALUM immunisation in Sm offspring recipients compared to within OVA-ALUM immunised naïve mice (Fig. 3.20D). This yielded trends towards lower proliferation and division index (metrics of total divisions based on division profile and total numbers of retrieved cells) (Fig. 3.20E), with less OT-I achieving later (3+) divisions in Sm offspring recipients after OVA-ALUM (Fig. 3.20F).

As shown in Fig. 3.9, CD44 upregulation is found on activated cells, and therefore proportionally varies according to the ratio of divided cells, including those with multiple divisions, to non-activated cells, which are $CD44^{lo}$ as originally flow-sorted. As such, although lower CD44 MFI could be indicative of lower overall division as already analysed above, the first peak itself has a lower CD44 MFI in OT-I activated via OVA-ALUM immunisation in Sm offspring recipients compared to naïve recipients, and indicates altered activation even prior to many cell divisions (Fig. 3.20G).

As such, not only did maternal schistosomiasis imprint reduced inflammatory T cell responses in offspring to heterologous antigens, but the effects of this maternal priming can be observed in exogenously derived T cell that have only been present within the maternal infection-primed mouse for a few days, and appear to already inhibit the initial T cell activation process. These results further suggest that alterations in T cell reactivity in Sm offspring using various immunization models most likely do not result from pre-set, CD8+ T cell-intrinsic changes, and instead that maternal schistosomiasis imprints a regulatory signature upon the offspring immune system that remains to be explored outside of the naïve T cell compartment.

3.3.2 Early Schistosomiasis Primes Shift in Offspring B Cells

3.1.12.1 Steady-State Priming of Homologous Antibodies to Schistosome-Antigens

The responses of Sm offspring to allergic sensitization, as associated later with reduced inflammation, were characterized by an increase in IL-4 production from T cells, as well as enhanced activation of B cells, including increased proportions and activation status of B cells, as well as higher OVA-specific antibody titres. This type 2 skewing of CD4+ T cell responses, plus enhanced B cell responses induced

in Sm offspring appeared to have an inverse relationship to IFNγ responsiveness in T cells (as indicated by differential IL-4+/IFNγ+ T cell ratios), and to the immunization-induced activation of CD8+ T cells. Both type 2 helper T cell responses and B cell activation can have inhibitory or antagonistic effect on pro-inflammatory type 1 immune responses, and linked to how helminth exposure can not only skewing of CD4+ responses, but also inhibited expansion of CD8+ CTL cells, otherwise required to efficient antiviral immunity (Shollenberger et al. 2013). Previous investigations of the effect on maternal schistosome infection in murine systems found the presence of anti-schistosome antibodies, that decreased from birth until 8-weeks of age (Attallah et al. 2006), although these experiments used acute, not chronic maternal infection. Given the weight of data on the stimulatory effects on B cells of schistosomes, their eggs, and derived-antigens, Sm offspring were evaluated for changes to B cell proportions, and schistosome-specific antibody titres.

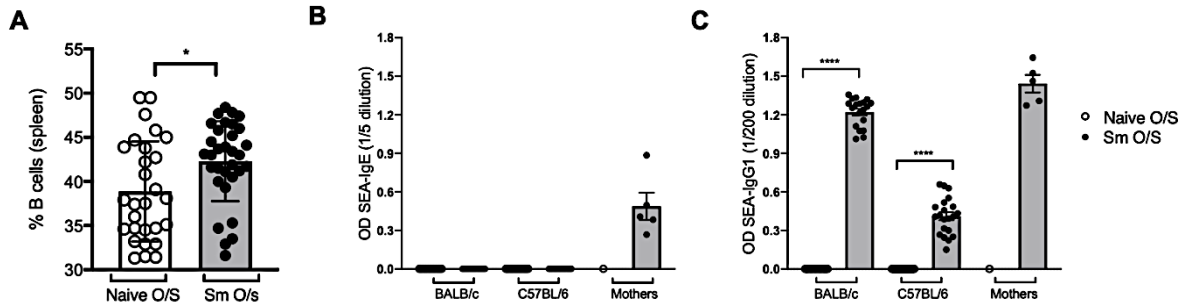


Figure 3.21 Steady-state Modifications to B Cell Compartment and Antibody Titres
(A) Proportions of B cells (B220+) within spleens of BALB/c Sm offspring and naïve controls as measured during steady state via flow cytometry. (B) SEA-specific IgE titres in the sera of BALB/c and C57BL/6 Sm offspring and naïve controls across steady state and immunization models (sampled from cohorts in Figs. 3.2, 3.3, 3.6, and 3.7), as well as SEA-IgE as measured in BALB/c and C57BL/7 infected mother mice, detected using ELISA. (C) Serum SEA-specific IgG1 titres in these same mouse cohorts.

Indeed, the spleens of Sm offspring displayed increased proportions of B220+ B cells (Fig. 3.21A). Initial functional changes to the B cell compartment were also assessed through detection of schistosome-specific antibody titres. No SEA-IgE was detected in the sera of Sm offspring, neither in BALB/c nor C57BL/6 mouse strain, and as expected, these were also absent from naïve offspring, but present in the sera of infected mother mice (Fig. 3.21B). In both mouse strains, however, all Sm offspring show significant levels of SEA-specific IgG1, even into adulthood and including mice beyond the 10

weeks of age. BALB/c Sm offspring mice even displayed high ODs very similar to the chronic infected mice themselves (Fig. 3.21C). Both IgE and IgG1 are demonstrative of antibody subclasses that required type 2 cytokine-based help via IL-4 for example. As such, the detection of such antibodies is not surprising, considering the earlier discovery of an expanded population IL-4+ producing CD4+ T cells during steady state, and enhanced IL-4 in response to immunisation. The long persistence of these IgG1 antibodies into adulthood suggest their continued de novo expression from plasma cells, rather than only passive transfer, although this remains to be investigated. The relatively long-term detection of anti-schistosome antibodies, alongside other less-specific changes to the B cell compartment including general expansion of numbers, suggests the likelihood of further modifications, including those that could mimic those induced regulatory changes seen in chronic infection itself.

3.1.12.2 Bystander Effect on Heterologous Antigen-Priming of T Cells

The presence of changes to B cells in response to maternal schistosome infection suggested the possibility of further regulatory marks within the B cell compartment of Sm offspring. The potential involvement of B cell changes within effects on CD8+ T cell activation was assayed in co-culture, where naive CD8+ T cells were activated in vitro by BMDCs loaded with SIINFEKL, in the presence of B cells sorted from Sm or Naive O/S (as per scheme below, in Fig. 3.22A).

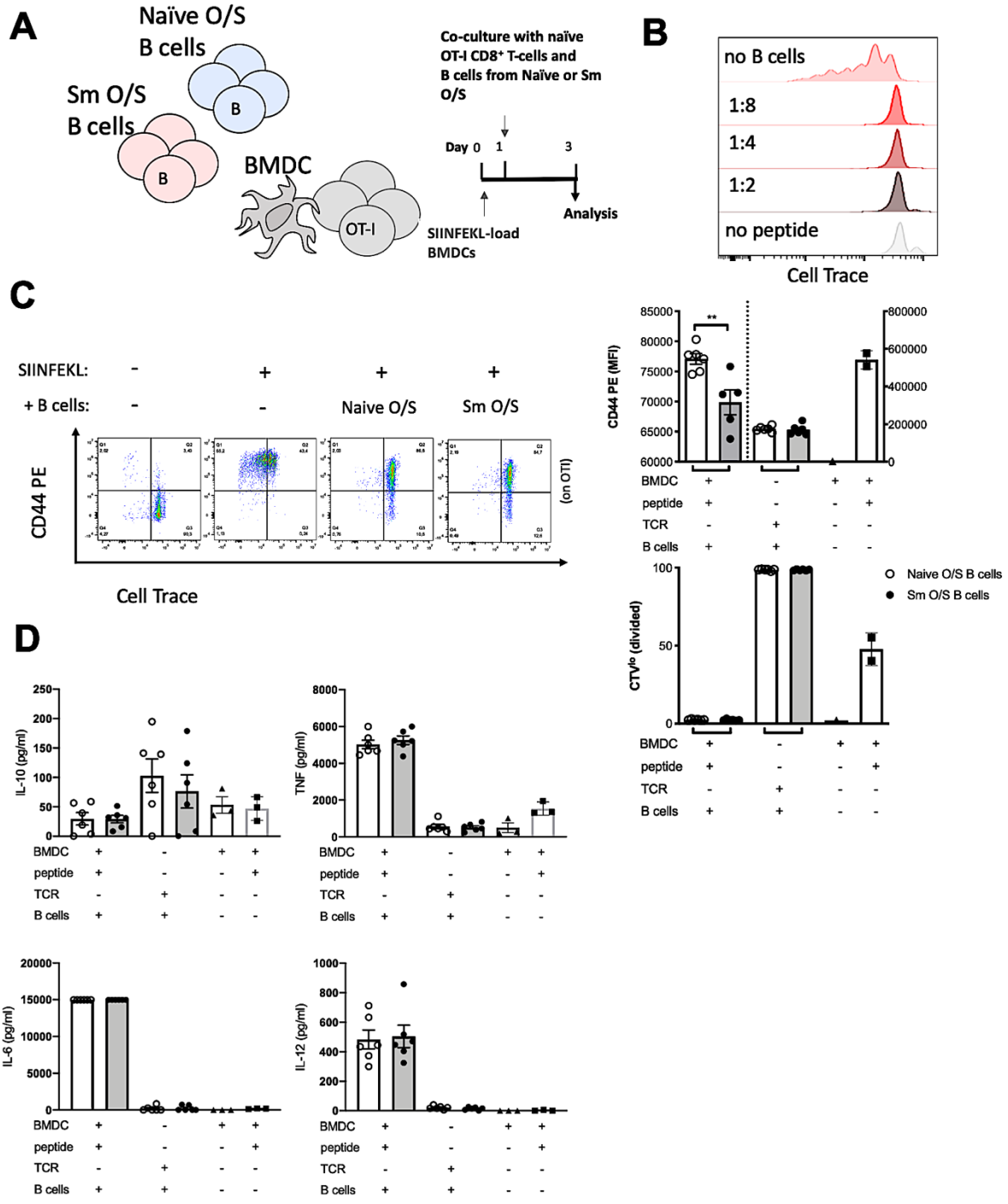


Figure 3.22 CD8+ Suppression by B Cells via DC Interactions
(A) Scheme for co-culture of B cell sorted from offspring cohorts with BMDCs + naïve OT-I cells + SIINFEKL peptide (with anti-CD3/CD28 used as a DC-free activation system for comparison). **(B)** CellTrace-based assessment dose-dependency of B-cell driven changes to DC-peptide induction of naïve OT-I proliferation. **(C)** OT-I proliferation (assessed using CellTrace dilution), and acquisition of CD44, via 72h activation +/- offspring B-cells as per (A), *right*: comparison B-cell effect on DC-peptide versus anti-TCR activating conditions. **(D)** Supernatant levels of IL-10, TNF, IL-6, and IL-12, as measured via ELISA, from 72h activation of OT-I under listed conditions. Pilot experiment with 6 naïve and 6 Sm C57BL/6 offspring per group, mothers: n = 3.

Interestingly, the additional presence of B cells alone was sufficient to largely inhibit the proliferative response from the OT-I CD8+ T cells in all cases (otherwise observed in co-culture with BMDCs + peptide, as in Figs. 3.22 B and C). This was rather on/off, with only some minimal dose-dependent effects across B:T cell ratios from 1:2 – 1:8 (Fig. 3.22B). A small yet distinct drop in CD44 MFI was evident in the OT-I cultured in the presence of Sm offspring-derived B cells compared to those cultured with naïve offspring-derived B cells, hinted at an effect on CD8+ suppression. This was not the case with TCR-based OT-I activation (Fig. 3.22C, *right*), indicating possible interactions between the B cells and the BMDCs used to prime the OT-I. Indeed, while there were no clear cytokine shifts between B cell co-cultures based on their origin from Sm or control offspring, the addition of B cells to the BMDC-OT-I-peptide co-culture yielded massive increases in TNF, IL-6, and IL-12, without changing IL-10 levels (Fig. 3.22D), further suggestive of drastic changes to immune interactions that may have impacted T cell proliferation under these conditions, and potentially severe triggering of DC responses of these cytokines, but none that provided further evidence for differences based in exposure to maternal infection. As such, while these initial results suggest a possible B-cell-driven effect on CD8+ activation, potentially in interaction with DCs, these would require further optimisation and experiments to draw meaningful conclusions.

