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# The role of ICOS in Foxp3$^{+}$ Treg responses induced by parasitic helminths

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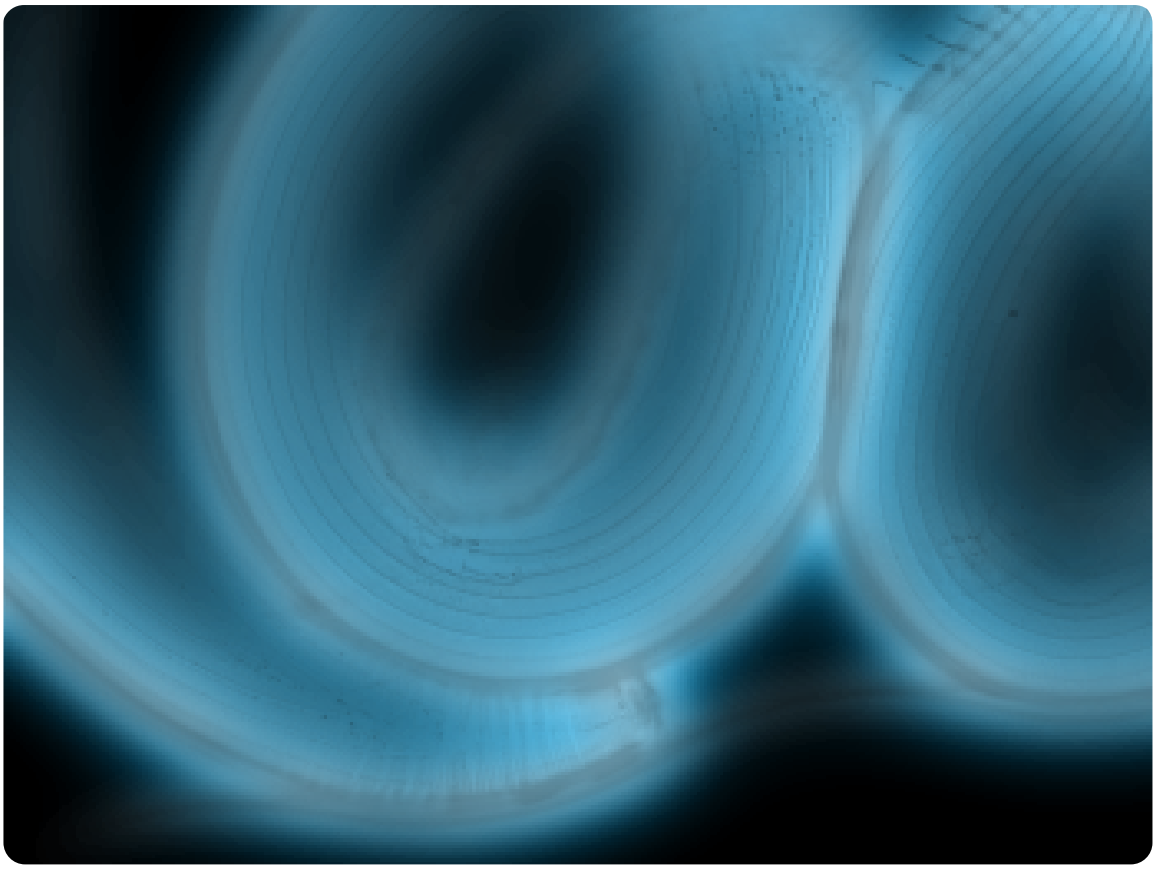

***Declaration***

I hereby declare that this thesis was composed by myself and that the work described herein is my own, unless otherwise stated. Front cover photographs courtesy of Janice Murray.

Stephen Alexander Redpath

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***Acknowledgements***

I would especially like to thank my mum and dad for everything they have done for me over the years. This thesis would not have been possible without your continual support, and I dedicate it to you. Mum, I am sorry that after retiring from your job as a Psychiatrist you have been continually employed in counseling me over the course of this PhD. Dad, thank you so much for the continued supply of decent bottles of red wine, your services as a removal man, and of course all the help and advice. You really are the best parents that anyone could wish for.

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***Abstract***

Helminth parasites excel at subverting the host's immune regulatory pathways resulting in immunosuppressed hosts harbouring chronic infections. This immune suppression forms a major barrier to the acquisition of protective Th2 immunity, both in regard to natural infections and potential vaccinations. At the same time, immune downregulation plays a beneficial role in protecting the host from pathology during chronic infection, and epidemiological links between helminth infections and the amelioration of allergy and autoimmunity diseases indicate that helminth-induced immune suppression can be therapeutically applied to the treatment of these conditions. $Foxp3^+$ regulatory T cells (Treg) play central downregulatory roles in controlling reactivity to self-antigens and preventing autoimmune diseases, as well as in limiting inflammatory responses during infection. Helminths induce dominant $Foxp3^+$ Treg responses that play key roles in inhibiting protective immunity and alleviating immunopathology, and that can protect against allergic inflammation. Thus, $Foxp3^+$ Tregs are a fundamental mechanism of immune regulation during helminth infections, and an understanding of the mechanisms governing the induction of $Foxp3^+$ Treg responses is of principal importance for the design of both prophylactic helminth treatments and therapies for allergies and autoimmunity. However, the nature of the T cell co-stimulatory signals driving Treg generation during helminth infection is largely unclear. Recent evidence suggests that the inducible co-stimulator (ICOS) contributes to Treg control of autoimmune inflammation. Further, ICOS expression is upregulated by $Foxp3^+$ Treg during infection with the filarial nematode *Litomosoides sigmodontis* suggesting ICOS is important for Treg during helminth infection. Therefore, we investigated the role of ICOS in helminth-induced Treg responses.

Similar to *L. sigmodontis* infection, $Foxp3^+$ Treg increased ICOS expression in response to infection with the intestinal nematode *Heligmosomoides polygyrus* and with the blood trematode *Schistosoma mansoni*. Functionally, ICOS was required for the optimal

expansion of lymphoid Treg numbers during early stage *H. polygyrus* infection and following the onset of the acute egg phase of *S. mansoni* infection suggesting common pathways for Treg induction by diverse helminth species. Whilst helminth induced proliferation and activation of $Foxp3^+$ Treg was ICOS independent, ICOS was essential for Treg survival in settings of homeostasis and helminth infection. In contrast to the lymph node, Treg responses in the intestinal lamina propria (LP) of $ICOS^{-/-}$ mice were increased due to expanded natural Treg. Following *H. polygyrus* infection $Foxp3^+$ $Helios^-$ $CD4^+$ T cells preferentially expanded in wild-type (WT) mice but not in ICOS deficient mice suggesting ICOS is required for the expansion of adaptive Treg at the site of intestinal nematode infection.

Functionally, ICOS supports Treg, but not effector T cells (Teff), *H. polygyrus* induced IL-10 production suggesting ICOS differentially regulates Treg and Teff. At the *H. polygyrus* infection site, ICOS acted to downregulate $CD4^+$ T cell Th2 cytokine production. Conversely, in the reactive lymph node ICOS signalling promoted Th2 immune responses, possibly by maintaining the pool of IL-4 secreting type 2 follicular helper T cells. Thus, ICOS has different effects on Th2 immunity depending on tissue location. Because Th2 immunity governs expulsion of *H. polygyrus* parasites, the differences in Th2 responses between lymph node and infection site could explain why ICOS deficiency did not impact worm burden.

Protective immunity to long-lived helminth infection can be quenched in the initial days of infection by the action of Treg. Whether Treg expand and suppress protective immunity during *S. mansoni* larvae lung transit has not been investigated. We found that in contrast to *H. polygyrus* and *L. sigmodontis* infection, early *S. mansoni* infection did not induce a Treg response suggesting other mechanisms are employed for immune subversion. During the acute egg-phase of *S. mansoni* infection, $Foxp3^+$ Treg protect the host from

damaging egg-induced hepatic immunopathology. Despite reduced $Foxp3^+$ Treg responses, ICOS deficiency did not impact egg-induced immunopathology.

Thus, ICOS co-stimulation contributes to early expansion and the continued maintenance of Treg during helminth infection, both in the local lymph node and at the infection site. ICOS is required for Treg function during helminth infection by promoting IL-10 production, whilst its contribution to Th2 effector immunity is tissue specific. In addition, ICOS is dispensable for protective immunity and pathology during helminth infection. As ICOS controls both positive and negative immune responses and can have opposing roles depending on tissue location, an understanding of the consequences of these contradictory effects will be important when considering targeting ICOS therapeutically.

***Abbreviations***

**Ab** – Antibody

**AHR** – Airway hyper reactivity

**APC** – Antigen presenting cell

**BAL** – Broncheo-alveolar lavage

**BTLA-4** – B and T cell lymphocyte attenuator-4 (CD272)

**cAMP** – Cyclic adenosine monophosphate

**CTLA-4** – Cytotoxic T lymphocyte antigen-4 (CD152)

**CTL** – Cytotoxic T lymphocyte

**CXCR5** – C-X-C chemokine receptor type 5

**DALY** – Disability adjusted life years

**DC** – Dendritic cell

**EAE** – Experimental autoimmune encephalomyelitis

**Ebi3** – Epstein-barr virus induced gene-3

**GALT** – Gut associated lymphoid tissue

**GC** – Germinal centre

**GI** – Gastrointestinal

**HES** – *H. polygyrus* excretory/secretory products

**HVEM** – Herpesvirus entry mediator

**ICOS** – Inducible costimulator (CD278)

**ICOS-L** – Inducible costimulator ligand (CD275)

**IDO** – Indoleamine 2, 3-dioxygenase

**IFN-γ** – Interferon gamma

**Ig** – Immunoglobulin

**IHC** – Innate helper cell

**IL** – Interleukin

**IL- 4R** – Interleukin-4 receptor

**IPEX** – Immunodysregulation polyendocrinopathy enteropathy X – linked

**iT$_R$35** – Inducible Treg IL-35

**LCMV** – Lymphocytic choriomeningitis virus

**LP** – Small intestine lamina propria

**LPS** - Lipopolysaccharide

**L3** – larval stage-3

**MHC** – Major histocompatibility complex

**MLN** – Mesenteric lymph node

**NK** – Natural killer cell

**NOD** – Non-obese diabetic

**PBMC** – Peripheral blood mononuclear cells

**PD-1** – Programmed cell death-1 (CD279)

**RELM-β** – Resistin like molecule beta

**SCF** – Stem cell factor

**TCR** – T cell receptor

**Teff** – Effector T cell

**Tfh** – Follicular helper T cell

**Th** – T helper cell

**TLR** – Toll like receptor

**TNFR** – Tissue necrosis factor related

**Treg** – Regulatory T cell

**Tr1** – Type 1 regulatory T cells

**TSLP** – Thymic stromal lymphopoietin

**TSLPR** – Thymic stromal lymphopoietin receptor

**WT** – Wild type

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## *Chapter 1. Introduction*

### *1.1 The global consequence of human helminth infections*

Parasitic helminth infections are a major global health burden infecting approximately 2 billion people worldwide resulting in severe morbidity that is manifest by anaemia, malnutrition, limb and genital deformities and blindness (1). The disease impact of helminth infections ranks among the highest of the neglected tropical diseases, affecting not just global health but also economic development in the poorest regions of the world where helminthiases are most prevalent (2, 3). The full impact of the negative consequences of helminth infection have only recently become appreciated, and one of the chief reasons for this neglect is the relatively low mortality incurred by helminth infection when compared to diseases such as HIV/AIDS and malaria (4). In fact, when the chronic morbidity resulting from the global spectrum of helminth infections is considered in terms of disability-adjusted life years (DALYs), it ranks higher than that of HIV/AIDS and malaria (4). Despite the global impact of helminth infections, only a limited number of anti-helminthic drugs are available and no effective vaccine has been developed. Mass treatment of human populations with anti-helminthic drugs can be successful (5) but economic, political and logistical problems, the frequency of re-infection and the threat of emergence of drug resistance limit the efficacy of this treatment. Therefore, the design of novel therapeutics for human helminthiases is essential.