3.1.13 Regulatory Network 2: Early Schistosomiasis Priming Modifies T Cell Activation Capacity of Antigen Presenting Cells

3.1.13.1 Steady-State Transmaternal Priming of Antigen Presenting Cells

Prior exposure to maternal schistosome infection was able to modify the activation of exogenously-derived, transferred antigen-specific T cells via immunisation of Sm-primed recipients relative to responses to immunisation of naïve recipients, including within a short time period after transfer, indicating the presence of alterations to factors regulating the activation of T cells. This immunisation-induced activation likely involves antigen-presentation via professional APCS, with DCs being largely responsible for de novo activation of naïve T cells, such as those transferred. This led to further analysis in steady state of B cell activation profile, already identified to differ with maternal exposure during immunisation, as well as that of cDCs (CD11c+MHCII+), and their major cDC1 (XCR1+) and cDC2

(CD11b+) subsets (as per FACS gating strategies in Fig. 3.23A). Given the plasticity of the APC compartment in response to environmental exposures already discussed, and potential mechanistic role in retaining imprinting via early life exposures via features of innate training, the previously identified markers of altered APC activation (MHCII and CD86) were further explored in steady-state in these APCs, within collagenase-digested spleens of offspring cohorts from both C57BL/6 and BALB/c mice offspring cohorts.

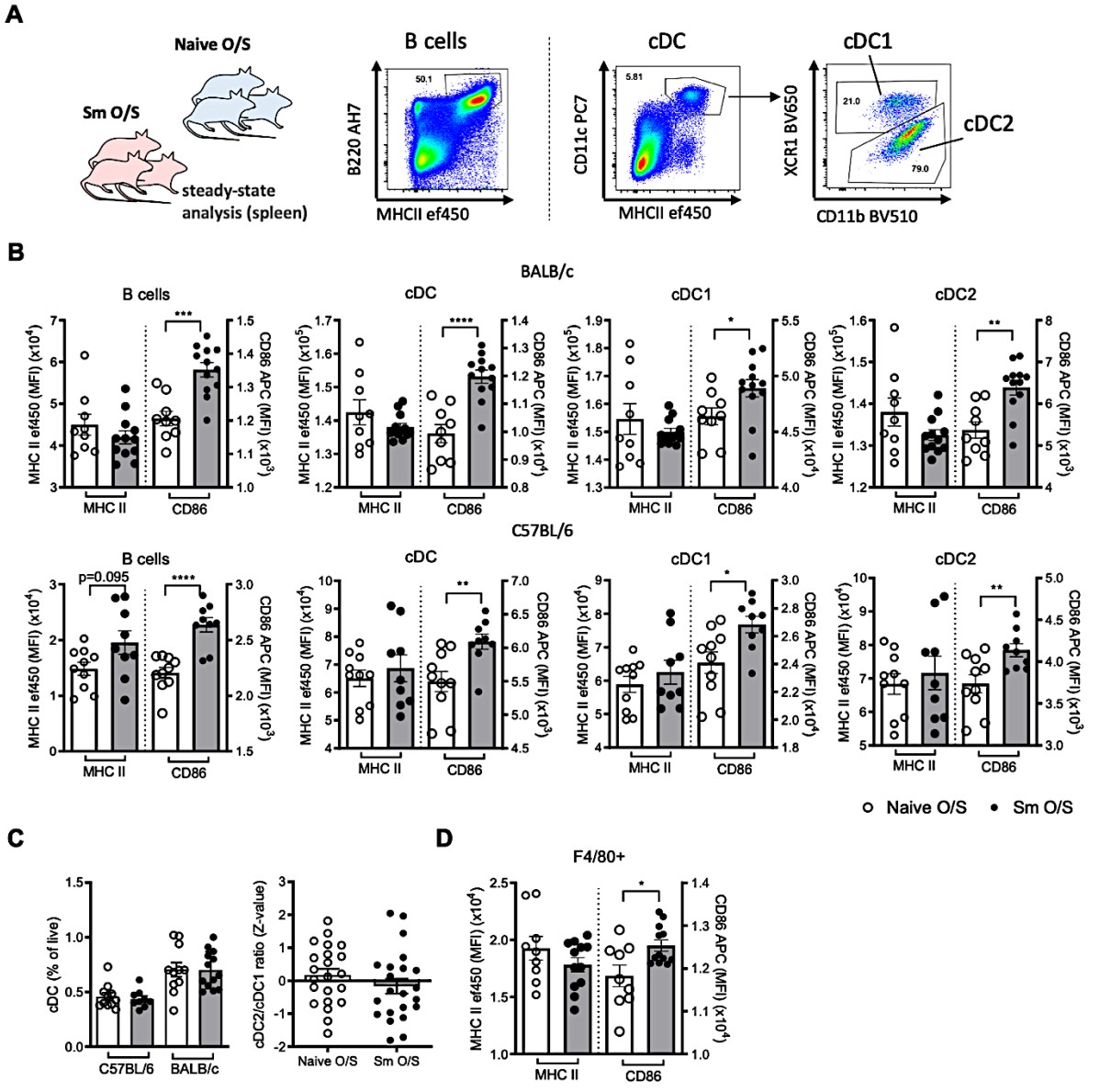


Figure 3.23 Altered Activation Profile of B Cells and APCs, including Steady-State DC Subsets
(A) Representative plots for steady state analysis of B220+MHCII+ B cells, CD11c+MHCII+ cDCs, including sub-analysis of XCR1+ cDC1 and CD11c+ cDC2 subsets. (B) Flow cytometric analysis of MHCII and CD86 MFI, for both BALB/c and C57BL/6 offspring cohorts. BALB/c data in (B) pooled from 2 independent experiments, totaling 9 naive offspring (mothers:

n = 4) and 12 Sm offspring (mothers: n = 6), all offspring 16+ weeks old. C57BL/6 data in (B) pooled from 2 independent experiments using offspring ranging from 6-15 weeks, n=9-10 (mothers: n = 4 per group).

Although upregulated on B cells during OVA-s.c, and to a greater degree in Sm offspring, MHCII expression showed no clear overall trend of altered expression across the APC subsets assayed, with only a trend towards higher expression on the B cells of C57BL/6 Sm offspring relative to controls. CD86 expression, however, as already noted on B cells and DCs during OVA-s.c, was elevated in Sm offspring on all APCs assessed, across B cells, total cDCs, cDC1s, cDC2s, in both BALB/c and C57BL/6 mouse strains (Fig. 3.23B). This demonstrates a clear and consistent alteration to modified activation status of these cells, and presents them as a key target of maternal priming via helminth infection. This is further supported by previous reports of CD86 in particular associated with schistosome-antigen-induced regulatory phenotypes and modified functionality on B cells and macrophages(Haeberlein et al. 2017). While there was no further shift in relative proportion of cDCs, or cDC1/cDC2 ratio to suggest additional fundamental developmental changes (Fig. 3.23C), the F4/80+ macrophages from Sm offspring further displayed higher CD86 levels (Fig. 3.23D), and further demonstrated this as a widespread feature indicating relatively global impact of maternal infection across APC cell types, with DCs a likely candidate as the intersection for these modifications and the altered *de novo* activation of naïve T cells.

3.1.13.2 In Vitro Assessment of DC-Enriched Splenocyte Functionality

To further explore the potentially enhanced regulatory network in Sm offspring, DC-enriched splenocytes (using CD11c+ MACS) were isolated from Sm and naïve offspring. As an initial measure of altered functionality, these were cultured for 72h and supernatants collected, both alone and in the presence of additional stimulation via LPS (as per Fig. 3.24A).

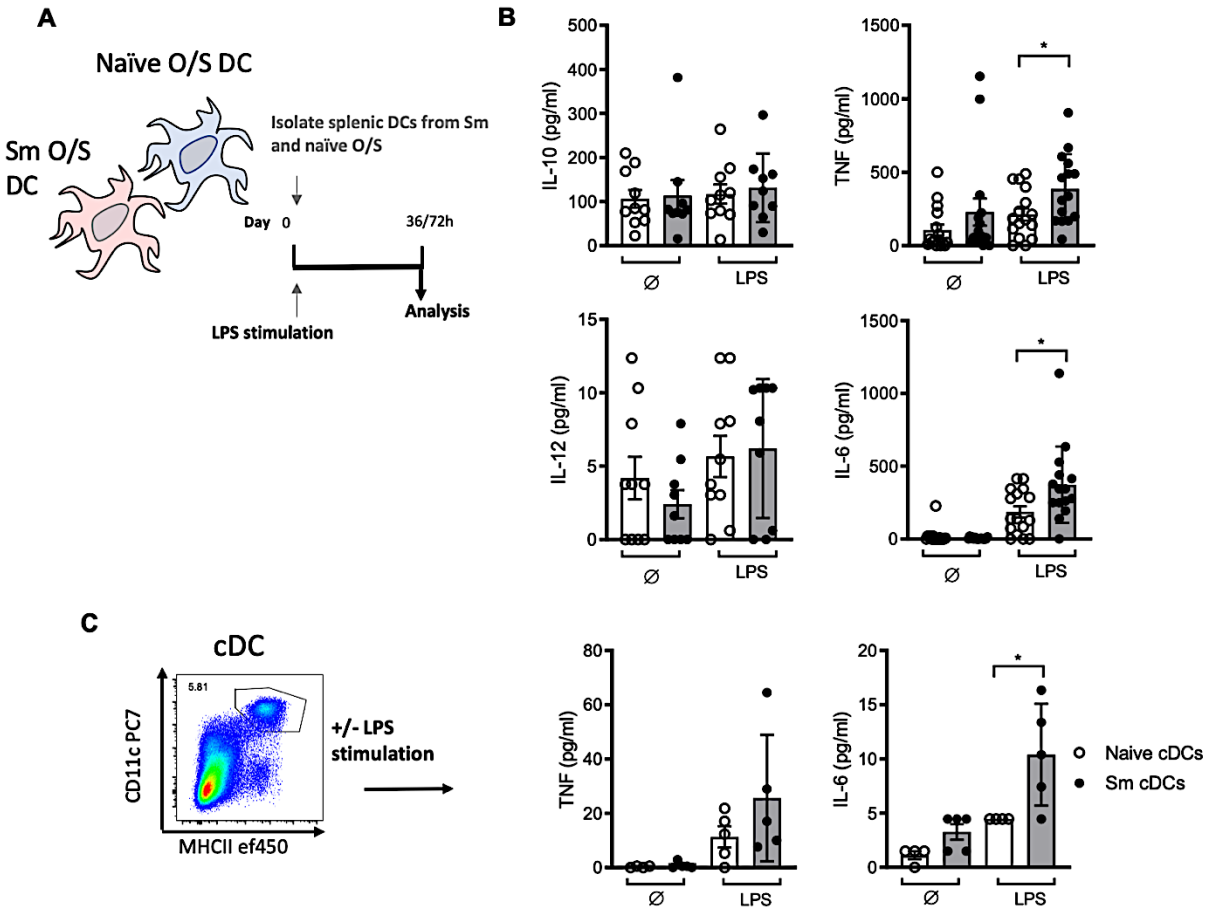


Figure 3.24 Imprinting of Modified Functionality in DC Compartment
(A) CD11c+ MACS positively selection was used to enrich DCs from spleen cell suspensions extracted from Sm offspring and naïve controls, with DC-enriched cells were then cultured for 36-72h, with or without additional treatment with LPS (2ng/ml), and supernatants collected then (B) assayed via ELISA for IL-10, TNF, IL-6 and IL-12 levels. (C) Additional flow sorting (as per representative plot, left) yielded purified cDCs from C57BL/6 offspring cohorts, *right*: ELISA data for TNF and IL-6 in culture supernatants after 24h stimulation with or without LPS. Data in (B) pooled from 2 independent C57BL/6 experiments, totaling n=9 per group (mothers: n = 3 per group), with additional pooled data from a third BALB/c offspring cohort, n= 6 per group (mothers: n = 4-6 per group) for TNF and IL-6. cDC were from C57BL/6 offspring cohorts (n = 5 per group).

In the simple culture setting, IL-10 and IL-12 levels from DC-enriched splenocyte cell preparations appeared largely unaltered between groups, and additionally not affected in these settings by LPS (Fig. 3.24B, *left panels*). A slight trend towards increased TNF was present in the supernatants of unstimulated Sm offspring DCs compared to naïve DCs, and Sm offspring DCs responded to LPS with higher TNF release that that of naïve DCS. IL-6 in supernatants was only detectable from DC-enriched splenocytes after LPS treatment, and was clearly enhanced responses from Sm offspring derived cells (Fig. 3.24B, right panels). Inclusion of both experiments from C57BL/6 and BALB/c offspring cohorts confirms the

consistency of this effect, as does the similar trends of increased TNF and clearly increased IL-6 from LPS-stimulation of highly purified flow-sorted cDCs (Fig. 3.24C), which further confirms this modulated functionality within those cells highly specialised for inducing activation of naïve T cells. These initial findings suggest that the offspring DC compartment can also bear long term modifications through priming via maternal schistosomiasis, with increased responsiveness in terms of cytokine production to LPS stimulation indicating further induced changes to maturation status, alongside the observed changes to co-stimulatory molecules in Fig. 3.23.

These results direct further questions as to whether indeed the DC-associated modifications indicated by this altered cytokine production may play functional roles, in addition to the T and B cell modifications so far described, in T cell activation. To assess this, 5x10^5 DC-enriched splenocytes were loaded with SIINFEKL peptide and co-cultured with naïve (CD44lo) proliferation dye-stained OT-I cells for 72h (as per scheme in Fig. 3.25A).

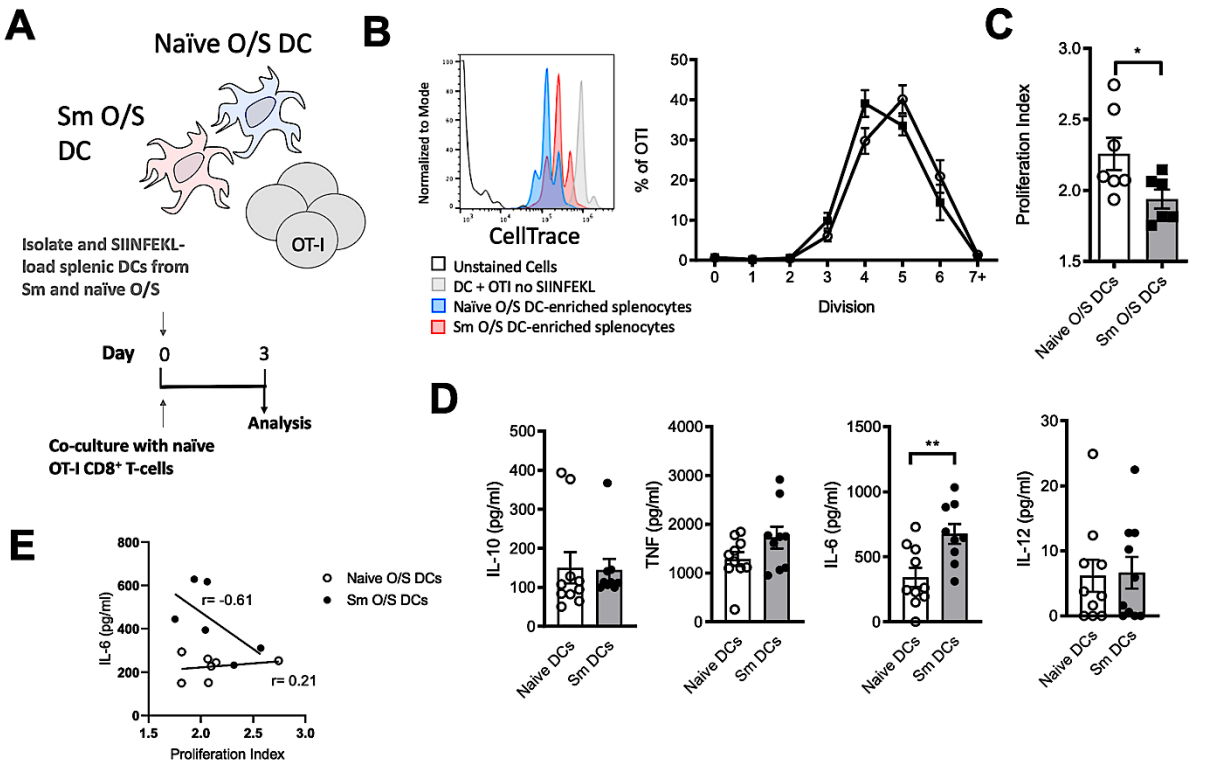


Figure 3.25 Maternal Schistosomiasis Impacts *In vitro* Capacity of DCs to Activate OT-I T cells
(A) Scheme for functional assessment of activation capacity of CD11c+ DC-enriched splenocytes, performed by peptide loading with OVA257-264 SIINFEKL (1µg/ml) and co-culturing with 1x10^5 CD44loCD8+ OT-I T cells stained with CTV for 72h. (B) *Left:* Representative histograms of proliferation curves acquired through assessment of CellTrace dilutions after

72h, *Right:* % of OT-I within each Cell Trace dilution peak, indicating successive cellular divisions. (C) Comparison of Proliferation index of OT-I cells within co-culture reactions. (D) Levels of IL-10, TNF, IL-6, and IL-12 in co-culture supernatants after 72h, as measured by ELISA. (E) Assessment of correlation between IL-6 in supernatant and OT-I proliferation. Data pooled from 2 independent experiments, each with n=3-6 per group (mothers: n = 3 per group). FACS data shown pooled with normalization from female-only offspring subset.

Here, the proliferative response was compared, with the CellTrace division curve of OT-I cultured with cells from Sm offspring displaying a shift to indicate less average divisions, when compared to the more forward shifted curve of OT-I cells activation using naïve CD11c+ cells as APCs (Fig. 3.25B). This effect on proliferation was confirmed by the relatively lower proliferation index of OT-I activated via Sm offspring CD11c+ cells (Fig. 3.25C). As such, Sm offspring-derived CD11c+ DC-enriched APCs in isolation were able to reproduce an OT-I activation phenotype to reflect the *in vivo* lower activation of OT-I cells within Sm recipients, and support a role for modified T cell inducing capacity of DCs as a link between maternal regulatory priming and modified T cell responses to heterologous antigens. This further contrasts the otherwise similar *ex vivo* CD8+ responsiveness of naïve CD44lo CD8+ T cells, which was unaltered by developmental helminth exposure.

The co-cultures of OT-I CD8+ T cells with DC-enriched splenocytes from Sm offspring again produced clearly higher amounts of IL-6 compared to those co-cultures where naïve offspring-derived CD11c+ cells were used as APCs. A similar trend for increased TNF was also seen, but again as with the LPS-induced TNF as observed in the sorted cDCS, this was less clearly enhanced in Sm offspring cultures and not statistically significant in this setting. Detectable levels of IL-12 and IL-10 were consistent across groups. This provides further evidence that observed CD8+ responses to immunization derive from alterations to the network of cells involved in regulating T cell responses, and highlights a role for antigen presenting cells including the involvement of DCs with a modified phenotype that is retained into adulthood following early life exposure to the modified developmental environment skewed by maternal helminth infection.

An additional preliminary experiment with DC co-cultured with OT-II T cell (transgenic $CD4^+$ with Ova-epitope-specific TCR), plus cognate peptide $OVA_{323-339}$ (as per scheme 3.26A), assessed the potential further association with modified DC and the CD4+ priming phenotype. Such previous

observations were an IL-4-skewed shift in CD4+ T cell responses, in steady-state and systemic immunisation, although not within type 2 inflammatory context of AAI in the lung.

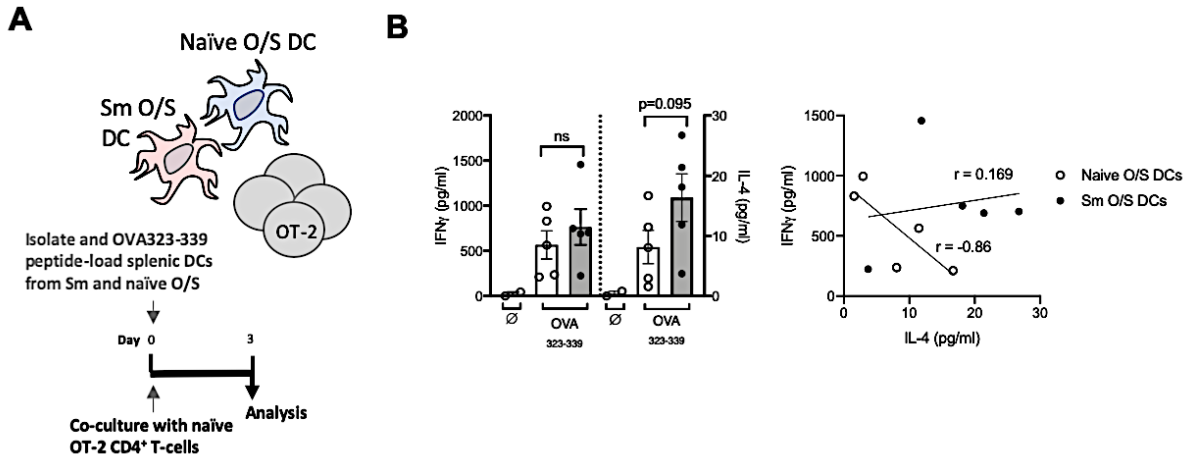


Figure 3.26 Effects of Transmaternal DC Priming On CD4+ Polarisation
(A) Scheme for co-culture of CD11c+ DC-enriched splenocytes derived from offspring cohorts with naïve (CD44lo) OT-II CD4+ T cells (B) *Left:* Amount of IFNγ or IL-4 measured in culture supernatants after 72h activation, *Right:* Correlation between IFNγ and IL-4 levels. Pilot experiment with CD11c+ DCs from 5 C57BL/6 offspring per group, mothers: n = 3.

DC-peptide-driven activation of OT-2 over this time yielded clear increases in both IFNγ and IL-4 in supernatants, compared to those of non-activation cells, where no peptide was given. A slight, non-significant increase in mean levels of IFNγ were secreted from OT-2 activated by Sm offspring-derived $CD11c^+$ DC-enriched cells, relative to those activated with naïve APCs. By contrast, there was a clear trend towards higher IL-4 secretion in the OT-2 cultured with Sm offspring derived DCs (Fig. 3.26B). Further, there was a (non-significant) negative correlation between IFNγ and IL-4 levels across individual samples in the naïve group, which was not present in the Sm group, which could point to further complexity in the priming of DCs that impacts their effects of $CD4^+$ priming, specifically that could be addressed in relation to an IL-4 bias as may relate to enhanced type 2 processes across cellular compartments after maternal helminth infection, that remains to be explored further.

3.2 Partial-Recapitulation of Priming with Infection-Free Parasite Egg-Exposures

One hypothesis is that regulatory immunological parameters are induced by continued exposure to schistosome eggs in mothers, which contributes to driving a tolerogenic phenotype in their offspring.

To explore this in an initial pilot experiment, murine dams were treated with intraperitoneal injection of schistosome eggs prior to mating, in multiple protocols previously shown to induce regulatory phenotypes. As shown in Fig. 3.27A, this included: A: 2,500 eggs two weeks before mating, previously shown to suppress AAI (Pacifico et al. 2009); or B: 5,000 eggs two weeks before mating, plus or minus a boost of 5,000 eggs (C) one week prior to mating, previously shown to induce regulatory B cells (Haeberlein et al. 2017)), to investigate the effect upon offspring allergic imprinting.