The most prevalent helminth infections of humans are the intestinal nematodes, and amongst the most common intestinal nematodes of humans are the hookworms *Necator americanus* and *Ancylostoma duodenale*. Of the hookworms, *N. americanus* accounts for about 85% of all infections with *A. duodenale* taking up the remainder. Most infections occur in the Asian countries of Indonesia, Bangladesh and India (60-70 million people in each), followed by the Democratic Republic of the Congo and Nigeria in Africa, and Brazil

(30-40 million people in each) (4). The other major intestinal nematodes are the roundworm *Ascaris lumbricoides* (800 million infections), the whipworm *Trichuris trichuria* (600 million infections), and the threadworm species *Strongyloides stercoralis* (30-100 million infections), and like the hookworms these species are most prevalent in developing regions of Asia, Africa and Latin America (1). Intense intestinal nematode infections frequently occur in children and can result in anaemia, malnutrition, growth retardation and cognitive dysfunction (6). During pregnancy, the severe anaemia caused by intestinal nematode infections leads to low birth weight and increased maternal and infant mortality. In Africa, the anaemia resulting from intestinal helminth infection exacerbates the disease symptoms of falciparum malaria, especially in woman and children (7).

The platyhelminth schistosome species rank next in global prevalence, and include the species *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum.* Of the 200 million global cases of schistosomiasis, around 90% occur in sub-Saharan Africa. *S. haematobium,* the causative agent of urinary tract schistosomiasis, accounts for approximately two thirds of global schistosome infections, whilst *S. mansoni,* one of the agents of intestinal schistosomiasis, takes up most of the remaining third. Alongside sub-Saharan Africa, *S. mansoni* infections are also common in Latin America, especially Brazil. In Asian regions, *S. japonicum* infects around one million people to cause intestinal schistosomiasis (4). Chronic schistosome species infections can last for five to seven years and the liver fibrosis, portal hypertension and intestinal bleeding that characterises disease pathology induced by schistosomiasis arises as a consequence of the host immune response to the parasite eggs (8).

Eight species of filarial nematodes have human hosts, and three of these account for the majority of disease causing infections (9). *Brugia malayi* and *Wuchereria bancrofti* are responsible for lymphatic filariasis, infecting some 120 million people in endemic areas throughout the tropics (9, 10). Lymphatic nematode infections are long lived and are

notable for causing heavy infections with no overt symptoms of clinical disease. However, around a third of those infected suffer from clinical manifestations of the disease, and infection can result in disabling deformities as seen in elephantiasis and severe genital swelling (9, 10). The third most important filarial nematode species affecting humans is *Onchocerca volvulus,* which infects almost 37 million people, and is most prevalent in Africa but also occurs in regions of central and southern America (9). *O. volvulus* is the etiological agent responsible for the debilitating disease river blindness, so called because it is transmitted by black flies and the prevalence of infection is greatest in communities adjacent to areas of black fly riverine breeding (9, 10).

A key feature of human helminth infections is their ability to persist within infected hosts for long periods of time without eliciting strong disease symptoms. When disease pathology does occur it is often as a result of over exuberance on the part of the hosts own immune system. That the host immune system is fully capable of mounting a strong response to the parasite, but generally does not gives some indication that host immunity is regulated during helminth infections. Indeed, the first evidence of immunosuppression by helminths came from studies on human filariasis, which demonstrated that peripheral T cells from infected individuals were frequently unresponsive to antigen stimulation, and immune responses to bystander antigens were reduced (11). It is now known that helminth parasites are capable of inducing strong immune regulatory responses, and in doing so they are endowed with the capacity to evade protective host immunity and survive within the infected host for decades thus favouring their continued transmission (12). Helminths achieve immune suppression by infiltrating the hosts own immunoregulatory network. Regulatory T cells (Treg) are one of the key components of this network and their actions can form a major barrier to the acquisition of protective immunity. By targeting the co-stimulatory receptors that govern Treg action it is possible to reverse immune suppression and this has been shown to enhance protective immunity and enable parasite elimination

(13-15). However, Treg mediated suppression of over-exuberant anti-helminth immunity inhibits the development of immunopathology and interventions that target Treg must be undertaken with caution (16, 17). For this reason, a complete understanding of the mechanisms that preside over Treg induction, function and maintenance is fundamental for the development of safe, effective immuno-therapeutics for helminth parasite infections.

### *1.2 Mouse models can be used to study immune responses to helminth parasites*

#### *1.2.1* **Trichuris muris** *as a model of protective anti-helminth immunity*

Despite the classic chronic signature of human helminth infections, epidemiological evidence exists that demonstrates the development of host protective immune responses (18). Difficulties in delineating the determinants of protective immunity in genetically outbred populations subject to large environmental fluctuations necessitated the development of defined laboratory models of human infection. Seminal work using the model murine gastrointestinal (GI) nematode *Trichuris muris* described the immune conditions necessary for resistance to helminth infection (19-21). The *T. muris* model is unique in that the reciprocal effects of Th cell subsets determine whether an infection will become acute or chronic in nature (19-21). In mice, and in humans, $CD4^+$ T helper (Th) cell were initially grouped into two distinct subsets; Th1, marked by expression of the cytokines interferon gamma (IFN-γ), interleukin (IL)-2 and lymphotoxin, or Th2 characterised by expression of IL-4, IL-5, IL-9 and IL-10 (22, 23). Whereas Th1 dominated murine strains are susceptible to *T. muris* and harbour chronic infections, Th2 dominated strains are resistant and expel *T. muris* parasites before they establish (20, 21). Moreover, antibody (Ab) depletion of IFN-γ in susceptible strains rendered these mice resistant to infection, and conversely depletion of IL-4 in resistant strains of mice rendered them

susceptible to infection (19). Thus, the Th2 immune response driven by IL-4 is required for protective immunity to helminth infection.

### *1.2.2 Heligmosomoides polygyrus*

Mouse model systems can be also be used to study the mechanisms governing immune regulation induced by helminth parasites. *Heligmosomoides polygyrus* is a natural mouse parasite used as a model of the human hookworm infections *N. americanus* and *A. duodenale.* Primary infections persist for many months reflecting the chronic nature of human helminth infections, and immune protection against challenge infection can also be studied following drug cure of primary *H. polygyrus* infection. Experimentally, infective third stage larvae (L3s) are orally introduced to the host and within 24 hours they penetrate into the submucosa of the small intestine. Following two developmental moults they migrate back into the intestinal lumen between day 8-9 post-infection. Adult worms coil around intestinal villi to anchor *in situ* from whence they mate and produce eggs. The eggs are ejected in the faeces and hatch in the external environment becoming infective L3s, thus the life cycle is perpetuated.

### *1.2.3 Schistosoma mansoni*

The human blood fluke parasite *S. mansoni* is infective in mice allowing tractable systems for the study of immunity to trematode parasites. *S. mansoni* has a complex life cycle; water molluscs act as intermediate hosts and higher order vertebrates as definitive hosts. Upon contact with fresh water, *S. mansoni* eggs hatch and release free swimming miracidium that infect specific fresh water snails. After around 30 days, in response to sunlight exposure, motile cercariae extrude from infected snails and seek out their vertebrate mammalian definitive host. Infective *S. mansoni* cercariae penetrate the skin of their host and migrate via the circulation to reside as adults in the mesenteric veins (24). Skin penetration is facilitated by enzymatic secretions from the acetabular glands located

in the head of the cercariae (25). Larvae begin to leave the skin through the venules around day 4 post infection from whence they arrive at the pulmonary vasculature, peaking here at day 8 (24). First transit through the lungs is a slow process and represents a major obstacle for parasite migration (24). Parasites pass from the lungs through the vasculature and splachnic capillary beds to the hepatic portal system where they mature. After 3 weeks adult parasites mate and migrate up the mesenteric veins to the intestinal wall and reside thereafter in the distal portal system laying eggs, some of which become trapped in the liver sinusoids triggering inflammatory foci (24, 26).

### *1.3 The type 2 protective immune response to helminth infection*

Separated by phyla and 600 million years of divergent evolution, *H. polygyrus* and *S. mansoni* are helminths of distinct nature. Whilst *H. polygyrus* is exclusively enteric, *S. mansoni* resides principally in the vasculature. Despite these differences, the nature of the immune response induced by these parasites is remarkably similar. The canonical immune response invoked by helminth parasites is typically Th2 in nature, orchestrated by the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (27). These cytokines activate a suite of downstream effector mechanisms including immunoglobulin (Ig)G1 and IgE isotype switched B cells, mast cells, eosinophils, basophils, alternatively activated macrophages, innate helper cells and increased permeability, smooth muscle contractility and mucus production in the gut (27). Whilst Th2 cells predominantly orchestrate the immune response to helminths, other non-T cells also contribute so collectively they will now be referred to as the type 2 response. Both *H. polygyrus* and *S. mansoni* induce host type 2 immunity but the consequence of this response is dramatically different. During *H. polygyrus* infection the type 2 immune response primarily promotes parasite expulsion and protective immunity (28). In contrast, the role of type 2 immunity in *S. mansoni* infection is one of dual host protection, functioning to wall-off and neutralise toxic egg products in the liver whilst suppressing damaging pro-inflammatory Th1 immune responses (29, 30).

### *1.3.1 The role of type 2 immunity in protection to intestinal nematode infection*

*H. polygyrus* infection induces the Th2 cytokines IL-3, IL-4, IL-5, IL-9, IL-10 and IL-13 in the gut associated lymphoid tissue (GALT) (31-33). Of these cytokines, IL-4 and IL-4 receptor (IL-4R) signalling are key to protective immunity to both primary and secondary *H. polygyrus* infection (28). Whilst innate lymphoid cells are an important early source of type 2 cytokines (34-37), the principal producers of type 2 cytokines during helminth infection are $CD4^+$ T cells (38, 39). More specifically, the main $CD4^+$ T cell subset producing IL-4 in the reactive lymph node following *H. polygyrus* infection are $CXCR5^+$ follicular helper T cells (Tfh) (40). IL-4 signalling activates a variety of cells that contribute to *H. polygyrus* stress and expulsion. The mechanisms of protection can be broadly divided into two phases dependent on location of the different *H. polygyrus* stages: those active against the intestinal submucosa dwelling larvae, and those that bring about expulsion of adult worms from the intestinal lumen.