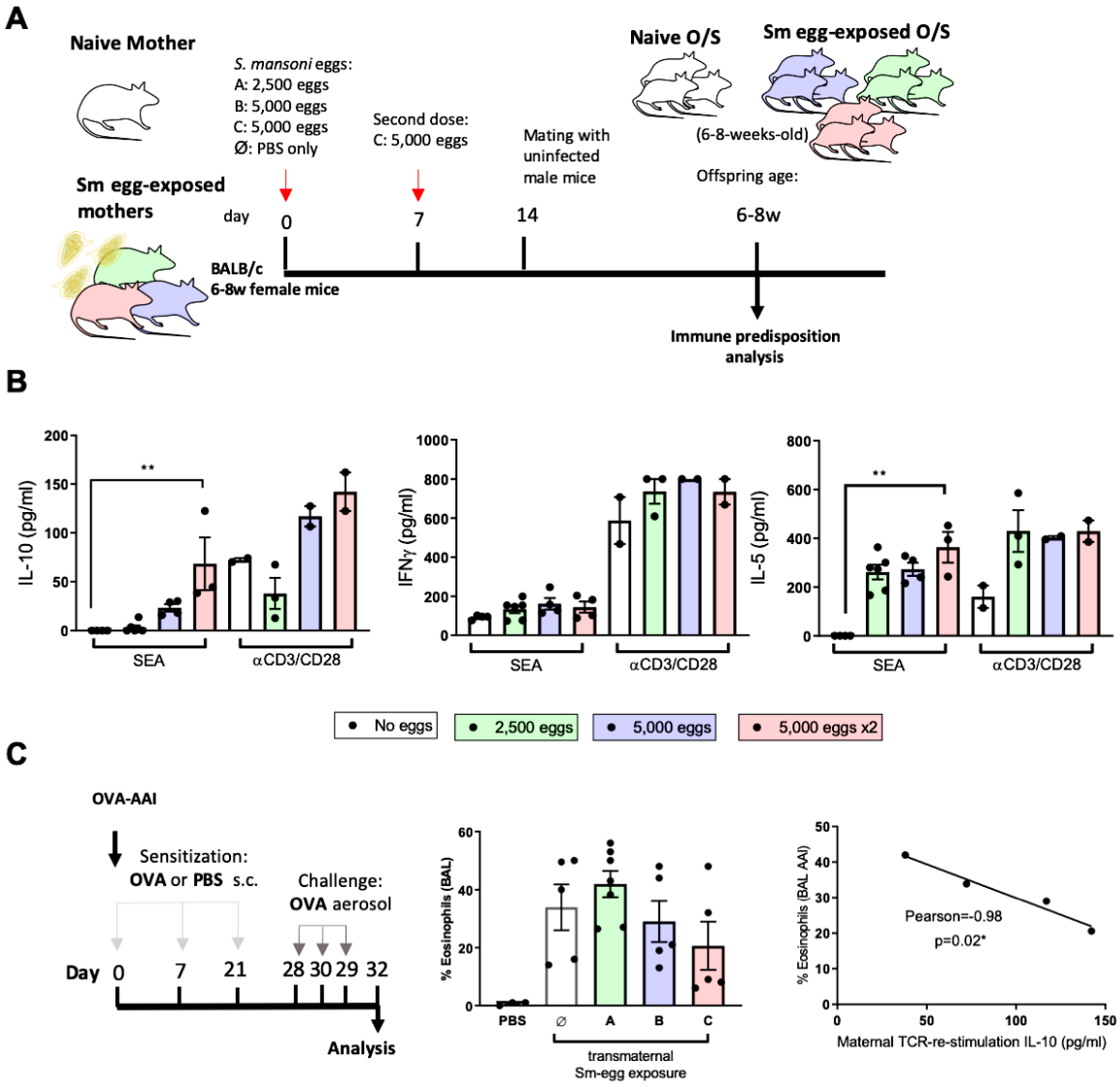


Figure 3.27 Transmaternal Schistosome-Egg Exposure and Allergic Responses
(A) Scheme for transmaternal exposure to schistosome eggs via i.p delivery to mothers prior to mating. (B) IL-10, IFNγ, and IL-5 levels in culture supernatants of maternal splenic cell suspensions after weaning, in response to 72h SEA (20µg/ml) or TCR-based stimulation. (C) Offspring BAL eosinophilia following OVA-AAI, right: correlation between maternal TCR-induced IL-10 levels and offspring AAI-induced eosinophilia. Pilot study with 5-7 offspring per group, and mothers: n = 2-3.

Analysis of maternal splenocyte responses to *ex vivo* SEA or TCR-based re-stimulation post-weaning (i.e.: 7-8 weeks after final schistosome egg treatment in vivo) revealed that all three egg-injection protocols induced IL-5 production, which was absent from unexposed mice. While there was minimal change in IFNγ, there was exposure-dose-dependent production of IL-10 under similar conditions (Fig. 3. 27), showing that increased egg-exposures tended towards closer replication of the "regulatory / modified Type 2" immune response typical of the chronic, regulatory phase of murine schistosomiasis. Initial readouts of offspring AAI responses, as determined by percentage eosinophilia in BAL, show that eosinophilic AAI in male offspring from these egg-exposed dams has a strong inverse correlation with egg-induced maternal IL-10 levels (as determined by TCR-re-stimulated splenocytes). Additional investigation of such egg-exposed offspring would reveal whether this infection-free induction of a maternal immune response to *S. mansoni* eggs and antigens is able to recapitulate the T cell, B cell, and APC/DC modulating effects for offspring, and further the facilitate studies into the relative contribution of direct parasite compound exposure versus via induction of maternal immune responses.

4. DISCUSSION

4.1 SHIFTED ALLERGIC PRIMING AND LOWERED ALLERGIC INFLAMMATION FOLLOWING EXPOSURE TO CHRONIC MATERNAL SCHISTOSOMIASIS

Schistosomes, as noted for other infectious agents that induce strong regulatory responses for survival in their hosts, can modulate bystander responses to heterologous antigens involved in immune disease, allergy, as well as vaccination efficacy. As discussed, maternal chronic schistosomiasis has documented effects in mediating offspring immune response heterogeneity, particular with regard to allergic and atopic inflammation, for both human children and within experimental murine systems. This maternal infection maintains direct concrete relevance, with roughly 40 million infected women of childbearing age, and in endemic areas, for example in Lambaréné, rural Gabon, 10% of pregnant women still test positive for schistosome infection ((Mombo-Ngoma et al. 2017). The breadth of these effects, however, has not been clearly profiled by previous work. Here, early life exposure to maternal schistosome infection uniquely combines the presence (during development) of strong parasite-derived immune stimuli, including potent foreign molecules and antigens, altered maternal cytokine and antibody profiles, as well as induced populations of regulatory cells, all during formative stages of immune ontogenesis. Specific exploration of whether, and how, maternal schistosomiasis affects the development of antibody titres in humans, for example, following Hepatitis B vaccination (Bassily et al. 1997) or in experimentally-induced allergy models (Straubinger et al. 2014), remains unclear, with data on T cell responses further lacking.

The murine system of chronic maternal schistosome infection has demonstrated modulatory effects on bystander responses to heterologous antigen OVA within the context of AAI. This therefore provided a unique system for exploring the mechanisms leading to lasting immune priming effects through early life exposure, including a potentially broad range of immunological consequences. Clear effects have been previously observed within the chronic stages of maternal infection, that is, with mating of mice from 16 weeks after initial delivery of cercariae. This chronic state further represents a more accurate

depiction of endemic populations, particularly from an evolutionary point of view of human life prior to drug-based interventions, and was therefore further chosen as the optimal exposure for examining transmaternal regulatory priming effects.

This thesis evaluated these priming effects using the OVA-driven experimental allergic airway inflammation model as the key inflammatory context to observe the effects of prior exposure to maternal infection. The involved changes to the associated process of allergic priming sensitisation, and by extension, across further vaccination modalities, provided further avenues for studying the more general impact of these effects for T cell priming behaviour. This resulted in further examination of the involved cellular changes and shifted immune behaviour at steady state to identify the extend of these priming effects.

The initial experiments provided observations that, despite some altered presentations of infection between C57BL/6 and BALB/c host mice with regard to *Schistosoma mansoni*, regarding immune response and its dynamics, the cellular changes to BAL composition following OVA-AAI were replicated across both strains of murine offspring after pre-exposure, including reduced allergic eosinophilia following immunization and challenge as previously observed in only BALB/c schistosome-exposed offspring (confirming a similar effect across strains, despite the noted possibility for heterogenous immune responses across strains that can in certain contexts be observed, such as disproportionate connections between in AAI, with BALB/c more predisposed to OVA-Ig, and C57BL/6 to greater eosinophilic responses (Kodama et al. 2010)).

The relevance of these findings within additional immune contexts was revealed through analysis of the associate T cell compartment changes during AAI. Here, the normal response observed in both BALB/c and C57BL/6 mice is local accumulation of CD4+ T cells that, following re-stimulation with cognate OVA antigen *ex vivo*, provide strong responses mixed/type-2 skewed inflammatory responses, with IL-4 and IL-13, as well as IFNγ. These responses were observed in both murine strains as clearly reduced in Sm-offspring, and indicated changes to T cell responsiveness that could underlie the observed attenuations to allergy as previously observed.

Despite previous reports of lower associated antigen-specific OVA-Ig levels during AAI (Straubinger et al. 2014), the observed effects across both strains and pooled experimental settings was an increase in allergen-specific antibody titres, clearly in terms of increased OVA-IgE in the BALB/c setting, and reflected in other Ig-classes present within the C57BL/6 cohorts. This indicates a complexity in the immune modification that is not reflective of a generalized induced immunosuppression. The finding of increased class switched antibodies, which would require enhanced presence of type 2 pathways, including most likely IL-4, potentially indicates a priming effect with systemic relevance. Interestingly, previous findings by our group identified placental vitamin D receptor regulation in association with chronic schistosome infection, and similar vitamin D knockout mutant mice have an allergic phenotype that to some degree mimics that of schistosome-primed offspring, with elevated Th2 cytokine levels and IgE, with lower AAI responses and lung inflammation, indicating the possibility that similar processes that decouple sensitisation from local inflammatory responses (Wittke et al. 2004).

That this systemic effect may be of a type 2 skewed nature is further supported within the AAI-derived data from the steady state polarisation effect on CD4+ T cells in the lung. Despite the much lower cytokine-producing potential of steady state CD4+ cells compared to those during lung inflammation, there was a clear skewing effect observed by pooling C57BL/6 and BALB/c offspring results, with an increased ratio of IL-4/IFNγ-producers supporting the presence of an imprinted priming effect impacting T cell responses (with such cytokine ratios frequently used to indicate skewing of T cell immune priming, as in (Yang et al. 2019)). This may inhibit pro-inflammatory effects, including locally induced inflammation, while simultaneously priming general type 2 or humoral responses in a more systemic manner.

Despite the almost paradoxical relationship between increased steady-state IL-4 capacity alongside reduced allergic inflammation, it is interesting to note the growing understanding of complexity within the T_H2 cell compartment and type 2 responses in general. These have alternatively have been termed pro- or anti-inflammatory cells dependant on context, as highlighted by the traditional description of IL-10 as both a type 2 and a regulatory cytokine, and the complex interplay between processes that mediate clearance of extracellular foreign bodies and also maintain tissue homeostasis (Gause, Rothlin, and Loke

2020). IL-5, a key component of driving the eosinophilic inflammation observed in the AAI model used in this thesis, has been found to be produced by only a certain subset of T_H2 cells which may be more associated with atopic disease states (Upadhyaya et al. 2011). This indicates a possible divergence between the mixed type 2/regulatory type 2 cells found at steady state in the Sm offspring, capable of producing IL-4 and bearing enhanced B cell activation, including greater amounts of class-switched antibodies, from the IL-5-producing CD4+ T cells required for allergic state, which have also been shown to produce higher amounts of IL-4 and IL-13 than cells that could not also produce IL-5 (Upadhyaya et al. 2011), further indicating a role supporting stronger effector responses and feedback loops to drive inflammation. The specific nature of the type 2 priming in schistosome-exposed offspring, and its connection to a regulatory rather than pro-allergic signature, therefore offers a further mechanistic opportunity for future work, such as further characterisation and subtyping of the induced IL-4 producing T_H2-like cells.

RNA-seq-based evaluation of transcriptional profile during AAI further identified that allergic priming responses, in particular those occurring within the local igLN draining site of injection, showed signs of modified inflammation co-existing with an enhanced B cell activation signature, most likely indicating a shift in trafficking or humoral priming more evident in Sm-exposed offspring. CXCL13 expression dropped in igLN during AAI in naïve mice, that may indicate the altered movement of Tfh or B cells during this model. The more stable expression of this gene in Sm offspring during AAI may indicate their delayed exit from this priming site during the inflammatory challenge phase (presumably into circulation or towards the inflamed lung). The more normal expression of CXCL13 in the igLN of Sm offspring during AAI, could comparatively indicate reduced “pull” towards a less inflamed lung, or alternatively processes leading to retaining these cells in the lymph node, where, complex interactions within the B cell follicle, between DCs, B cells, and T cells, has been discussed as modifying T cell priming and fate in terms of polarisation and migration (Leon, Ballesteros-Tato, and Lund 2014). Similarly, the increased H2-Aa in Sm offspring igLN relative to naïve mice during AAI (which otherwise also show a drop in H2-Aa during AAI), may further indicate an altered dynamic of B cell trafficking (a highly proportion of the MHCII+ cell type in igLN) towards retaining or even expansion

of B cells, further supported by lower decrease in CXCL13, that suggests altered priming of allergic responses in Sm offspring that could be further investigated through special analysis of cellular dynamics and interaction in the lymph node, and further characterisation of divergence T and B cell populations and behaviour at this priming site during this model.

Indeed, analysis of related cellular features within the allergic priming phases on this model, prior to lung challenge, revealed a similar skewed profile after maternal priming. This included enhanced B cell responsiveness, including higher levels of activation markers MHCII and CD86 on B cells at the allergic priming site. The simultaneous alteration to the IL-4/IFNγ ratio within activated lymph nodes further indicated skewing of enhanced type 2 processes with signs of lower pro-inflammatory response, and was functionally evident in higher OVA-specific IgE titres for immunised Sm-offspring. In non-immunized mice, the presence of a similar skewed type 2 versus type 1 cytokine profile in the T cell compartment again suggest a steady-state priming effect, that drives changes to the induced responses to bystander antigen challenge.

The initial observations of the original hygiene hypothesis, as relate to hay fever and allergic incidence, put forward a case that the increased bacterial and viral infections, particularly faeco-oral routes, train the immune system to limit unnecessary type 2 responses to harmless antigens, and thereby reduce the allergy-prone state of an unchallenged immune system (Strachan 1989). However, in the case of maternal schistosomiasis, the signs of increased type 2 priming, which fit with a history of helminth-based immune priming, in antagonism or dampening of pro-inflammatory T cell responses, indicate a different kind of immune balance shift. Here, it may instead be more likely that exposure to a strong modified type 2 / regulatory maternal immune response, and the parasitic molecules involved in maintaining such a state, expand a set of type 2-skewed regulatory circuits in offspring. The direct mechanistic link remains to be clarified between these and alleviated AAI, and in particular the noted drop in eosinophilia. However, this priming effect clearly skews induced T cell responses and cytokines, as well as APC and B cell activation, including Ig levels, at least within the context of allergy. Additionally, as evading lung inflammation is key to the survival of the migratory early stages of schistosomula (which pass through the lung and induce processes including IL-6 production which

antagonises eosinophilia (Angeli et al. 2001)), and CD4+ infiltration into the lung being involved in a preliminary step of the cascade towards pulmonary eosinophilia (Gonzalo et al. 1996), it is interesting here to note that schistosome-induced imprinting may specifically be targeting those pro-inflammatory processes that mediate local recruitment of T cells, and in this way, persistence parasite-induced modulations, even across generations such as through transmaternal effects, may mediate host inflammation enabling mutual survival.

OVA-carrying DCs are also present in the draining igLN following subcutaneous delivery, with similar fluorescent OVA-labelling showing that these migratory DCs in the lymph node that carry antigen display a characteristic $CD11c^{+}MHCII^{hi}$ phenotype, it is also of interest to note that in the Sm offspring, the modified T cell response is further accompanied by changes in the DC compartment, which as the with B cell responses, show altered rather than absent responsiveness, in this case similarly showing trends towards higher co-stimulatory MHCII and CD86. These results, taken in light of the subsequent lower levels of inflammation upon challenge, and T cell profile in steady state, allergic priming, as well as locally during inflammatory challenge, appear to indicate an induced tolerogenic status that could impact novel induction of T cell responses. This could be further explored through subset-based phenotyping, e.g., single-cell transcriptional profiling, and further functional analysis.

4.2 MATERNAL SCHISTOSOMIASIS HAS IMPLICATIONS FOR MODIFIED VACCINE-INDUCED T CELL RESPONSES

Next, through tracking the activation and proliferation of OT-I and OT-II adoptively transferred T cells, it was confirmed that the immunization strategy used to elicit AAI does in fact trigger local antigen-specific T cell activation within the igLN draining site. This confirms further that the observed shifts to B cell activity, as well as steady-state skewing and shifted DC activation as found in Sm offspring, can be localised site of T cell activation in response to OVA-s.c., and directly poised to modify T cell responsiveness to bystander antigens, or even may signify modified priming events.

Through an evaluation of broader immunization strategies, inducing adjuvants driving type 2 focused responses (alum), mixed type 1 responses (as aided by bacterial-based adjuvant CFA), as well as with the MVA viral-vector, a clearer picture arose that maternal schistosome-induced priming impacts pro-inflammatory bystander T cell responses, and that across vaccine modalities the CD4+ compartment of Sm-offspring shows a hyporesponsiveness in this regard. As occurred with the type 2-activating OVA-s.c. allergic model already implemented, this lower proinflammatory response may be due to an antagonistic type 2-skewing present already within the CD4+ compartment.

CD8+ activation is vital for effective vaccine-induced protection, particularly towards viruses. With regard to CD8+ T cell responses of Sm offspring to vaccination, however, there appears to be a specific modulatory relationship between activated CD8+ responses and the modality of delivery. CFA and alum, as well as unadjuvanted OVA, were not able to trigger the same magnitude antigen-specific CD8+ responsiveness in Sm-offspring as from naïve offspring. Both of these adjuvants trigger inflammatory pathways to enhance responsiveness to extracellular antigens, which, in the example of CFA, prolongs antigen presence and simultaneous activates innate responses using bacterial products (Billiau and Matthys 2001). The reversal of this effect using the MVA-vector indicates the possibility of an inhibitory effect towards CD8+ priming that is reversible, or at least amenable to further modulation dependent on context of antigen delivery. For example, transmaternal effects in this setting may prime changes to processes involved chiefly in extracellular antigen but not intracellular virus-associated antigens, or MVA-driven pathways may be able to override primed changes in DCs that present viral-encoded antigens after direct infection of these cells with the vector. MVA may also more widely counter tolerogenic maternal priming by driving pro-inflammatory maturation of bystander DCs (through producing cytokines including type I IFNs) (Pascutti et al. 2011). In each case, it appears that systemic CD4+ responses largely follow the steady state type 2-priming skewing effect. CD8+ responses are more clearly readily observed as strongly induced relative to non-immunized mice for the immunisations used here. In Sm offspring, the observed CD8+ responses follow the more general trend towards lower specific pro-inflammatory responses, and mirror the lower reactivity to local-inflammation observed in AAI (with the caveat of MVA-induced overriding effects). This effect, with differential responses across

immunisation modalities, demonstrates a clear impact of perinatal helminth priming as an environmental factor that creates lasting modulation of T cell function, particularly within the context of mounting specific immune responses to previously unseen antigens.

Indeed, these lower pro-inflammatory responses carry over to functional effects regarding hepatitis B virus vaccination and challenge. CD8+ T cell activation in particular is required for an effective anti-viral response to Hepatitis B leading to viral clearance (Thimme et al. 2003), and involves the expansion and persistence of effective memory CD8+ T cell populations. Following model HBV challenge after only an adjuvanted protein pre-immunisation, Sm offspring show reduced T cell responses and, not surprisingly, lower clearance of viral DNA than control offspring. This is agrees with previous data of schistosome infection aggravating Hepatitis C infection prognosis (Loffredo-Verde et al. 2015), as well as modulating Hepatitis B vaccine efficacy (Ghaffar et al. 1990). It further reflects variable responses to traditional protein-adjuvant Hepatitis B vaccines in children born to schistosome-infected mothers (Ghaffar et al. 1989). Here, the lower ability to mount effective CD8+ and IFNγ responses, and lower levels of clearance, may reflect a type of selectively compromised immunity. At minimum, this shows a propensity towards lower inflammatory responses, as observed in helminth-infection, and often cited for the lower inflammation-driven morbidity in endemic populations to parasites themselves. Further, this adds support to the concept that inflammatory factors, such as CD8+ responses, as susceptible to the effects of maternal priming.

The ability of the MVA viral vector, as observed for model antigen OVA, to normalise the IFNγ levels as well as viral clearance in Sm offspring, further identifies regulation of CD8+ T cell responses as an important axis for maternal helminth-induced immunomodulatory effects. The mechanism of how MVA rescued protective HBV effects by overcoming early life schistosome priming, however, remains elusive. Effective CD8+ T cell responses are induced through either cross presentation of exogenous antigens, or, through endogenous pathways as is the case with viral infections and intracellular pathogens. Viral vector-based strategies make use of this endogenous route to elicit strong CD8+ responses to antigens which would otherwise not be naturally processed through these pathways, and may therefore utilize pathways unaffected by maternal schistosomiasis to prime strong anti-viral

responses, or provide sufficient stimulus to override modified DC maturation status to yield strong CD8+ activating capacities. Indeed, the application of heterologous boost vaccinations with MVA-based or similar viral vectors are of interest particularly in difficult to vaccinate populations and show efficacy in overcoming tolerogenic contexts, such as tolerance induced by high amounts of antigen (Backes et al. 2016). Additionally, there are reports that CD4+ T cell help may be dispensable for initial CD8+ T cell activation, but such "unhelped" CD8+ T cells shift towards a short lived, effector cell phenotype (more CD127- and KLRG1+) away from a memory precursor phenotype (CD127+ KLRG1+) and are less likely to provide adequate recall responses to, for example, subsequent viral challenge (Hwang et al. 2015). Sm offspring consistently exhibit reduced CD4+ T cell IFNγ responses, at steady state and as induced by immunization, including with MVA-vectors. This indicates a potential lack of type 1 CD4+ T cell help may indeed be a possible downstream effect. The MVA-vector, however, may provide a strong enough stimulus to overcome blockages associated with lack of CD4+ help which occur in other immunization modalities following schistosome priming. The MVA vector has also been shown to induce immune responses more polarized towards IFNγ / IL-12 production (Olszewska et al. 2004), potentially accounting for the "overriding" effect with regard to the inhibitory type 2 priming found in Sm offspring, with the supplementation of IL-12 previously shown to counteract type 2 responses within schistosome-infected mice and restore pro-inflammatory responses (Wynn et al. 1995). One possibility for increased responsiveness for MVA-vectors observed in Sm offspring, in terms of CD8+-derived IFNγ in particular, may be that inherent susceptibility to viruses (i.e.: through dampened pro-inflammatory T cell responses) leads to greater infection and more persistent immune stimulus provided to the Sm offspring through MVA than occurs in control mice, although this remains speculative. Other factors may be as-yet-described modulatory roles of other factors, for example, the potential for enhanced Sm-offspring IL-10 levels within this system, as this anti-inflammatory cytokine has demonstrated in certain contexts differential suppressive effects on CD4+ versus CD8+ T cells (Brooks et al. 2010). In the end, defining the link between modified regulation of induced specific CD8+ T cell responses and the steady-state immune shifts observed in Sm offspring, such as specific modifications in factors priming APC and DC priming, may prove to be a significant next step in defining how this

potential network of regulatory changes modulates the induction of pro-inflammatory responses to bystander antigens.

An initial hypothesis was that steady state changes could be present within the naïve T cell compartments themselves, whether these were maintained through interactions within other primed immune processes, or if indeed these arose through an altered environment during development and immune ontogenesis. The suggestion that maternal schistosome-induced alterations to naïve T cell compartments (in terms of *in vitro* capacity to respond to activation or polarizing stimuli) would reflect *in vivo* T cell responses was based on initial work previously published showing modified epigenetic status (more closed histone profile in terms of pan acetylation at lysine residues) and differential cytokine production under polarizing conditions. The latter, through re-analysis, reflects increased baseline cytokine production within the CD4+CD62L+ T cell compartment of Sm offspring compared to naïve offspring, which produced higher IFNγ levels under T_H1 polarising conditions (where IL-4 was blocked), and higher IL-4 levels under neutral conditions (termed T_H0), where both IFNγ and IL-4 activity was blocked.

This CD4+CD62L+ compartment is considered to consist predominantly of naïve T cells, essentially consisting of the largest fraction of the total CD4+ minus the CD62L- effector and effector memory subsets. However, significant memory cells still remain within CD4+ sorted via MACS for CD4+CD62L+ selection, including roughly 5% of $CD44^{hi}$ central memory CD4+ T cells. Repeating the *in vitro* polarisation experiments under further sets of conditions to drive T_H17 and Treg induction revealed no further shift in terms of induction of FoxP3 or RORγt expression between cells from offspring groups, although these were strongly upregulated under specific polarising conditions compared to T_H0 neutral culture, reproducing typical results of these established protocols for inducing T cell polarisation (Flaherty and Reynolds 2015). Baseline cytokine levels in T_H0 conditions were again different between groups, with proportions of cells making IFNγ and IL-17 higher in those cells from Sm offspring. This provides further evidence for baseline CD4+ T cell activation, along with previous results for IL-4 production, show pluripotent effects of previous activation across T helper subtype. These priming effects are reminiscent of the mixed T cell response induced through the schistosome infection itself, the includes IFNγ and IL-17 producing cells alongside T_H2 cells (Rutitzky and Stadecker

2011). Further epigenetic analysis of this compartment was performed at 3-weeks, an earlier timepoint to complement the previous work performed at 6-8 weeks in order to closer identify development effects during early life. Here, all cytokine loci examined showed lower histone acetylation within the samples from Sm offspring. This revises the previous supposition of earlier work that such epigenetic closed states were only found on T_H2 cytokine loci, and disagrees with the associated conclusion that the epigenetic state of the cells indicates a balance shift from type 2 to type 1 polarisation, an interpretation with the potential to mechanistically be linked to the reduced type 2 AAI response. As already detailed, the systemic baseline *ex vivo* T cell response, even in lung CD4+ T cells, is more IL-4-prone in Sm offspring, further supported by higher *in vitro* IL-4 levels under neutral conditions. Instead, the more global closed epigenetic status across this CD4+CD62L+ compartment may be indicative of a general trend towards lower accumulation of memory cells, as proportionally these cells have been found to be lower in Sm offspring. This would appear to reflect a complex dynamic affecting T cell maturation at a population level, such as priming effects in APC populations that inhibit memory cell formation, or suppressor cell-based deletion of strongly activated pro-inflammatory cells. Those memory cell populations which are present in the steady-state following maternal infection, show heightened cytokine production.