When larvae are resident in the intestinal mucosa, aggregates of innate and adaptive immune cells form granulomas around the invading larvae. The granulomas comprise macrophages, neutrophils, dendritic cells, eosinophils and in secondary infections, $CD4^+$ T cells (41-43). In response to IL-4, macrophages within the granuloma take on the characteristic type 2 phenotype distinguished by expression of the IL-4R, the mannose receptor CD206, Ym-1, Fizz1 and arginase-1 (41). These macrophages secret arginase-1 which acts directly on the larvae in the host-parasite interface to induce parasite stress (41). Therefore, in the intestinal tissue, it is thought that macrophages are the principal mediators of protective immunity to *H. polygyrus* larvae.

Once adult nematode parasites are present in the intestinal lumen they are subject to a barrage of assaults that aim to flush them out. IL-4 and IL-13 from innate and adaptive sources act directly on gut epithelial cells and resident effector cells expressing IL-4R$\alpha$ to

alter the physiology of the gut. For example, expulsion of luminal *T. muris* parasites is enhanced by an increased rate of intestinal epithelial cell turn over following IL-13 signalling (44). IL-4R driven goblet cell hyperplasia results in increased mucus secretion (45), which creates a barrier to nematode establishment in the intestines (46, 47). Specific components of the mucus, such as the mucin Muc5ac can have a direct effect on the vitality of *T. muris*, as well as maintaining the intestinal mucus layer in a resistant composition (46). Resistin-like molecule-β (RELM-β) is also present in intestinal mucus, which can inhibit the migration of *H. polygyrus* and *Nippostronglyus brasliensis* to the intestinal wall so they are unable to feed, and this results in parasite stress and eventual expulsion (48). Mast cells are also important players in type 2 immune responses in the gut. For example, depletion of mast cell function with antibodies to stem cell factor (SCF) demonstrated that intestinal mast cell mastocytosis is essential for expulsion of the intestinal nematode *Trichinella spiralis* (49). Mast cell proteases have a further role in protective immunity by disrupting gut epithelial cell tight junctions to enhance luminal flow (50), and in conjunction with the IL-4R activated increases in intestinal smooth muscle contractility (51), they constitute the 'weep and sweep' response contributing to parasite expulsion from the intestinal lumen.

### *1.3.2 Type 2 immunity protects against disease pathology during trematode infection*

Protective immunity to *S. mansoni* infection is less clear-cut and here the Th2 response is more important to protect the host from damaging egg-induced immunopathology. The worms themselves are poorly immunogenic and the early Th1 bias induced by invading *S. mansoni* larvae gives way to a dominant Th2 response at approximately week 5 of infection when egg deposition begins (52). The target destination for the eggs is the external environment via the intestinal mucosa. However, some are carried by venous flow

to the liver where they are trapped in hepatic sinusoids (52). Trapped eggs become a focal point for the development of type 2 immune granulomas consisting of collagenous fibres and type 2 macrophages, eosinophils and $CD4^+$ T cells (52). Similar to the granulomas induced during *H. polygyrus* infection, the formation of *S. mansoni* egg induced granulomas is orchestrated by IL-4 from $CD4^+$ T cells (53-55). Paradoxically, the formation of type 2 granulomas can be both beneficial and detrimental to the infected host. A chronic granulomatous response and the accompanied fibrosis can eventually result in portal hypertension, the development of portosystemic shunts, intestinal bleeding and in some cases death. Therefore, granulomatous lesions are responsible for much of the disease morbidity and mortality during schistosomiasis (56). However, the formation of type 2 granulomas also benefits the host two-fold by containing dangerous egg hepato-toxins and suppressing inappropriate inflammatory Th1 immune responses (29). For example, in the absence of IL-4, a Th1 response develops in response to the eggs resulting in increased levels of the inflammatory mediators IFN-γ, TNF-α and nitrous oxide leading to greater morbidity and mortality (57). The suppressive cytokine IL-10 is also critically important for the regulation of pathogenesis during schistosomiasis and its ablation results in increased immunopathology denoted by larger granulomas associated with the development of a mixed Th1/Th2 response (58). Thus, an appropriately balanced type 2 immune response is required for *S. mansoni* infection, but in contrast to *H. polygyrus* infection, during *S. mansoni* infection the type 2 immune response functions not for worm expulsion but instead serves to protect the host from damaging immune pathology.

Whilst a robust Th2 immune response is required to protect against helminth infection, the extent of this response must be regulated to prevent detrimental immunopathology. However, as a consequence of immunoregulation sterilising immunity can be compromised and this in turn favours parasite survival and transmission. Thus, in a paradox similar to the type 2 *S. mansoni* egg-induced granuloma, immune regulation can

be both beneficial and detrimental to the infected host. However, to the invading parasite, regulation of host immunity appears to be only of benefit, serving to prevent its own immune ejection whilst protecting its requisite host from lethal immune pathology. Perhaps for this reason, helminth parasites have evolved to infiltrate the hosts own immunoregulatory network (12). The regulatory network is comprised of numerous types of immunosuppressive cells including $CD4^+$ $Foxp3^+$ Treg, regulatory B cells and suppressive macrophages. Acting in concert with these cells are the suppressive cytokines IL-10 and TFG-β, which can be produced by cells of both lymphoid and non-lymphoid origin. Within this network, $CD4^+$ $Foxp3^+$ Tregs are the most well characterised population of suppressive cells. Initially described for their ability to maintain peripheral tolerance, protecting against autoimmunity (59, 60) and suppressing chronic inflammatory diseases (61, 62), it is now known that $Foxp3^+$ Tregs are also induced by a wide variety of infectious pathogens, including helminth parasites. In summary, on one hand $Foxp3^+$ Tregs can benefit the host by protecting against over exuberant immune responses, but conversely, the suppressive effects of $Foxp3^+$ Tregs can be of detriment to the host, by limiting protective immunity to infectious pathogens (63, 64) and endogenous malignancies (65).

### *1.4 Regulatory T cell subsets*

Treg encompass a number of cell types with different phenotypes that are able to suppress effector immune responses (Fig 1.4.1). $Foxp3^+$ Treg are the principal subset of regulatory T cells and these can be either CD25 positive or negative (66, 67). $Foxp3^+$ Treg can be further subdivided into natural or adaptive $Foxp3^+$ Tregs. Natural Tregs develop in the thymus through high affinity major histocompatibility complex (MHC) class II-dependent T cell receptor interactions to produce a population of long lived antigen specific $Foxp3^+$ Treg in the periphery (68, 69). Adaptive $Foxp3^+$ Treg are generated outside the thymus during the normal homeostasis of the gut in response to TGF-β and

retinoic acid (70, 71), during chronic inflammation and following subimmunogenic antigen presentation (72). The two following experiments of nature have demonstrated that Foxp3 is a master transcriptional regulator for this class of Treg, being essential for their development and function: i) Spontaneous deletion of Foxp3 in mice resulted in the Scurfy phenotype characterised by a complete loss of Treg and fatal autoimmune lymphoproliferative disease (73, 74). ii) In humans, mutations in the Foxp3 gene results in the development of IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome) that presents with multisystem autoimmune disorders (75).

In addition to $Foxp3^+$ Treg, a number of other $Foxp3^-$ T cells are able to suppress immune responses. For example, IL-10 secreting T regulatory Type1 (Tr1) cells can be defined by their cytokine profile, producing high amounts of IL-10 along with TGF-β, IL-5 and variable amounts of IFN-γ and no IL-4 (76). The specific cytokine profile can vary depending on the conditions governing their generation but the hallmark of Tr1 cells is high IL-10 secretion. Tr1 cells suppress antigen specific effector T cell responses in a cytokine dependent manner, and unlike natural $Foxp3^+$ Treg which originate in the thymus, Tr1 cells are induced by antigen in the periphery (76). A relatively new type of $Foxp3^-$ Treg has been described which is generated under inflammatory conditions and suppresses effector immune responses through the cytokine IL-35, these cells have been termed $iT_R35$ (induced regulatory T cells IL-35) (77). Furthermore, T cells producing high levels of the suppressive cytokine TGF-β have been labelled Th3 cells, and whilst the majority of Treg are $CD4^+$, suppressive $CD8^+$ $Foxp3^+$ T cells have also been defined (78).

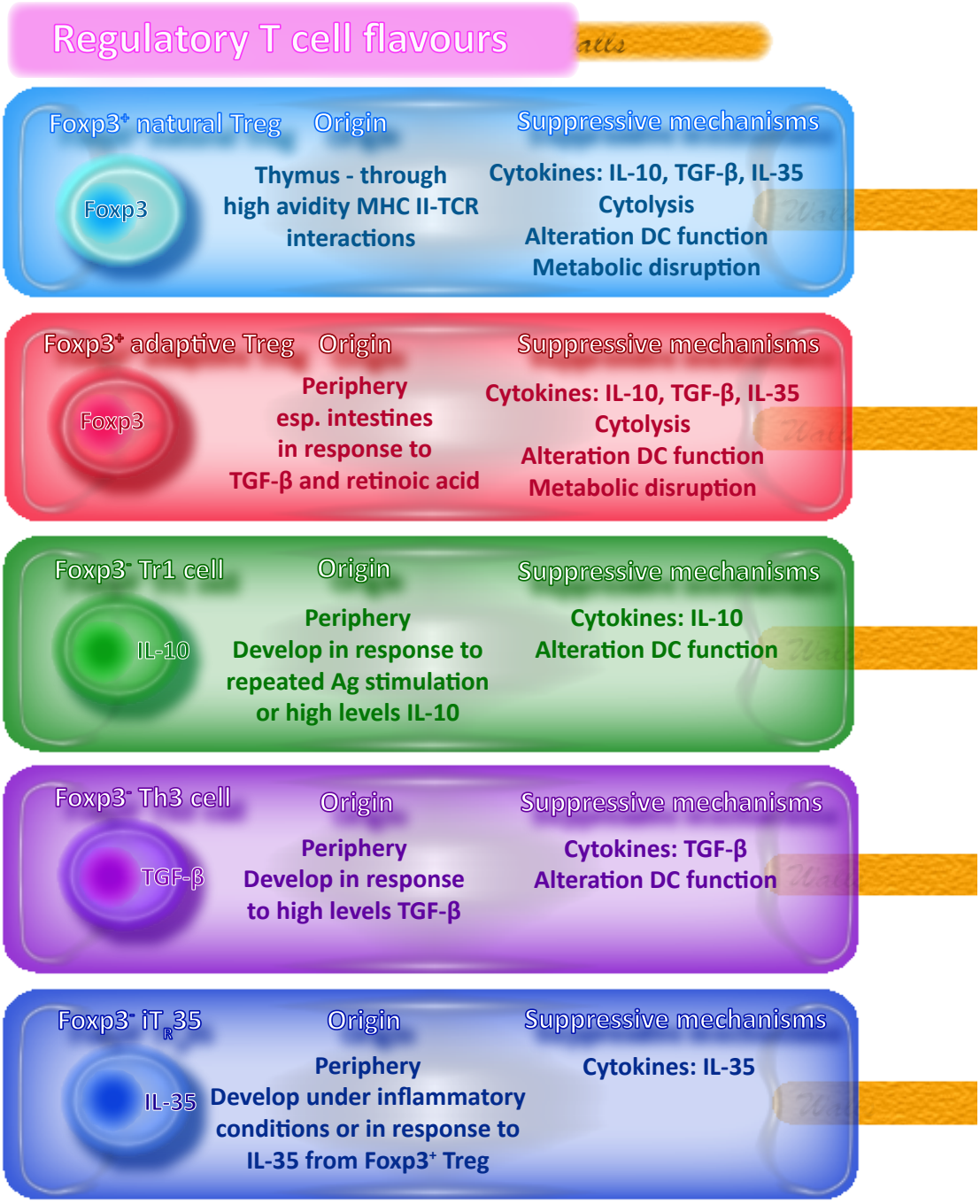



***Figure 1.4.1 Regulatory T cell subsets***

### *1.5 Regulatory T cell modes of immune suppression*

Foxp3$^+$ Treg can suppress the activation, proliferation and effector functions of a number of immune cell types including CD4 and CD8 T cells, B cells, natural killer (NK) and NKT cells, dendritic cells (DCs), macrophages and mast cells (79). To bring about suppression, Foxp3$^+$ Treg have been purported to utilise a number of mechanisms and these can be grouped into four main inhibitory processes; a) suppression by secretion of inhibitory cytokines, b) suppression by cytolysis, c) suppression by metabolic disruption and d) suppression by modulation or inhibition of antigen presenting cell (APC) function and maturation.

#### *1.5.1 Suppression by secretion of inhibitory cytokines*

IL-10, IL-35 and TGF-β are produced by Foxp3$^+$ Treg to suppress effector cell function in both autoimmune and infection settings, although the importance of these cytokines for Foxp3$^+$ Treg suppression is controversial and context dependent. The function of IL-10 as a Treg suppressive factor seems to be of most importance at the environmental interface of the mucosa. For example, IL-10 produced by Treg is essential for the prevention of colitis in the mouse transfer model of inflammatory bowel disease (80). Mice with a Foxp3$^+$ Treg specific IL-10 deletion spontaneously develop intestinal inflammation, and following induction of local antigen specific immune responses they exhibit increased inflammation and tissue pathology in models of lung and skin hypersensitivity (81). However, the Treg specific IL-10 deletion does not result in early onset systemic autoimmunity (81). Thus, the principal action of Treg IL-10 is to constrain hyperactive immunity at environmental interfaces. This has been further demonstrated in the *Leishmania major* model where Treg IL-10 contributes to the regulation of local epidermal effector cell function at the site of parasite inoculation promoting parasite persistence (82). Nevertheless, in a number of other infectious settings where Treg activity has been reported, the role of IL-10 produced

directly by Treg in suppression of immunity is less clear. In this regard, whilst the activity of Treg impedes the development of protective immunity to the Th1 inducing pathogens *Mycobacterium tuberculosis*, and *Toxoplasma gondii,* this occurs independently of IL-10 (83). Furthermore, in both human (84) and murine (85-88) helminth infections, $Foxp3^+$ Treg and IL-10 function separately in the suppression of host immune responses. Indeed, the principal source of IL-10 during helminth infections appears to be $Foxp3^-$ $CD4^+$ T cells of the Th2 (89) and/or Tr1 lineage (87, 90), which will be discussed later.

The role of TGF-β in Treg suppression has also been controversial, early *in vitro* studies showed that neutralisation of TGF-β did not reverse Treg suppression of effector cells suggesting that TGF-β is not required for natural Treg suppressor function (91). However, alternative evidence from *in vitro* studies suggests that Treg membrane tethered TGF-β is crucial for this process (92). Furthermore, a number of studies have demonstrated a role for Treg TGF-β suppression of effector immunity *in vivo*. $Foxp3^+$ T cells expressing TGF-β are essential for the inhibition of Th1 mediated inflammatory bowel disease in a transfer model of colitis (93). In a mouse model of diabetes islet derived Tregs express high levels of TGF-β on their surface which correlates with a delay in the onset of diabetes suggesting membrane bound Treg TGF-β is required for suppression of auto-reactive T cells (94). In addition, TGF-β produced by Treg has also been shown to impede anti-tumour immunity in head and neck squamous carcinoma (95), and in follicular lymphoma (96). Although evidence for a direct effect of Treg TGF-β in the suppression of effector immune responses during infectious disease is lacking, an increase in the levels of Foxp3 has been associated with greater TGF-β activity following *Plasmodium falciparum* infection which resulted in higher rates of parasite replication due to suppression of protective Th1 responses (97).

IL-35 is a Treg specific heterodimeric cytokine consisting of Epstein-Bar-virus-induced gene 3 (Ebi3, which encodes IL-27β) and IL-12α, which is required for Treg suppressive

function both *in vitro* and *in vivo* (98). In the intestines of *T. muris* infected mice, Foxp3$^+$ Treg produce high levels of IL-35 resulting in the generation of iT$_R$35, and tumour infiltrating Foxp3$^+$ Treg express high levels of IL-35 and can suppress the proliferation of tumour responder cells in vitro (77), suggesting that IL-35$^+$ Foxp3$^+$ Treg are active under inflammatory conditions and can further contribute to the suppression of effector immunity by generating Foxp3$^-$ iT$_R$35. Thus, the inhibitory cytokines IL-10, TGF-β and IL-35 are all key mediators of Treg function but the extent to which they are utilised by Treg can vary depending upon the nature of immune insult.

### *1.5.2 Suppression by cytolysis*

Tregs can directly kill Teff through release of granzymes and perforins which act together to perforate cell membranes and activate apoptotic pathways. Human Tregs can induce cytolysis of target effector cells through secretion of granzyme A and perforin to control immune responses (99). In mice, Treg secrete granzyme B to kill and suppress the activity of a number of immune target cell types including Teff (100) and B cells (101). Treg granzyme B cytolysis of NKT cells and cytotoxic T lymphocytes (CTLs) results in the suppression of antitumour immunity (102). Interestingly, in the context of human helminth infection, within the immunosupressed environment of onchocercoma nodules containing adult *O. volvulus* parasites, Foxp3$^+$ Tregs expressed granzyme A, albeit at low levels (103). Furthermore, mice deficient in both granzyme A and B, and granzyme B alone, showed increased resistance to infection with the filarial nematode *L. sigmodontis*, and this was associated with enhanced protective type 2 immune responses indicating impaired immune regulation in the absence of granzymes (104).

### *1.5.3 Suppression by metabolic disruption*

To impede target cell metabolism, Foxp3$^+$ Treg are thought to denude local concentrations of IL-2, possibly due to their high expression of the high affinity chain IL-2Rα (CD25), resulting in cytokine-mediated deprivation of effector cells and Bim-mediated apoptosis (105). Recently it has been suggested that Treg target the release both of intra and extracellular adenosine nucleotides. Treg cell surface expression of the ecto-enzymes CD39 and CD73 results in the generation of pericellular adenosine, which can suppress Teff cell function through activation of the adenosine receptor 2A ($A_{2A}R$) (106). Treg can also directly introduce the potent inhibitor of T cell proliferation and IL-2 synthesis cyclic AMP (cAMP) into effector cells via membrane gap junctions to impede Teff function (107). Intriguingly, *H. polygyrus* excretes an apyrase enzyme that hydrolyses ADP to cAMP suggesting that the parasite has directly hijacked a host mechanism to perturb the immune responses directed against it (108).

### *1.5.4 Suppression by modulation or inhibition of antigen presenting cell (APC) function and maturation*

To indirectly suppress Teff cell responses Foxp3$^+$ Treg interfere with antigen presenting cell function and/or maturation. For example, through a mechanism dependent on Treg surface expression of CTLA-4, DCs are conditioned to express indoleamine 2,3-dioxygenase (IDO) resulting in the production of pro-apoptotic metabolites from tryptophan catabolism and the suppression of effector T cells (109, 110). Treg may also disrupt DC expression of the co-stimulators CD80 and 86 resulting in fewer activating signals for Teff (111). New data suggests Treg block DC maturation through expression of the CD4 homologue LAG3 which binds MHCII on immature DC with high affinity initiating an inhibitory signalling cascade that blunts Dc maturation and immunostimulatory capacity

(112). Further, Treg, but not Teff, express neuropilin-1 (Nrp-1) that prolongs Treg-DC interactions giving Treg a competitive advantage over Teff for interactions with DC (113).

### *1.5.5 Bystander suppression and Infectious tolerance*

Other regulatory mechanisms used by Treg include bystander suppression and infectious tolerance. Bystander suppression refers to the ability of Treg to suppress in an antigen-nonspecific manner. Although initial activation of Treg requires TCR engagement with its cognate antigen, once activated, Treg can suppress effector T cells with TCR specificities to an array of antigens distinct to the initial Treg activating antigen. For example, transfer of Treg from *H. polygyrus* infected mice suppresses the development of OVA induced type 2 allergic airway inflammation, demonstrating that Treg activated by *H. polygyrus* can suppress immune responses initiated by OVA (88). The manifestation of infectious tolerance occurs when a particular population of regulatory cells influence the immune environment to induce the expansion of a new population of Treg with antigen specificities singular to the original inducing antigen. This can be demonstrated in the context of type 1 diabetes where transfer of *in vitro*, DC-expanded islet specific Treg into diabetic mice dampens diabetes symptoms through expansion of the recipient $Foxp3^+$ Treg repertoire (114).

## *1.6 The two sides of immune regulation in infectious disease*

The studies highlighted above show that Treg employ a multitude of mechanisms to suppress effector immunity, and that they can target not just Teff cells but a number of immune cell types to do so. Whether all of these mechanisms are in active use by Treg or if they exhibit a degree of redundancy remains to be resolved, but together they contribute to control peripheral immune homeostasis. The maintenance of a balanced immune response is not just important for tolerance to self-antigen, it is also crucial for host survival and the development of an appropriate immune response following challenge from

infectious pathogens, and in this context Foxp3$^+$ Treg play a leading role. In a number of infectious settings, Foxp3$^+$ Treg function to inhibit the collateral tissue damage that can result from over-prolific immunity. For example, depletion of Treg prior to ocular herpes simplex virus infection results in increased severity of viral immunological lesions indicating heightened immunopathology (115, 116). In addition, Treg deficient mice infected with the fungal pathogen *Candida albicans* are better able to clear Candida cells but paradoxically fail to survive the infection as a result of exaggerated Th1 mediated inflammation (117). Similarly, Tregs are an important factor in the control of immune pathology during infection with the protozoan parasite *Leishmania amazonia* (118). However, in this model the action of Tregs allows for better control of pathogen replication by down regulating the development of inflammatory leishmania specific Th1 cells (118).