Maternal infection-induced T cell priming in Sm offspring was most clearly evident in terms of priming of IL-4-producing CD4+ cells, as within total igLN or lung cell suspensions, and with isolated CD4+ under T_H0 conditions. This was found to be restricted to CD44+ cells within the CD4+CD62L+ compartment, and supports that the earlier effects, likely including those relating to IFNγ and IL-17, derived from the presence of pre-activated central memory T cells. Microchimeric cells have previously been shown to be transferred from helminth-infected mothers and confer a level of protection against cognate infection, including recently within the context of maternal infection with the nematode *Heligmosomoides polygyrus* (Darby et al. 2019). Through congenic marking of offspring cells using CD45.1+ male mice during mating (Fig. 3.14), analysis of the total CD4+CD44+ compartment detected the clear capacity for IL-4 production which was not present in cells from naïve mice, and clarify that offspring-derived cells, and not chimerically transferred cells from the infected mothers, bear a signature

of type 2 activation that functionality translate to active cytokine production. The presence of these cells (and therefore the likelihood of increased IL-4 levels) concurrent with other priming events, such as *de novo* activation of naïve CD4+ and B cell priming to initial immunisation challenge with OVA, may act to skew type 2 responses, enhance humoral responsiveness and class switching, and antagonise pro-inflammatory type 1 responses. Alongside direct effects on lymphocytes, this may further operate via the APC compartment, skewing towards more alternative activation of macrophages and DCs, or tolerogenic states, to modify their capacity to expand pro-inflammatory T cell populations. Co-culture with SEA-pulsed BMDCs led to a trend of higher proliferation for CD4+CD44+ memory cells from Sm offspring, indicating the potential for schistosome-specific T cell responses to have been primed through maternal exposure. However, SEA is not a specific single antigenic epitope, nor even a pool of them, and is instead a complex mix including proteins, glycoproteins and lipids (van der Kleij et al. 2002; Everts et al. 2009), with multiple noted immunomodulatory activities, particularly with regard to APC and DC function (Kaisar et al. 2018). This assay for schistosome-specific T cell responses therefore may require alternative approaches and refinement (as in more epitope-specific assays, as previously used in the context of vaccine development (Fonseca et al. 2004).

A further hypothesis regarding such antigen-specific effects was that transmaternal schistosome exposure would confer a tolerance to cognate infection in this setting, whether that would translate to a higher survival of worms and higher burden of infection, or modified less pro-inflammatory cytokine profile during infection. Previously, exposure to early (T_H1) acute pre-patent maternal schistosome infection previously was to lead to reduced worm burden, lowered Ig titres and lower liver pathology in subsequently infected offspring (Attallah et al. 2006). This was not suggested for those exposed to chronic maternal infection, and there was no clear functional protection towards schistosome infection of Sm offspring compared to naïve offspring never previously exposed. The only effect hinted at was a weak trend towards lower IL-5 production to SEA-re-stimulation after infection of Sm offspring, most clearly in the mesenteric LN, but mirrored also in the spleen and across both organs in slightly lower mean level of IL-10 production, although this again was not significant.

Remaining questions include when CD4+CD44+ IL-4-producing memory T cells are primed by maternal infection, as well as their mechanism of priming, for example, whether *in utero* or during nursing. The mechanisms of persistence and deeper profiling (e.g.: transcriptome) of these cells remain unknown. Whether maternally-primed IL-4-producing CD4+CD44+ indeed modify the activation of naïve CD4+ T cells to acquire a type 2 skewed phenotype, could be directly assessed by activating naïve CD4+ T cells (for example, via TCR stimulus or APC + peptide exposure of transgenic OT-II or DO11.10 cells) in the presence of these Sm offspring-derived CD44+ cells, whether in *in vitro* co-culture experiments or adoptive transfer experiments *in vivo*. Similarly, the effect of transmaternally primed CD4+CD44+ T cells on APC phenotype, DC activation status (and subsequent capacity to induce T cell priming), or B cell responsiveness could also be further elucidated in a similarly manner. Such experiments would further clarify the potential role of these cells within the regulatory network that appears imprinted via exposure to maternal infection, and in particular offers avenues for studies that would uncover mechanistically how immune skewing across interacting cellular compartments can persist into later life.

Removal of CD44+ memory CD4+ cell prior to polarisation assays further revealed no differences in FoxP3 or RORγt positivity, neither at T_H0, T_H17, nor Treg-inducing conditions. Assays of cytokine production when using this highly purified naïve compartment, including IL-4, IFNγ and IL-17, which previously shown differences prior to full memory cell removal, also now revealed no differences between offspring cohorts. The observed T cell functional effects of maternal schistosome exposure, including those from *ex vivo* analysis and following allergic inflammation, therefore likely do not derive from developmental shifts to the naïve antigen-responsive T cell themselves, as these in isolation do not show major shifts under neutral conditions or capacity to response to polarising stimuli.

The RNA-seq profile of these naïve CD4+ T cells, and also for CD62L+CD44- naïve CD8+ T cells, also confirm the high degree of similarity within these compartments regardless of maternal schistosome status. The antigen-specific CD8+ response appears to be a focal point for heterogeneity of immune responses to antigenic stimuli in Sm offspring, which would involve the activation of naïve CD8+ T cells. However, as with the transcriptional profile, the epigenetic landscape of these is likewise not

drastically altered in ways that can explain the observed differences during *in vivo* models. Further functional analysis of these cells examined their response to general stimulation with mitogens, and to TCR stimulus in the presence of co-stimulatory DCs, with and without the MVA-stimulus already shown in vivo to deliver differential CD8+ responses in vivo from Sm offspring. Across these conditions, representing a range of signal strength and modes of T cell activation, no functional difference in terms of IFNγ production was detected between cells of differing offspring groups. This further confirms that naïve T cells themselves are not directly altered with a clear functional imprint by maternal infection in this setting. Instead, the persistence of activated cells or other changes regulating the checkpoints that control T cell activation, as already observed within the activated CD44+ memory T cell compartment or in antigen presenting cell populations, are more likely sources underlying the functional effects observed in the form of modified T cell responses induced *in vivo*.

4.3 MATERNAL INFECTION PRIMES OFFSPRING REGULATORY NETWORKS INTO ADULTHOOD THAT MODULATE T CELL RESPONSES

Challenge-free allergic sensitisation and other immunisation models clarified that induced specific T cell responses are altered by prior exposure to maternal helminth infection. The results highlight the induction of CD8+ T cell responses as a clear candidate readout for exploring the mechanisms underlying these effects. As detailed above, steady-state analysis of naïve CD8+ T cells from Sm offspring did not reveal any clear alterations that could be linked to their *in vivo* functionality, neither in terms of transcriptional or epigenetic state, nor *in vitro* functionality across a range of stimulatory conditions. Alongside the absence of any previously observed CD4+ differences following the removal of memory T cell compartments, and similarly no differences in their transcriptional profile, this suggested that the presence of activated cells, and including potential modifications to the cellular compartments that regulate the induction of antigen-specific activation of naïve T cells, may instead bear immune imprinting following early life schistosome exposure that is capable of effecting T cell functionality.

This was confirmed through the use of exogenous transfer of naïve CD44^{lo} OT-I CD8+ T cells into Sm offspring and naïve recipients prior to immunization, and builds on work evaluating transferred transgenic T cells to evaluate immunization methods especially as used to experimentally induce allergy such as with alum (McKee et al. 2009) (Kool et al. 2008). Transfer of congenic-labelled OT-I facilitated tracking of antigen-specific responses through the clear identification of antigen-specific cells. Additionally, assessment of these cells, which were not exposed developmentally to any maternal infection effects, demonstrated that modified activation was transferable through endogenous factors that modified CD8+ activation, with those cells readily assimilating to the differential response pattern previously observed for endogenous T cells of Sm offspring. This was shown in the context of OVA-s.c. immunisation with no adjuvant, where low cell number transfer led to the marked absence of OT-I from Sm offspring spleens, with lower OT-I presence in the draining igLN, alongside lower CD4+ and CD8+ T cell IFNγ response. Transfer of much higher numbers of OT-I cells facilitated their retrieval splenic retrieval from Sm offspring, although these cells that were found in circulation showed clearly lower proportions of CD44+ activated cells, with both settings providing evidence for a block in CD8+ activation and expansion into a circulating memory population. Activation using proliferation dye-labelled OT-I cells, at shorter time points using alum adjuvanted OVA or MVA-OVA, allowed observation of OT-I proliferation as well as CD44 acquisition. Both of these signs of activated expansion were lower in Sm offspring in response to OVA-ALUM, the same protein + adjuvant stimulus earlier observed to yield lower IFNγ, less pro-inflammatory responses, and lower viral clearance after maternal priming. Again, mimicking the endogenous responses earlier observed, MVA-OVA induced normalised responses from transferred OT-I cells regardless of the maternal priming status of recipients. These results further confirm that altered activating or regulatory capacity is located within factors extrinsic to the naïve CD8+ T cells themselves. For example, that cross-presentation pathways through which exogenous antigens are presented to CD8+ T cells via MHCI molecules could be impacted by maternally-primed maturation status. Viral vectors such as MVA may either directly interfere with such priming via changes to maturation status and strong inflammatory signalling, or circumvent these via presenting antigens via the usual cell-surveillance mechanisms of endogenous cellular antigens via MHCI molecules.

The previous results of altered responses to immunization aside from changes to T cells, namely B cell responsiveness as well as activation of APCs, indicate a range of induced modifications across cellular compartments. This further includes activation within CD4+CD44+ cells, and chiefly their steady-state enhanced capacity to produce IL-4 and other cytokines. In all likelihood there is an interconnectedness between these changes, and these may operate within a network to shift immune set points in ways that modify bystander immune responsiveness and subsequent inflammation.

In terms of steady-state induced changes, existing previously unpublished data suggest baseline B cell activation are elevated in Sm offspring, for example on the basic level of proportions within the spleen. This enhanced B cell profile is then already present prior to the increases to activation induced during allergy priming, which were increased MHCII and CD86 expression alongside higher levels of antigen-specific Ig titres. Further analysis in this thesis revealed that schistosome-specific IgG1 antibodies were strongly present in Sm offspring, which had previously not been shown to persist at ages beyond 8 weeks in more acute maternal infection setting (Attallah et al. 2006). Particularly BALB/c Sm offspring, perhaps by virtue of the increased humoral responsiveness of this strain overall, even approached high specific Ig titres that were similar to those of infected mice themselves, even post-partum. At this point, whether these are long-lived transferred maternal antibodies (although the normal 6-8 day half-life of murine IgG1 (Vieira and Rajewsky 1988) would make this unlikely) or continually produced by induced or transferred schistosome-specific plasma cells, remains to be directly demonstrated. These could be induced, for example, through early life antigen exposure, and altered DC-follicular helper T cell interactions to enable class switching. CD45.1/2 congenic labelling could be used to discriminate maternal from offspring-derived cells, as well as being investigating any functional significance of their appearance, which are likely minimal based on the similar response to infection in this setting in Fig. 3.15, with major parameters of cognate schistosome infection of exposed offspring showing no major effect.

The observance of such striking anti-schistosome Ig titres, including within the small groups of steady-state offspring mice included as controls within the previous AAI and immunization experiments, warranted further experiments using larger cohorts of offspring mice to elucidate additional possible

effects with regard to baseline activation. These revealed an altered activation phenotype of increased CD86 expression across the APC compartments measured. These included B cells, DCs, including $CD11c^{hi}MHCII+$ conventional DCs and their major cDC1 and cDC2 subsets, and macrophages. For B cells and DCs, these patterns were verified across both BALB/c and C57BL/6 strains of offspring cohorts. Unlike B cells, however, there was no overall proportional shift in the numbers of DCs, nor changes to the ratio of their subtypes. A previous study supports that altered APC function may be altered by maternal schistosomiasis in other settings (Santos et al. 2016), although this was only during immunisation. It interesting to note, however, that increased CD86 in particular has already been identified as a hallmark of schistosome-induced regulatory priming in similar cell types. SEA has been shown to induced alternative cytokine production (e.g.: TGFß, IL-10, and IL-6) and transcription factors (including Arginase-2 and Fizz-1), can modify how APCs prime T cells (leading to IL-4, IL-10, and IFNγ+/IL-10+ T cell induction) (Zaccone et al. 2010), as well as changes to inflammasome activation (Ritter et al. 2010). SEA can also directly induce regulatory changes to B cells, making them more likely to produce IL-10, as well as changing the activation profile of macrophages, with both latter effects associated with cellular states where CD86 was upregulated, and further associated with particular subcomponents molecules including IPSE/alpha-1 (Haeberlein et al. 2017). Schistosome antigens have been found to persist in offspring from infected mothers after birth, including in livers and kidneys (Attallah et al. 2006), and breastmilk has been found to transfer antigen-specific offspring responsiveness in humans (Eissa et al. 1989), so it is possible that in the chronic maternal infection used in this thesis, generally characterised by the accumulation of high and sustained numbers of parasite eggs in maternal livers, that exposure to continuous release of SEA could be involved in such priming within the APC compartment of offspring and elicit modulatory effects. Additionally, it is possible that the induced IL-4 producing population of CD4+CD44+ in Sm offspring could bear SEA-epitope specificity, and this further would support the presence of priming in the B cell compartment.