In some instances, Treg can be of benefit to the infected host by contributing to the generation of a protective memory response. Following *Leishmania major* infection of mice, natural Treg suppress the activity of effector cells at the infection site and this impairs full eradication of the parasites, but in doing so they allow the persistence of antigen for long enough to generate a memory response that confers life long immunity to re-infection (82). However, the Treg response can also have a negative impact on the host when effective control of the pathogen is compromised allowing disease reactivation to occur. In this regard, transfer of Treg from *L. major* infected donor mice into chronically infected recipient mice can impede the protective memory response in the recipients and provoke disease re-activation (119). Moreover, over-zealous immunosuppression by Treg can lead to excessive pathogen replication which is detrimental to host survival. For example, depletion of Treg during infection with the lethal malarial strain *Plasmodium yoelli* protects mice from death by recovering an efficacious effector immune response that is able to eliminate the parasite (120). Therefore, the actions of Treg during infection are paradoxical in that they can be benefical in the control of immunopathology and the

maintenance of memory, but also detrimental when control of effector immunity is excessive and results in pathogen expansion. These dualistic actions of Treg have been described in bacterial, viral and fungal infections (63) but they are perhaps most prominent during parasitic helminth infections.

### *1.7 Evidence for Treg activity in human helminth infections*

#### *1.7.1 Filarial nematodes*

Immune suppression by helminth parasites was initially noted in studies of human filariasis in which T cells from infected individuals were characteristically hypo-responsive. For example, T cells isolated from patients infected with filarial parasites were unresponsive to *ex vivo* restimulation with parasite antigens and expressed high levels of the suppressive cytokine IL-10 (121-123) suggesting a regulated phenotype. Disease manifests in individuals infected with filarial worms in two categories: the first are asymptomatic individuals with circulating microfilariae, and the second are those with overt symptoms of disease such as lymphedema but no microfilariae. Infected patients that show no clinical symptoms of disease frequently possess high levels of the Treg associated antibody isotype IgG4 (123-126), suggesting that regulatory responses are associated with low pathology. Conversely, patients with the overt filarial disease symptoms of lymphedema and elephantiasis had high levels of the type 2 antibody isotype IgE (124), but reduced expression of Foxp3 and the Treg associated markers GITR, CTLA-4 and TGF-$\beta$ (127). Moreover, stimulation of lymphocytes from patients with patent filariasis with live *B. malayi* upregulated expression of Foxp3 indicating heightened Treg activity (128). Depletion of Treg from the peripheral blood mononuclear cells (PBMC) isolated from individuals infected with *B. malayi* recovered T cell responses to parasite antigens suggesting Treg actively suppress Teff cell responses in human filariasis (129). In addition, T cells producing high amounts of IL-10 (reminiscent of the Tr1 phentoype) have been isolated

from onchocercomas of onchocerciasis patients (130) suggesting that Treg are present in the host-parasite interface. More recently it has been shown that individuals from a region of Mali endemic for the filarial worms *W. bancrofti* or *Mansonella perstans* have expanded proportions of $CD4^+CD25^+Foxp3^+$ T cells, directly demonstrating that filarial nematode infections induce Treg in human populations (84).

### *1.7.2 Intestinal nematodes*

Peripheral $Foxp3^+$ Treg proportions are generally stable in intestinal nematode infected individuals, but it is possible that local increases in $Foxp3^+$ Treg occur. Despite the lack of evidence for changes in the peripheral Treg populations, there is evidence of heightened Treg activity in people harbouring intestinal parasite burdens. For example, T cells isolated from GI nematode infected children of a Tenek Indian community exhibited increased expression of CTLA-4, which is suggestive of immune suppression (131). In areas of Cameroon hyperendemic for *A. lumbricoides* and *T. trichuria*, lymphocytes isolated from infected children expressed high levels of IL-10 and TGF-β and were characteristically unresponsive to antigen indicating an immunoregulatory phenotype (132). Evidence for bystander suppression of immunity has come from humans in which intestinal helminth infections impede immunity to extraneous antigens (133). This bystander suppression can have negative consequences for vaccination against viruses and bacteria. Indeed, intestinal nematode infections are associated with reductions in immune responses to BCG (134) and cholera vaccines (135).

### *1.7.3 Schistosomiasis*

Immune regulation is also a key feature of human schistosome infection. In individuals infected with the trematode *S. hematobium,* their T cells are hypo-proliferative and produce reduced levels of IL-5 following re-stimulation with parasite antigen, demonstrating that cellular immune responses are depressed (136). Those individuals

who are employed in sand harvesting or car washing in Lake Victoria, and therefore occupationally exposed to *S. mansoni* infection, exhibited increased frequencies of Treg which were significantly decreased following drug cure, suggesting that Treg are expanded during human *S. mansoni* infection (137). A relationship between $Foxp3^+$ Treg activity and protective immunity in human schistosomiasis has been described in a study of *S. hematobium* infected Zimbabwean children; here, the ratio of $Foxp3^+$ Treg to activated effector cells increased only in younger children at an age susceptible to peak infection intensity. In older, more resistant children, the proportion of Treg declined with infection intensity (138). Thus, increased proportions of $Foxp3^+$ regulatory T cells are associated with increased susceptibility to *S. hematobium* infection (138).

### *1.8 Evidence for Treg activity in mouse models of helminth infection – immune protection versus prevention of pathology.*

Evidence of immune regulation in human helminth infection has resulted in extensive investigations into the mechanisms of helminth induced regulation in mice. In mouse models, diverse helminth species have been shown to promote Treg expansion, proliferation, activation and suppressive function. Further, these helminth induced murine Treg can directly inhibit protective immunity, immune pathology and suppress bystander immune responses to allergens. Indeed, the opposite ends of the spectrum of host consequences resulting from Treg activity have been observed in murine helminth infections. In this regard, in mouse models of filarial nematode infection, Treg can prevent the development of protective immunity and their depletion reverses immune suppression and recovers Th2 effector immune responses without the danger of incurring immunopathology (13-15). During intestinal nematode infections, removal of Treg can result in stronger Th2 immunity and enhance parasite eradication but at the risk of developing damaging pathology at the site of infection (17). At the far end of the scale, depletion of Treg during blood trematode infection can boost Th2 effector responses but

this has no effect on parasite clearance and comes at the cost of damaging immune pathology (16).

### *1.8.1 Host immune protection - Filarial nematodes*

Infection of BALB/c mice with filarial L3 stage *Brugia pahangi* results in expansion of $CD25^+$ expressing Foxp3 and these cells are enriched for the cytokine IL-10. Depletion of Treg with antibodies to CD25 restores cellular responsiveness *in vitro* demonstrating that filarial induced $Foxp3^+$ Treg actively suppress the Th2 immune response (139). Similarly, $Foxp3^+$ Treg expand and become activated in response to both the larval and adult stages of the filarial nematode *B. malayi*. This only occurs following infection with live parasites suggesting that induction of Treg is an active process. In addition, infection with *B. malayi* results in Foxp3 expression in naive T cells specific for an irrelevant antigen indicating that filarial nematodes elicit bystander Treg responses (140). Filarial infection with *L. sigmodontis* invokes an early wave of $Foxp3^+$ Treg proliferation and activation that inhibits early type 2 priming allowing establishment of infective larvae (15). Depletion of Treg prior to larval inoculation bolsters protective immunity during the later stages of infection (15). However, removal of Treg following establishment of infection is not sufficient to recover protective immunity. During active infection, both Treg cells and Teff cells must be targeted to recapitulate immunity; this is achieved by combining Treg depletion with boosting of co-stimulatory signals to supplement the action of effector cells (13, 14). Similar to *B. malayi* infection, *L. sigmodontis* infection can suppress immune responses to non-parasite antigens and in doing so inhibit the development of allergic airway inflammation (141).

### *1.8.2 Protection and pathology - Intestinal nematodes*

Expansion and activation of Treg is also a common feature of mouse models of intestinal nematode infection. In this context Treg have been shown to block the development of protective immunity, but at the same time they protect against tissue damage that can

result from vigorous effector responses. *H. polygyrus* infection results in the development of a strong regulatory response in the GALT (31, 32). This is characterised by an increase in the number of highly activated $CD25^+CD103^+$ Treg, and these cells are highly suppressive *in vitro* (31). Treg from *H. polygyrus* infected mice are also highly suppressive *in vivo* protecting mice from atopic inflammation in the airways of the lung. Here, transfer of Treg from the MLN of *H. polygyrus* infected mice prevented inflammatory cell infiltrate and eosinophilia in the lungs following experimental induction of allergy with ovalbumin or the dust mite allergen Der p 1 (88, 142). Further, *H. polygyrus* infection impedes colitic inflammation in IL-10 deficient mice suggesting that *H. polygyrus* induced Treg suppress colitis independently of IL-10 (143). Intriguingly, *H. polygyrus* excretory-secretory products (HES) convert naïve T cells into $Foxp3^+$ adaptive Treg *in vitro* in a manner dependent on TFG-β signalling (144). HES induced Treg are functionally suppressive *in vivo* inhibiting effector cell proliferation and airway induced inflammation (144). Thus, helminth products can directly generate Treg from naïve T cells to bring about suppression of host immunity.

Furthermore, inhibition of TGF-β signalling during *H. polygyrus* infection resulted in reduced parasite burdens (144), and because this intervention targeted the induction of Treg during late stage infection it could be argued that adaptive Treg contribute to suppression of protective immunity. Early depletion of Treg during *H. polygyrus* infection does not significantly reduce worm burdens despite enhanced type 2 cytokine production and effector cell activation in the local lymph node (17). Instead, removal of Treg resulted in the development of pathology at the infection site highlighting the requisite for Treg control of tissue damage during intestinal nematode infection (17). However, expulsion of *H. polygyrus* adult worms in naturally resistant mice is not evident until day 21 or 28 of infection and in this study enumeration of worms at day 14 may have pre-empted later expulsion. Similar to depletion of Treg during *H. polygyrus* infection, anti-CD25 treatment during infection with the Treg centric S isolate of *T. muris* had no effect on worm burden

but led to the development of increased gut pathology, further demonstrating the importance of Treg in the suppression of immune pathology during intestinal nematode infection (145). Interestingly, in this model blockade of GITR enhanced Th2 immunity and significantly reduced worm burden suggesting that inhibiting co-stimulatory activity in Treg can re-invigorate protective immune responses (145). However, whilst GITR blockade successfully reduced the parasite load, like CD25 depletion, this was at the expense of enhanced intestinal pathology (145). Depletion of Treg during intestinal nematode infection can in some cases successfully recover protective immunity without incurring pathology. For example, depletion of Treg prior to and during the initial stage of *Strongyloides ratii* infection results in a dramatic reduction in intestinal worm burden associated with an increased type 2 response and mast cell degranulation, but without the development of excessive immunopathology (146). Therefore, Treg cells actively suppress protective immune responses during intestinal nematode infection and inhibit worm clearance but at the same time act to protect the host from damaging pathology.

### *1.8.3 Prevention of pathology – trematode infection*

In mouse models of *S. mansoni* infection Treg serve primarily to protect the host from egg-induced immunopathology. Following the onset of egg deposition, $Foxp3^+$ Treg at the site of infection and within the draining lymphoid organs develop an activated phenotype and limit the production of egg-induced Th2 cytokines (85). Indeed, $Foxp3^+$ Treg expressing activation associated genes such as granzyme B and neuropilin-1 are present within the egg-induced granuloma (147). When Treg populations are diminished during *S. mansoni* infection, either by the absence of Toll-like receptor (TLR)-2 mediated priming or anti-CD25 treatment, egg- induced hepatic immunopathology is exacerbated (16). Conversely, over expression of Foxp3 by retroviral transfer actively suppresses the development of hepatic immune granulomas during the egg-phase of *S. mansoni* infection (148).

Accordingly, Foxp3$^+$ Treg are an essential component in the control of *S. mansoni* egg-induced hepatic pathology.

Foxp3$^+$ Treg are not, however, exclusive suppressors of hepatic immunopathology during acute *S. mansoni* infection. Much of the control of egg-induced granulomas has been attributed to the suppressive effects of IL-10 (30). In this respect, immune suppression by IL-10 is superior to that of Foxp3$^+$ Treg in that lethal egg-induced immunopathology only occurs when Treg are depleted in the absence of IL-10 (115). Whilst IL-10 production is cited as a principal mechanism of Treg suppressive function (149), and both IL-10 and Foxp3 have been implicated in the down-regulation of anti-helminthic immune responses, the two appear to act independently during *S. mansoni* infection (85). In *S. mansoni* infection, the main sources of IL-10 are most likely activated Th2 cells expressing CD25 and/or Foxp3$^-$ IL-10$^+$ CD25$^+$ CD4$^+$ T cells with characteristics of Tr1 cells (87, 150).

### *1.8.4 Evidence for Tr1 cells in helminth infections*

Foxp3$^-$CD4$^+$IL-10$^+$ cells are also key suppressors of immune inflammation in other helminth infections. During *T. spiralis* infection, inflammatory immunity and tissue pathology was tempered by IL-10 from CD25$^-$CD4$^+$ effector T cells (86). In filarial infected humans, CD4$^+$ CD25$^-$ putative Tr1 cells are the dominant source of IL-10 (90). Similarly, T cell clones from onchocerciasis patients secreted high amounts of IL-10 and had a Tr1 phenotype (151). Reacting to *S. mansoni* egg antigens, Foxp3$^-$CD25$^+$CD4$^+$ T cells expressing the chemokine receptor CCR8 were the foremost producers of IL-10 (150). In addition to Tr1 cells, the appearance of Foxp3$^-$CD4$^+$ IL-35 producing T cells during *T. muris* infection (152) suggests that iT$_R$35 may be important in the control of helminth-induced immune responses.

### *1.9 T cell activation requires two signals*

The above studies demonstrate that the generation of a properly regulated immune response is essential to maintain homeostasis during helminth infection, striking the balance between protective immunity and damaging immunopathology. To generate an immune response, naïve T cells must first be activated by cognate antigen in the presence of non-cognate co-stimulatory signals before they can begin to proliferate and differentiate. However, it is now understood that co-stimulators do not just act as a molecular on-off switch for T cells, but instead function as a complex signalling network of receptors and ligands that can quantitatively and qualitatively configure immune responses, affecting T cells, B cells and DCs. CD28 was the first T cell co-stimulator to be characterised and recognised as the prototypical co-stimulatory molecule in that its ligation allows TCR generated signals to result in effective T cell activation, and in its absence a state of anergy ensues that cannot be recovered by further stimulation, even in the presence of other co-stimulatory molecules (153). Both resting and activated T cells express CD28 and following engagement with one of its two ligands (CD80 and CD86 which are only expressed by APCs) the signals generated drive T cell proliferation, survival, differentiation and cytokine production (154). Since the initial description of CD28 the field of co-stimulation has expanded dramatically with the discovery of many additional co-stimulatory pathways, the relative importance of which depends on the T cell subset, activation status, tissue location and the particular stage of the immune response. For, example, alongside CD28, the members of the tissue necrosis factor receptor (TNFR) family GITR, OX-40, CD40L and 4-1BB provide important signals for a complete T cell response (155). Furthermore, the discovery of inhibitory co-stimulators that can have antagonistic effects on the immune response added to the complexity of the co-stimulatory domain. Accordingly, the CD28 family of co-stimulatory receptors now includes the inhibitory co-

stimulators cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-ligand 1 (PD-1) (156).

### *1.9.1 Negative co-stimulatory signals inhibit T cell action*

CTLA-4 shares the same ligands as CD28, but unlike CD28 which is constitutively expressed by T cells, CTLA-4 is only upregulated after T cell activation and delivers a negative signal antagonizing the positive signals of CD28 (157). CTLA-4 signalling inhibits T cell IL-2 synthesis, cell cycle progression and proliferation so that ligation of CTLA-4 results in the dissolution of T cell responses (157). The important contribution of negative co-stimulatory signals to the control of peripheral tolerance is dramatically demonstrated in Ctla-4$^{-/-}$ mice who succumb to a fatal lymphoproliferative disease a few weeks after birth (158). Moreover, mutations in the CTLA-4 gene have been associated with increased susceptibility to a variety of autoimmune diseases in mice and humans (159). CTLA-4 signalling can also inhibit the development of pathogenic Th17 responses. For example, antibody blockade of CTLA-4 results in increased Th17 differentiation and increased IL-17 production *in vitro* and *in vivo* (160). Importantly, Teff cell expression of CTLA-4 can impair anti-tumour immunity and lead to tumour progression (161) so that targeting CTLA-4 signalling may prove an effective strategy for cancer immunotherapy.

Similar to CTLA-4, the primary role of PD-1 is to attenuate effector immune responses and when the suppression of immunity by PD-1 is lifted it leads to the development of autoimmunity. Indeed, deletion of PD-1 in diabetes prone mice speeds the development of disease (162). In addition, blockade of PD-1 and PDL-1 interactions following transplantation results in the break down of immune tolerance and accelerates graft rejection (163). Furthermore, PD-1 signalling is implicated in the attenuation of anti-tumour immunity (164), and tumour cells themselves have been shown to express one of the ligands of PD-1 to suppress immunity (165). Disruption of this interaction can recover

tumour specific immune responses and improve survival rate (164, 165). In the context of helminth infection, inhibtion of PD-1 signalling during *L. sigmodontis* infection reverses the hypo-responsive state of Th2 cells to promote protection to infection (Dr N. Van der Werf, Dr Matthew Taylor, submitted manuscript). Alongside CTLA-4 and PD-1 a number of new coinhibitory pathways have now been described. For example, B7-H3 is a member of the B7 family of co-stimulators that is expressed by T cells, DCs and monocytes (166). The ligand of B7-H3 is proposed to be the triggering receptor expressed on myeloid like cells (TREM)-like transcript 2 (TLT2), which is expressed by $CD4^+$ and $CD8^+$ T cells after antigen activation. Treatment with an antagonistic B7-H3 mAb leads to early onset experimental autoimmune encephalomyelitis (EAE) and more severe clinical disease associated with increased brain infiltrating $CD4^+$ T cells (167), and B7-H3 deficiency can lead to greater airway inflammation under Th1 polarising conditions (168) suggesting a role for B7-H3 in negative regulation of T cell responses. B7-H4 is a recent addition to the B7 family associated with downregulation of T cell responses. Stimulation of B7-H4 on the NOD background reduces insulitis and increased pancreatic $Foxp3^+$ Treg infiltration suggesting B7-H4 contributes to suppression of autoinflammation (169). Other inhibitory molecules include B and T cell lymphocyte and attenuator (BTLA-4), herpesvirus entry mediator (HVEM) and LIGHT which can interact with each other with HVEM acting as a central regulator that can direct negative or positive signals depending on ligand binding (166). CD160 can also bind HVEM but little is yet know regarding the function of this interaction (166). In summary, inhibitory co-stimulatory signals counter the effects of positive co-stimulators to shut down T cell responses and mediate tolerance, but in some cases suppression of host immunity through co-inhibition impairs immunity to infectious pathogens and host malignancies.

### *1.10 Co-stimulatory signals drive Treg generation and function*

Similar to Teff, Treg express an antigen specific receptor and are dependent on co-stimulatory signals for their development and function (157, 170, 171), and in this regard co-stimulatory molecules originally shown to promote T cell responses are now also implicated in negative immune regulation, whereas inhibitory signals from CTLA-4 and PD-1 can potentially augment immunity by shutting down Treg. As is the case for Teff, the most well characterised Treg co-stimulator is CD28 and its importance as a regulator of Treg homeostasis attributes this positive co-stimulator with the capacity to maintain peripheral tolerance. This was initially observed following CD28 deficiency on the Non-obese diabetic (NOD) background, which results in the development of an aggressive form of diabetes that was associated with a dramatic loss of Treg in the periphery (171). Control of disease could be restored by addition of WT Treg, demonstrating that CD28 signalling is critical to maintain Treg populations and prevent auto-immune disease (171, 172). Furthermore, blockade of the CD28 pathway leads to a rapid reduction in the proportion and numbers of peripheral Treg (173) highlighting the importance of CD28 in the maintenance of Treg survival in the periphery. In addition, CD28 is also essential to promote Treg cell proliferation and expansion *in vitro* (174) and *in vivo* (173). Thus, CD28 is emerging as an important mediator of Treg development and function and in this manner contributes to the maintenance of tolerance in the peripheral tissues. However, the role of other co-stimulatory molecules in this regard is less clear, and the effects of other co-stimulatory molecules on Treg function are still being explored and in some cases remain controversial. For example, some studies have shown that the TNFR family members OX-40L and 4-1BB can promote the generation, survival or expansion of Treg (175-178), yet other studies suggest that OX-40L and 4-1BB signalling inhibit the generation of Tregs, or that they alter Treg suppressive function (179-181). Other members of the TNFR family, such as CD40L and GITR, have also been proposed to

contribute to the homeostasis and function of Treg, but they may have a greater influence on Teff than Treg (182-185). Indeed, GITR expression can render effector cells more resistant to suppression (186), which is consistent with the co-stimulatory activity of GITR. Therefore, studies are beginning to show that co-stimulatory molecules are of principal importance in regulating the actions of Treg, but much remains to be learned regarding the contribution of some of the more recent additions to the co-stimulatory families. Because Treg are fundamental to the control of autoimmune disease and allergy, and for the suppression of host immunity and pathology during helminth infection, and understanding of the co-stimulatory mechanisms governing their induction and function is essential for the development of new therapeutics to treat these disorders. Recently, the third member of the CD28 family of co-stimulatory receptors, the inducible co-stimulator (ICOS), has been implicated in the development and function of Treg in the context of autoimmune disease, but little is known of the mechanisms underpinning its contribution to Treg, and it is not known if ICOS is required for the Treg generated during infectious disease.