Such priming may drive non-specific suppressive or tolerogenic responses to bystander antigen, as a complement to the specific induction of adaptions induced by maternal priming, already explored in the context of potentially specific CD44+ T cells, and persistence of anti-schistosome antibodies. Evidence

for this comes in the form of Sm offspring B cells, which in some settings demonstrated effects on CD8+ activation. This was in terms of CD44 acquisition of co-cultured naïve CD8+ T cells in the presence of BMDCs and peptide, and was not replicated using TCR-direct antibodies, nor associated with increased IL-10 in culture supernatants, as could be expected from previous studies on schistosome-induced regulatory B cells. As such, this effect appeared during combination of B cells and DCs, indicating a potential effect of Sm offspring B cells on CD8+ activation that may operate through modified interactions with DCs. The general finding of inhibited CD8+ proliferation with any B cells presence in co-culture, as well as high levels of cytokines within the B cell / DC / CD8+ co-cultures signify complexity within such a co-culture system, that would warrant optimisation, for example, to ensure viability across co-cultured cell types, and appropriate ratios and timepoints to observe functional interacts. Previous studies have evaluated B cells / DC / CD8+ interaction and observed proliferation of CD8+ T cells in vitro, although activation states of B cells (e.g.: via previous LPS-induced stimulation) have previously been shown to modify in vitro DC-CD8+ interactions, where B cell contact deactivated DCs and regulating CD8+ activation (Boldison et al. 2020). After optimisation, further analysis may be possible for interpretation of specific effects of interactions between primed cells (i.e.: primed DCs or primed B cells) on the activation of naïve cells.

Intravenous polyclonal immunoglobulin has been shown to modulate T cell responses and dendritic cell function, and has therefore been evaluated as an immunomodulator, with specific dampening effects already shown in murine models of allergic airway inflammation (Kaufman et al. 2011). As such, the appearance of baseline type 2-priming, increased baseline immunoglobulin titres, and enhanced antigen-specific immunoglobulin titres, all in association with reduced inflammatory T-cell responses suggest that maternal helminth infection could alter steady state immune balance in a similar manner and through related high IgG-mediated mechanisms. This would need to be further clarified in specific experiments assessing the requirement for high Ig titres in this process (such as reproduction of this effects through serum or Ig transfer, and / or blocking or knocking out of FcR during this model, and may offer a further potential role for B cell / DC interactions within the mechanism of persistent regulatory changes. Clarification of whether the presence of IL-4-producing CD4+CD44+ further support this interaction

may uncover how distinct factors induced by maternal schistosomiasis operate within a network, and begin to simplify the complexity within this system of functional cellular changes.

Altered functionality within the DC compartment was confirmed in *in vitro* experiments using stimulation of isolated cells. Here, the key TLR agonist LPS elicited increased TNF and IL-6 responses from the CD11c+ DC-enriched cells of both BALB/c and C57BL/6 Sm offspring. The attribution of these effects to the cDCs, the APCs most commonly associated with priming of naïve CD4+ or CD8+ T cells, was further confirmed by stimulation of $CD11c^{hi}$MHCII+ DCs after additional purification. Modification within other subsets, such as the plasmacytoid dendritic cells known to produce high amounts of type I interferons, and also recently shown to cross-present to CD8+ T cells with the aid of cDCs, remains to be assessed and would require the inclusion of subset-specific markers (e.g.: BST2) for clear identification (Fu et al. 2020). The direct relationship of the altered activation status of DCs and the modified induction of CD8+ T cell responses was revealed through further co-culture experiments. Here, the lower proliferation of naïve OT-I primed with Sm offspring-derived DCs relative to those from naïve offspring replicated the lower OT-I proliferation already observed *in vivo* of naïve OT-I when activated within Sm offspring as recipients prior to immunisation. This was further associated again with increased cytokine production, notable IL-6, although with differential correlations between IL-6 in this setting with proliferation complicate direct interpretations of IL-6 directly inhibiting CD8+ proliferation, where IL-6 has previously also been shown to modulate CD8+ activation, for example inducing a more alternate polarisation that induces IL-21-producing CD8+ T cells that support follicular interactions and B cell isotype switching (Yang et al. 2016). This instead may be a sign of additional changes to Sm offspring DCs that impact their interactions with CD8+ T cells, with IL-6 often noted to antagonise other inflammatory pathways (for instance, antagonising IL-12 induction and inducing IL-10 through feedback loops following stimulus by schistosome eggs (La Flamme, MacDonald, and Pearce 2000)) and may therefore further indicate an induced altered activation status of DCs exposed to maternal schistosome infection. This is supported by altered CD86 levels on Sm offspring, matching those of SEA-exposed APCs, although a direct role for IL-6 could be further explored with antibody-based blocking of IL-6 or receptor knockout systems.

Although the enhanced release of these cytokines does not provide simple evidence for tolerogenic dendritic cells (as would, for example, increased IL-10 levels alone), the activities of IL-6, which is often described as pleiotropic, fit surprisingly well with those so far observed in Sm offspring, namely: antagonization of selected pro-inflammatory T cell responses while stimulating certain aspects of humoral responses. For example, IL-33 (an alarmin clearly related to the immune responses induced by parasitic helminths) stimulates DCs to produce more IL-6 (Rank et al. 2009), as part of the response of APCs to PAMPs and related signals. IL-6 has also been found to favour the polarization of helper T cells to a Th2 phenotype, including in response to schistosome antigens, through a cascade that inhibits type 1 / IFNγ-dominated responses, and that triggers regulatory loops including IL-10 upregulation (La Flamme, MacDonald, and Pearce 2000). IL-6 further enhances follicular helper-type phenotypes in CD4+ and CD8+ T cells (Eto et al. 2011) (Yang et al. 2016), and was originally discovered (original name: B cell stimulating factor, and B cell differentiating factor) for its demonstrated effect in maturation of B cells and enhancement of antibody production, with further links between these DC modifications to the other similar observed phenotypic features of Sm offspring remaining to be explored.

Parasite product-induced DC maturation has also specifically been previously shown (for *Trypanosoma cruzi*) to inhibit effective antigen-specific $CD8^+$ T cell priming (Ersching et al. 2016). It is therefore highly possible that maternal helminth priming could shape the CD8+ T cell vaccine response through altered maturation of innate and antigen presenting cells, given the evidence of similar cellular changes associated with priming effects in this setting, such as previously shown for schistosome-induced PD-L1 on macrophages driving CD4+ and CD8+ T cell anergy (Smith et al. 2004). A direct link to parasite antigens, as opposed to other mechanisms including maternal cytokines or antibodies, remains to be shown as a potential mechanistic follow-up to this work, such as whether exogenous cytokines or antibodies during pregnancy or nursing can reproduce the effects, or if effects change with selective knockout or inhibition of cytokines or cellular subtypes. Similarly, it remains to be determined whether signs of these changes are already present at birth, or whether they accumulate with time or in response to nursing (with SEA shown to be present in mother's milk (Attallah et al. 2006)). Study of such details

would also aid in comparing any difference in effects of early life exposure relative to later time points, and determining the specific significance of environmental shifts during the early life window coinciding with ontogenesis. For example, DCs are generally considered to only have a lifespan of up to 21 days. Persistent changes within these populations, then, would likely derive from immune training within precursor populations, such as within bone marrow, or via some other persistence stimulus, such as other more long-lived activated cells or their products, such as immunoglobulins. Training immunity, in terms of persistent myeloid cell priming via haematopoietic precursor priming has been shown most clearly to endure after BCG immunisation, even in human experimental vaccination groups (Cirovic et al. 2020), and has even been shown in murine schistosome infection (Cortes-Selva, Gibbs, Maschek, et al. 2021), although this was impacted by gender differences and located within male infected mice. Persistence changes in B cell maturation, potentially demonstrating altered priming in long-lived memory-type B cells, have been shown in uninfected infants exposed transmaternally to hepatitis C infection, in the form of specific IgG and IgA producing plasma cells and increased CD40 expression, in absence of direct haematopoietic training immunity in bone marrow precursors (Lutckii et al. 2020). Ongoing work exploring maternal schistosome priming maybe further uncover that long-lasting changes to such cell populations as T and B cells contribute to similar priming of more transitory cells through cell-cell interactions or similar immune networks, with other settings and time points of maternal schistosomiasis also demonstrating lasting effects in the B cell compartment that may disrupt immune responses (Cortes-Selva, Gibbs, Ready, et al. 2021).

Schistosome infection has an impact across a range of vital biological systems, especially within the abdomen and gastrointestinal tract, which may provide further, less direct, ways maternal infection impact fetal development. For example, although the adult parasites are not in the intestinal lumen, unlike *H. polygyrus*, the passage of schistosome eggs through the intestinal epithelia drives inflammatory processes that may impact the microbiota (Bhattacharjee, Kalbfuss, and Prazeres da Costa 2017). Maternal microbiota changes, as described earlier, are known to be key drivers of divergent offspring immune susceptibility, particularly through alterations to the developmental metabolomic profile. Additional studies on the metabolite profiles of urine from schistosome infected mice found

changes in association with altered liver function (Wang et al. 2004) (with the livers of heavily infected mice showing clear morphological changes that would impact their function). Such microbiota changes, changes to metabolites (e.g.: such as if these modify SCFA) or increased presence of damage or danger signals indirectly derived from the diseased maternal state, could impact immune development in this setting, particularly in ways that impact the maturation status of APCs. The polarisation APCs including macrophages and DCs is known to be heavily responsive to shifts in basic metabolism and the tissue microenvironment (as discussed in (Murray 2017)), and represent additional avenues of study (such as transmaternal priming through metabolite or metabolic danger signals) that may provide further insight into the transfer of these effects.

Modified DC phenotype therefore appears to be the linker between a complex set of steady-state alterations in immune set points and differential T cell responses. These modified factors are primed through early life exposure to maternal helminth infection, and may be induced by enhanced maternal immune features during a modified response to chronic inflammation, and / or those stimuli directly from the parasite and its eggs, although this remains to be directly assessed in detail. The presence of this complex stimulus drives: priming changes to T cell polarisation, inhibiting IFNγ production and characterised by the presence of IL-4 producing memory T cells, B cell activation set point and systemic levels of Ig, as well as a modified activation status across APC compartments. The mechanisms of the persistence of these effects remains to be explored, as does their interaction within a regulatory network that inhibits local allergic inflammation, as well as systemic inflammatory CD4+ and CD8+ T cell responses to bystander antigens. These represent fruitful avenues of further research into susceptibility to inflammatory dysregulation and its responsiveness to environmental cues.

4.4 OVERALL CONCLUSIONS

There is increasing evidence that predisposition for allergy and immune disease can be imprinted in early life, with weight given to maternal factors and exposures during pregnancy. Maternal parasite infection has bene linked to heterogenous immune responses, and the initial observations, of lowered susceptibility towards experimental allergic asthma in schistosome exposed offspring uncovered a system for studying the relationship between maternal infection and immune predisposition. This model

builds from concepts within the hygiene hypothesis that point to the root of hyper-susceptibility to immune dysregulation as related to molecular switches and set points associated with infectious agents that posed evolutionary pressures upon the mammalian immune system. Human studies of transmaternal helminth infection effects on immunisation responses and allergic predisposition are continually marred by considerable limitations in terms of effective sample size, and more troubling, sample management and effective acquisition and collation of diagnostic information within sufficient immune parameters to make clear observations, as in (Nash et al. 2017) and most recently in (Flugge et al. 2020). The murine model continues to therefore offer unique opportunities for studying clear effects of transmaternal immune priming.

The modified phenotype of these Sm offspring was found in this thesis to consist of clear modulations to antigen-responsive CD4+ T cell activity induced during localised lung inflammation. Transcriptionally links to changes at the priming site of immunization, as well as other signs that disagreed with general non-responsiveness, including enhanced allergen-specific antibody titres, indicated the complex activity of a modified immune response following maternal priming. Analysis of B cell and DCs during allergic sensitisation supported the presence of an induced altered kind of response from these offspring. Further assessment of lymphocyte responses during immunization found shifts along the IL-4/IFNγ axis that represented an induced difference that approximated a tolerogenic T cell response shifted away from pro-inflammatory outcomes. These changes, evidenced by overall type 2-skewed, tolerogenic outcomes, themselves stem from exposure to a strong modified type 2 / regulatory stimulus, that is: the environment provided by maternal chronic *S. mansoni* infection. This appears to provide effects that educate the immune response to newly encountered antigens and modify inflammatory susceptibility. This observably impacts both CD4+ and also clearly CD8+ T cell compartments in ways that extend not only to other forms of immunization with modalities also relevant for vaccination settings, but also functionally to reduced ability to control viral infection. Manipulation of vaccine modality, namely through use of the viral vector MVA, was able to counteract these inhibitory effects, and highlight the regulation of CD8+ T cell responses as a specific feature of prior schistosome exposure.