### *1.11 ICOS biology*

Similar to CD28, ICOS delivers positive costimulatory signals following engagement of its cognate ligand, but whilst the expression of CD28 is constitutive, ICOS is expressed at very low levels by naïve T cells and is only upregulated following T cell activation (187, 188). T cell ICOS expression has been shown to be enhanced by CD28 signalling (189). However, the presence of functional ICOS proteins on the surface of $CD28^{-/-}$ T cells suggests that upregulation of ICOS expression can occur independently of CD28 (190). Only one ligand for ICOS has been described, ICOS-L, which is constitutively expressed as a monomer on B cells, DCs, monocytes/macrophages, fibroblasts, endothelial cells and renal epithelial cells (191-195). Human monocytes can up-regulate the expression of ICOS-L in response to IFN-$\gamma$, but not to lipopolysaccharide (LPS) or CD40 ligation (191).

However, ICOS-L expression is up-regulated by murine fibroblasts following exposure to TNF-$\alpha$ and LPS (196). On B cells, the expression of ICOS-L is downregulated in response to B cell receptor engagement or IL-4R signalling; this BCR/IL-4 mediated downregulation can be reversed via CD40 ligation (192).

### *1.12 ICOS drives T cell effector responses*

Initial studies into the effects of ICOS co-stimulation on T cells described a role for ICOS in T cell proliferation and secretion of both Th1 and Th2 effector cytokines (188, 189, 197). Expression of ICOS was notably high on T cells within B cell follicles (187) and analysis of ICOS deficient mice demonstrated that the ICOS pathway is essential for T-B cell interactions (198, 199). Further work has gone on to show that ICOS is an important co-stimulator for the all major T cell effector subsets including Th1, Th2, Th17 and Tfh (200-204). In addition, novel innate helper cells induced by the cytokines IL-25 and IL-33 have been shown to express high levels of ICOS (34), the significance of which has yet to be described. Importantly, recent evidence has attributed ICOS to the action of Treg cells, including Tr1 cells (205, 206), Foxp3$^+$ Treg (207, 208) and most recently iT$_R$35 (209).

#### *1.12.1 ICOS signalling and Th1 immunity*

Early *in vitro* work showed that stimulation of ICOS resulted in the production of the Th1 associated cytokines IFN-γ and TNF-α, but also the Th2 cytokines IL-4, IL-5 and IL-10 (199) highlighting that ICOS is not specific to one type of immune response. *In vivo*, ICOS signals collaborate with CD28 to contribute to the Th1 response to Lymphocytic choriomeningitis virus (LCMV) infection (201). Similarly, ICOS deficiency leads to reduced IFN-γ from CD4$^+$ T cells during infection with *Salmonella enterica*, demonstrating that ICOS promotes Th1 responses to bacterial infections (202). Indeed, in human mycobacterium infection, ICOS expression correlated with T cell IFN-γ production and ICOS ligation augmented IFN-γ secretion (210). In a transfer model of colitis, stimulation of

intestinal $CD4^+$ T cells with ICOS-L induced high levels of IFN-γ production, and blockade of ICOS in the absence of CD28 prevented disease progression suggesting that ICOS contributes to Th1 mediated auto-inflammation (200). The protozoan parasite *Toxoplama gondii* polarises a strong Th1 response that controls parasite replication. During *T. gondii* infection, $CD4^+$ effector T cells producing IFN-γ have elevated ICOS expression (211); following ablation of ICOS signals, *T. gondii* antigen specific T cell IFN-γ production declined, indicating that ICOS contributes to Th1 immunity during parasite infection (203). Taken together, these observations suggest that the ICOS pathway is important for Th1 immune responses in both diverse infection models and settings of Th1 mediated auto-immunity.

### *1.12.2 ICOS signalling and Th2 immunity*

ICOS co-stimulation also aids polarisation and maintenance of Th2 immunity in both infection settings and allergic disorders. At the molecular level, ICOS signalling promotes expression of the Th2 associated transcription factors c-maf (212) and NFATc1 (213) and in doing so contributes to early IL-4 production following T cell activation *in vitro* (212). In addition, ICOS ligation enhances IL-4R signalling resulting in the phosphorylation and activation of the master regulators of Th2 differentiation, STAT-6 and GATA-3 (214). Thus, ICOS has been shown to contribute to early in vitro Th2 polarisation through enhancement of IL-4 signalling and induction of Th2 associated transcriptional regulators.

*In vivo* work has demonstrated a role for ICOS in the maintenance of Th2 responses in models of allergic airway inflammation. In ICOS deficient mice, following induction of Th2 airway inflammation using either OVA or *S. mansoni* eggs and egg antigen, lung T cells stimulated *ex vivo* produced severely diminished amounts of the key Th2 cytokines IL-4 and IL-13 (197, 215). A more intricate dissection of the effects of ICOS on Th2 airway inflammation was achieved using an ICOS blocking antibody at different stages of the Th2

response. *In vivo* blockade of ICOS during the priming phase of OVA induced airway inflammation had no effect on bronchiolar lavage (BAL) Th2 cytokines IL-4, IL-5. IL-10 and IL-13, but in contrast if ICOS was blocked once airway inflammation was established then BAL Th2 cytokines were significantly reduced (216). Similarly, ICOS blockade during *S. mansoni* egg sensitisation in an SEA model of Th2 driven airway allergy did not affect lung T cell cytokine production. However, if ICOS was blocked *in vitro* following *ex vivo* isolation of lung T cells then Th2 responsiveness was significantly reduced (217). This is in contrast with *in vitro* work suggesting ICOS aids early Th2 polarisation, and instead implies that ICOS is dispensable for Th2 priming *in vivo* but that it is required for an optimal Th2 response, once this has been established.

The ICOS pathway has also been shown to be important for the Th2 response to helminth infection. During *S. mansoni* infection, ICOS expression was associated with $CD4^+$ effector T cells secreting Th2 cytokines in the liver (211). Following infection with the GI nematode *N. brasiliensis* the levels of IL-4, IL-5, IL-10 and IFN-γ increase in the local lymph node. In this model, antibody blockade of ICOS signalling reduced the levels of both Th1 and Th2 cytokines, demonstrating that ICOS contributes to the development of a complete Th2 cell effector response (201). In addition, infection of $ICOS^{-/-}$ mice with *T. muris* results in decreased lymph node IL-4 and IL-13, whilst the levels of IFN-γ are increased (203). Furthermore, in mice infected with the GI nematode *T. spiralis,* treatment with an anti-ICOS antibody reduces levels of IL-4 and IL-5, yet IFN-γ production is increased (218). These studies highlight the importance of the ICOS pathway in the development of full Th2 effector responses in the lymphoid tissue following intestinal nematode infection.

T cells are not the only players in type 2 immunity and a number of innate effector cells can contribute to the induction, maintenance and regulation of the type 2 responses. New amongst these innate cells are the innate helper 2 cells (IHCs) which loosely encompasses a group of lineage negative cells which produce type 2 cytokines in response to the epithelial cell derived cytokines IL-25, IL-33 and TSLP (219). In the context of helminth infection, IHCs have been identified during infection with the intestinal nematodes *N. brasiliensis* (34, 35) and *T. muris* (36), and by providing an early source of IL-13 they are essential for the development of a competent type 2 response and parasite expulsion (34). Moreover, IL-13 is one of the key cytokines in the development of allergic asthma, and innate helper 2 cells have now been shown to be a dominant source of IL-13 during allergic lung inflammation, being sufficient to induce asthma in the absence of IL-13 producing $CD4^+$ T cells (220). Interestingly, IHCs can be identified on the basis of a number of cell surface markers including ICOS (34). However, the functional significance of ICOS expression by IHCs is not known.

### *1.12.3 ICOS signalling and Th17 immunity*

Th17 cells are important for the protection against of bacterial and fungal pathogens, but they also inadvertently mediate autoimmune diseases such as EAE, inflammatory bowel disease and collagen-induced arthritis (221). ICOS has been shown to contribute to T cell IL-17 production both *in vitro* and *in vivo* following stimulation with antigen (222). Mechanistically, ICOS has been shown to maintain Th17 responses through activation of the transcription factor c-maf which leads to IL-21 production (204). IL-21 enhances expression of the IL-23 receptor which acts on previously differentiated Th17 cells to maintain their phenoytpe (223). The demonstration that ICOS is required for the maintenance and function of Th17 cells implicates this co-stimulator in immunity to certain microbes, and in the development of autoimmune disease. Interestingly, similar to its role in Th2 driven allergic airway inflammation, ICOS has different effects on the pathogenesis

of the Th17 mediated disease EAE depending on the kinetics of ICOS blockade. Blockade of ICOS at a stage of EAE when immune priming is thought to occur exacerbated symptoms of disease, suggesting a regulatory role of ICOS at this stage (224). Conversely, blockade of ICOS during the efferent phase of EAE ameliorated disease pathology. Thus, in line with *in vitro* work, during EAE, ICOS maintains Th17 effector function once this response has been established and in doing so enhances the severity of disease (224).

### *1.12.4 ICOS signalling and Tfh*

ICOS expression was first noted in the follicular regions of the lymph nodes and in human tonsils. The initial characterisation of ICOS deficient mice showed defective germinal centre formation and poor T cell dependent antibody responses (198, 199, 225). Similarly, ICOS deficiency in humans has been associated with the development of common variable immuno-deficiency, characterised by a severe reduction in serum immunoglobulin, impaired specific antibody production and increased susceptibility to bacterial infections of the GI and respiratory tracts (226). Further investigations into ICOS deficient patients demonstrated that the formation of germinal centres is dramatically impaired and the proportion of circulating $CXCR5^{+}CD4^{+}$ germinal centre T cells is severely diminished (227). These observations are mirrored in $ICOS^{-/-}$ mice, where ICOS deficient T cells fail to up-regulate CXCR5 resulting in reduced numbers of Tfh and impaired germinal centre (GC) formation, demonstrating the dependency of $CXCR5^{+}CD4^{+}$ follicular helper T cells on ICOS co-stimulation (227). Mechanistically, it has been shown that ICOS is required for the production of the key Tfh cytokine IL-21 (204, 228), and that B cell expression of ICOSL is necessary for IL-21 production (229). Furthermore, ICOS signalling is critical for the induction of the Tfh master transcription factor Bcl6 suggesting a role for the ICOS pathway in the lineage commitment of Tfh (230). In contrast to ICOS deficient mice, in Sanroque mice, which have an inactive form of Roquin that degrades ICOS

mRNA leading to over expression of ICOS (231), increased ICOS signalling leads to increased IL-21 production, greater expansion of Tfh and the development of a autoimmune lupus-like syndrome due to high titres of auto-antibodies (232). Thus, ICOS is essential for the development, maintenance and function of Tfh cells. Moreover, the Tfh requirement for ICOS implicates ICOS as an important mediator of anti-helminth type 2 immunity following the demonstration that Tfh are an important source of IL-4 during both *H. polygyrus* infection (40) and *S. mansoni* egg immunisation (233). Whether ICOS is required for Tfh cell expansion during helminth infection has not been investigated.