The CD8+ T cell compartment showed little baseline modulation from maternal infection, and alongside further analysis of the highly purified CD44- naïve T cell compartment, their analysis demonstrated that previously uncovered *in vitro* priming effects were likely derived from changes observed within the CD44+ memory T cell compartment driven by maternal infection. After observing the ability exogenous CD8+ T cells to assimilate to the regulatory environment primed by maternal infection, without the need to direct developmental exposure of the CD8+ T cells themselves, it became clear that factors extrinsic to the naïve T cells themselves were driving a persistent change to bystander T cell responses. Through expanded analysis of other cell types, the observed differences of IL-4-producing CD4+CD44+ T cells were situated within an enhanced network of regulatory changes. Across cellular compartments, this included observations of induced altered functionality of APCs as well as B cells. Modifications with the DC compartment were specifically found to provide a link to altered T cell responses, showing a reduced capacity to elicit CD8+ responses, already observed to be key feature of the immunomodulation present in Sm-exposed offspring during the initial experiments.

In terms of direct translational outcomes, this work relates to ongoing projects that involve the development and application of microbial and helminthic extracts for tolerogenic immune education. Evidence from human cohorts of such treatments to manipulate the molecular switches underlying susceptibility to inflammation have been mixed, particular with regard to live experimental infection, and have frequently not reproduced the beneficial outcomes observed in closed systems of animal models. The murine system of chronic maternal infection and its outcome of offspring immune predisposition more specifically continues now to have an advantage with regard to determining the relative contribution of early life effects within a relatively closed experimental system. Continued usage of this and similar systems could help explore the significance of different time points and developmental stages through which environmental exposures, infection-based or otherwise, can be delivered for the greatest and safest effects. Perhaps more importantly, based on the specific findings of this thesis, particularly in terms of the range of cellular changes identified and their implications for immune heterogeneity, would be the continued assessment of how these not only operate together within the crosstalk of a cellular immune network, but also how this leads to a persistent effect of

immunomodulation. Exploration of this feature of deliberate and persistent immune imprinting has the potential to provide insight on how immune-based therapy, whether through environmental changes or medication-based therapeutics, could be itself optimised in order to delivery lasting positive change with regard to immune regulation, particularly in otherwise susceptible individuals.

Detailing the direct effects of the impact of helminth infection on pregnancy itself was outside the scope of this thesis, enabling instead a focus on the effects on offspring immune predisposition through early life priming. Future directions that would be tailored to more effectively yield clinically-minded outcomes regarding in infection during pregnancy would profit from examining details at the fetomaternal interface (including the placental tissues) during prenatal infection. In particular this could include histological and cellular evaluation of local changes to steroid hormone pathways and inflammation, already identified on a transcriptional work. These may further aid in generating informed prescriptions for pregnancy regarding infection or removal of infection, with current guidelines otherwise considering treatment to be safe during pregnancy. This may further aid in identifying factors downstream of helminth infection responsible specifically for the protective response regarding allergic disease and induced enhancement of immune regulation. This would be especially aided by discerning the relative contributions of the diverse elements of this complex maternal set of stimuli, such as between the effects of transferred parasite molecules, including components of SEA, and maternal cytokines, cells, immunoglobulins, and other induced immune factors, as well as potential inflammatory, steroidal, or hormone-based changes *in utero* and their effects on basic development and immune ontogenesis. This thesis has outlined a number of specific alterations, implicated in multiple cellular compartments, that are associated with enhanced immune regulation through transmaternal priming. These parameters, including striking levels of SEA-specific IgG1, offspring-derived IL-4 producing CD4+CD44+ cells, or B cells and APCs including DCS that show modified co-stimulatory profiles such as CD86 expression could be used (and even further refined) as surrogates for this enhanced immunoregulated status. As such, these features can now be used as readouts to direct more sophisticated approaches towards further investigation of the pathways leading to their priming-based induction and persistent maintenance. The discovery of these associated phenotypic details could also direct additional mechanistic projects to gain

insight into how these persistently alter the susceptibility to inflammatory disease, and methods that could be employed to replicate them. Such detailing of the underlying processes and effects within the documented, specific instance of immune programming via maternal chronic helminth infection in a murine system will help generalise these outcomes to other situations. In doing so, this may uncover effects and molecular switches that could potentially be mimicked and manipulated through other means, and further identification of triggers which could interact positively with these same pro-tolerogenic pathways in humans.

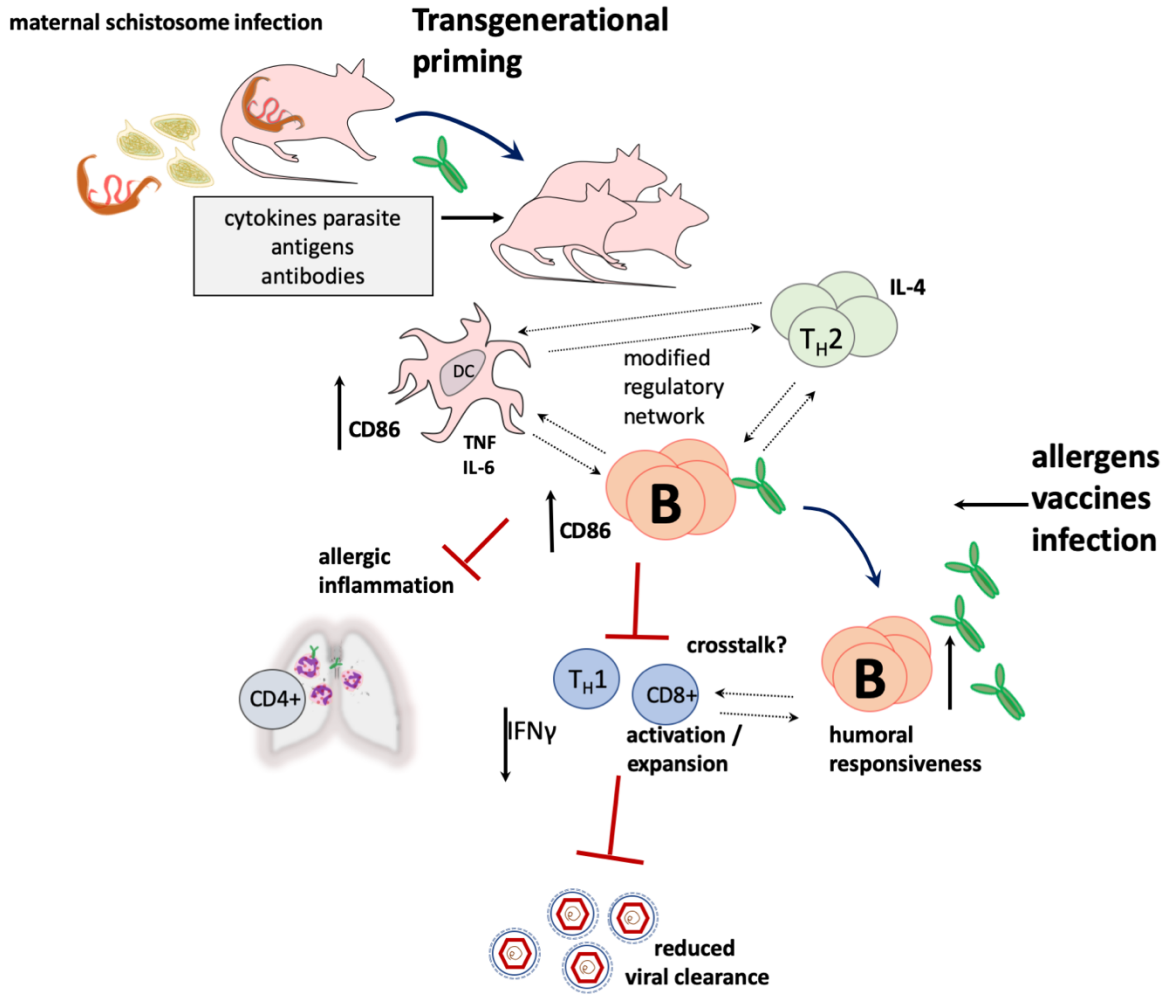
As such, the current study deepens our understanding of the longitudinal effects of maternal schistosomiasis on the development of offspring. It has yielded insight into host-parasite and immune crosstalk that will clarify the role of maternal inflammation in immune priming of offspring. In more general terms, these findings give further evidence of the role that immunological imprinting, inducible through environmental factors such as maternal infection status, can play in modulating inflammatory outcomes. This has uncovered factors that may be of relevant for allergic and immune heterogeneity, such as infection or vaccination strategies in helminth endemic areas, and also immune pathways associated historically with host-helminth crosstalk that will likely continue to be relevant for treatment and prevention of immune disorders.

This project has identified cellular outcomes of maternal helminth infections that show interfere with immune processes involved in many clinically-relevant areas, as diverse as inflammatory diseases and immune disorders, early development, vaccines and management of co-infections, and successful development following healthy pregnancy. As such, deeper, clearer investigation of how these microbial exposures and infectious challenges modulate immune memory in a more global sense will enrich our understanding of immune interactions with the environment, and provide tools for fine tuning of immune responses and modulation of immune disorders.

In populations where helminth infection is endemic, such considerations may impact interventions regarding infection including treatment and immunisation during and after pregnancy (including during the nursing period). In the wider movement towards personalising medicine as an aspect of population health, understanding the effects of past and current microbial and parasitic exposures, and the

dysregulated states that arise in the absence of such potentially modulatory stimuli, will change interpretations of individualised skewing or training of the immune system that present as a range of susceptibility to inflammatory trigger. This individual history of immunogenic exposures may be at the root of not only idiosyncratic responses to standard vaccine protocols (which have so far mostly eluded accurate study, particularly in the settings where helminths are endemic), but also the appearance of inflammatory and immune disease, meaning that practical application of continued work in this area extends far beyond support of de-worming or controversial re-worming practices. As such, deeper understanding of how schistosomes and their compounds can manipulate the immune system can teach us more general lessons about fine control over immune responses, and the developmental origins of dysfunctional states in later life. By examining these processes during the highly vulnerable *in utero* and early postnatal periods, we gain mechanistic insight into the influence wielded by environmental exposures. This work has uncovered that transmaternal imprinting can drive persistent phenotypic changes to cellular pathways involved in the regulation of allergic and inflammatory responses, including adaptive T cell responses related to inflammatory processes that underlie non-communicable diseases such as diabetes. These findings can be developed towards directing interventions during the impressionable window of early life and beyond, and highlight the lasting impact of environmental factors on immune health and disease.

4.5 GRAPHICAL SUMMARY



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6. Publications

Reviews:

Lacorcia, M., & Prazeres da Costa, C. U. (2018). Maternal schistosomiasis: immunomodulatory effects with lasting impact on allergy and vaccine responses. *Frontiers in immunology*, *9*, 2960.

Prodjinotho, U. F., Lema, J., **Lacorcia, M.,** ... & Prazeres da Costa, C. (2020). Host immune responses during Taenia solium Neurocysticercosis infection and treatment. *PLoS Neglected Tropical Diseases*, *14*(4), e0008005.

Primary Research Papers:

published:

Lacorcia, M., … & Prazeres da Costa, C. **Fetomaternal immune crosstalk modifies T cell priming through sustained changes to DC function.** *Journal of Allergy and Clinical Immunology*, 2021 Mar 5. pii: S0091-6749(21)00360-2. doi: 10.1016/j.jaci.2021.02.031.

Ludwig, E., Harder, J., **Lacorcia, M**., ... & Adegnika, A. A. (2019). Placental gene expression and antibody levels of mother-neonate pairs reveal an enhanced risk for inflammation in a helminth endemic country. *Scientific reports*, *9*(1), 1-12.

submitted:

Schick, J., **Lacorcia, M**., … Lang, R., Prazeres da Costa, C. (submitted – Journal of Clinical Investigation). IL-4 and helminth infection downregulate MINCLE-dependent macrophage response to mycobacteria and Th17 adjuvanticity.

in development:

Ludwig, E., **Lacorcia, M**., Kugyelka, R., … & Prazeres da Costa, C. (in development). "Schistosomiasis alters Maternal Cytokines at birth and modifies maturation of cord blood T and B lymphocyte subsets as well as haematopoietic stem cells".

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Anhang I

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung
Doktor der Naturwissenschaften (Dr.rer.nat)

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der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
Modulation of Fetomaternal Crostalk through Chronic Helminth Infection: Sustained Alterations to T Cell Responses and DC Functionality

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in Fakultät für Medizin, Institut für Mediziniche Mikrobiologie, Immunologie und Hygiene.
Fakultät, Institut, Lehrstuhl, Klinik, Krankenhaus, Abteilung

unter der Anleitung und Betreuung durch: Prof. Dr. med. Clarissa Prazeres da Costa ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Ab. 6 und 7 Satz 2 angebotenen Hilfsmittel benutzt habe.

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Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich

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