### *1.13 ICOS co-stimulation and regulatory T cells*

Numerous lines of evidence have demonstrated that ICOS is an important positive co-stimulator of $CD4^+$ T cell immune responses, and that the functional significance of ICOS expression is not limited to a particular Th subset, rather ICOS promotes Th1, Th2, Th17 and Tfh cell responses once they have been initiated. However, it is clear that some T cell subsets show a greater degree of dependency on ICOS signalling, as evidenced by the strict requirement of Tfh for ICOS co-stimulation. In contrast to its role in driving positive immune responses, relatively little is yet known in regard to the importance of ICOS expression on Treg. ICOS is expressed at higher levels on $Foxp3^+$ Treg than $Foxp3^-$ Teff (207) suggesting that the reliance of the $Foxp3^+$ Treg subset on ICOS signalling exceeds that of $Foxp3^-$ Teff. Thus, as a positive co-stimulator of Treg responses the ICOS pathway can also potentially act as a negative regulator of immunity, but the manner in which ICOS contributes to $Foxp3^+$ Treg responses is not known.

#### *1.13.1 ICOS signalling and $Foxp3^+$ Treg*

A growing body of evidence has suggested ICOS is of importance to $Foxp3^+$ Treg. For example, under homeostatic conditions, ICOS is expressed at high levels on $Foxp3^+$ Treg, and $ICOS^{-/-}$ mice harbour 30% fewer $Foxp3^+$ Tregs than WT mice, demonstrating the

importance of ICOS for Foxp3$^+$ Treg (207). The Foxp3$^+$ Treg reduction in ICOS$^{-/-}$ mice is specific to the periphery, and thymic proportions of Foxp3$^+$ Treg are intact suggesting that ICOS is dispensable for Treg thymic generation but that it contributes to their maintenance in the periphery (207). In humans, Foxp3$^+$ Treg can now be subdivided on the basis of ICOS expression, and both ICOS$^+$Foxp3$^+$ Treg and ICOS$^-$Foxp3$^+$ Treg exist within the thymus and peripheral lymphoid tissues. The two populations mediate suppression through distinct molecular mechanisms; ICOS$^+$ Treg utilise IL-10 in conjunction with TGF-β, whilst ICOS$^-$ Treg suppress through TGF-β alone (234). It was further demonstrated that two separate dendritic cell lineages govern the *in vitro* proliferation of ICOS$^+$ and ICOS$^-$ Foxp3$^+$ Treg. Plasmacytoid dendritic cells (pDCs) preferentially promoted the proliferation of ICOS$^+$Foxp3$^+$ Treg in a manner dependent on ICOS-L, whereas the proliferation of ICOS$^-$Foxp3$^+$ Treg was selectively promoted by myeloid dendritic cells (mDCs) through the co-stimulatory ligands CD80 and CD86 (234). Taken together, these observations suggest that ICOS is important for the maintenance of Foxp3$^+$ Treg in the periphery under homeostatic conditions.

### *1.13.2 ICOS and Tr1 cells*

ICOS co-stimulation is also important for the development and function of Tr1 cells. For example, T cells expressing ICOS at high intensity are potent producers of IL-10, which is a phenotype associated with Tr1 cells (235). In transgenic mice that express ovalbumin under the control of the rat insulin promoter, high levels of ovalbumin expression resulted in the development of hyporesponsive CD4$^+$ IL-10 producing cells, which expressed high levels of ICOS. Following ICOS blockade, the production of IL-10 was abrogated, and the ability of these cells to suppress T cell proliferation was lost (206). In a mouse model of asthma, adoptive transfer of IL-10$^{high}$ T cells inhibited the development of airway hyper-reactivity (AHR). Treatment with an anti-ICOS antibody reversed IL-10 dependent

tolerance and led to the development of severe AHR (205). Indeed, the generation of IL-10 producing Tr1 cells is mediated through pDCs in a manner dependent on ICOS-L (236). Thus, ICOS is required for the development and function of Tr1 cells.

### *1.13.3 ICOS controls Treg function in in vivo models of autoimmunity*

The requirement for ICOS in the function of regulatory cells has been demonstrated in a number of autoimmune models. For example, in BDC2.5 T cell transgenic mice expressing a T cell receptor (TCR) derived from a diabetogenic $CD4^+$ T cell clone, pancreatic Treg express high levels of ICOS and IL-10. Treatment with an anti-ICOS antibody disrupted the ratio of IL-10 producing Treg to Teff cells allowing the rapid development of diabetes (237). Further work with these mice showed that ICOS was preferentially expressed by islet $Foxp3^+$ Treg, and expression of ICOS endowed Treg with enhanced suppressor function and IL-10 production. In addition, ICOS deficient Treg were unable to inhibit the induction of diabetes in a transgenic transfer model suggesting that $ICOS^{-/-}$ Treg fail to control auto-reactive Teff cells (208). As previously mentioned, the observation that blockade of ICOS during the antigen-priming phase of EAE exacerbated disease symptoms could be attributed to an ICOS dependent Treg defect, resulting in an enhanced effector response and subsequent immune pathology (224). Together, these observations suggest that ICOS is required for $Foxp3^+$ Treg suppressive function in vivo and in doing so contributes to the maintenance of peripheral tolerance.

### *1.13.4 Evidence for ICOS in human Treg function*

In support of these observations made in mice, new clinical evidence suggests that ICOS is required for optimal Treg function in humans. In children recently diagnosed with insulin dependent diabetes mellitus, it was demonstrated that $CD4^+CD25^+CD127^{low}$ cells had reduced levels of ICOS mRNA, associating ICOS deficiency with a breakdown in the control of auto-reactive T cells in humans (238). Whilst most ICOS deficient humans

exhibit symptoms of common variable immuno-deficiency (CVID), a recent report describing the immunological phenotype of two ICOS deficient siblings demonstrated a reduction in the expression levels of Foxp3 and a reduction in the proportion of CTLA-$4^+$ IL-10 producing Treg (239). Interestingly, the female sibling showed signs of rheumatoid arthritis, inflammatory bowel disease, interstitial pneumonitis and psioriasis indicating a high degree of immune auto reactivity. Thus, evidence suggests that ICOS plays a part in the peripheral control of immune self-reactivity in humans.

### *1.13.5 Role for ICOS in Treg responses to infectious pathogens*

The evidence described above suggests an important role for ICOS in the function and maintenance of both $Foxp3^+$ Treg and Tr1 cells under conditions of autoimmune inflammation and homeostasis. However, little is known regarding the role of ICOS in the function and maintenance of Treg generated in response to infectious pathogens. Some evidence suggests that through Treg ICOS signalling contributes to the suppression of protective immune responses during infection with the genital tract resident Th1 inducing bacteria *Chlamydia trachomatis*. $ICOS^{-/-}$ mice had much stronger protective immunity to reinfection with *Chlamydia trachomatis* than their WT counterparts due to increased frequencies of IFN-γ producing $CD4^+$ T cells (240). This was, however, at the expense of increased immunopathology in the genital tract as a consequence of reduced Treg activity; levels of Foxp3 in the draining lymph nodes were much lower in the absence of ICOS (240). During *S. mansoni* infection, blockade of ICOS during the onset of the acute phase of disease led to enhanced egg-induced immunopathology suggesting impaired immune suppression (241). This was attributed to reduced levels of hepatic T cell IL-10, and increased levels of IFN-γ, but expression of Foxp3, and the phenotype of IL-10 secreting cells was not investigated (241). Infection with *L. sigmodontis* leads to a rapid increase in the proportion of $Foxp3^+$ Treg expressing ICOS suggesting ICOS is important for Treg function as these Treg were implicated in the suppression of protective immunity (15).

The studies highlighted above suggest a new role for ICOS co-stimulation in the down-regulation of immune responses in the settings of immune homeostasis and control of reactivity to self-antigen, and there are some hints of evidence that ICOS contributes to Treg during infection settings. However, the functional significance of ICOS expression on Treg has not been investigated and the mechanisms underlying immune regulation by ICOS co-stimulation are not known. Furthermore, very little is known regarding the role of ICOS in regulatory T cell responses generated during pathogenic infections. In the context of helminth infection, ICOS co-stimulation has only been investigated in regard to type 2 immunity and it is not known if ICOS is required for Treg responses to helminth infection. We have seen that during helminth infection, Treg responses play dualistic roles in the suppression of protective immunity and prevention of immune pathology. If ICOS co-stimulation is indeed required for optimal Treg responses following helminth infection then ICOS is implicated as a determinant in the outcome for the helminth infected host. We hypothesised, therefore, that alongside its more noted role as a positive co-stimulator of effector immune responses, ICOS is also important for the development and maintenance of $Foxp3^+$ Treg responses during helminth infection, and through these actions ICOS co-stimulation contributes to suppression of host protection to helminth infection and the development of damaging immunopathology.

## *Chapter 2. Materials and Methods*

### *2.1 Animals, infections, and parasites*

The following mice were used for *in vivo* parasite infections: C57BL/6, ICOS$^{-/-}$ mice (242), C57BL/6 RAG$^{-/-}$ and BALB/c mice were bred in-house. TIGER mice were a gift from Dr. R. A. Flavell (243) and Ly5.1 and Ly5.2 Foxp3 GFP mice were bred in house. TIGER mice were crossed with ICOS$^{-/-}$ mice to generate TIGER ICOS$^{-/-}$ in house. Mice were maintained under specific pathogen-free conditions at the University of Edinburgh. Mice were used at 6-8 wks (wk) of age, and all animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986. Male mice were infected with 200 *H. polygyrus bakeri* L3 larvae by oral gavage. *Biomphalaria glabrata* snails infected with *S. mansoni* were obtained from F. Lewis (Biomedical Research Institute, Rockville, MD). Female mice were infected percutaneously with 70 *S. mansoni* cercariae. The *L. sigmodontis* life cycle was maintained in gerbils using the mite vector *Ornithonyssus bacoti* and mice were infected with 25 *L. sigmodontis* L3 larvae sub-cutaneously (s.c.) on the upper back. C57BL/6, ICOS$^{-/-}$, C57BL/6 RAG$^{-/-}$, TIGER and TIGER ICOS$^{-/-}$ were used for *H. polygyrus* infections. C57BL/6, ICOS$^{-/-}$ and TIGER mice were used for *S. mansoni* infections. BALB/c mice were used for *L. sigmodontis* infections.

To enumerate adult *H. polygyrus* worms, small intestines were removed and worms were excised with forceps. For faecal egg counts, faeces was dissolved in saturated NaCl solution and eggs were counted under a microscope using a counting chamber. The average of three faecal egg counts were taken. To isolate and count adult *S. mansoni* parasites, mice were hepatically perfused with ~ 30 ml sterile and ejected worms were collected. *S. mansoni* eggs liver eggs were counted following digestion in 4% potassium hydroxide buffer/g liver tissue at 37$^{0}$C. For measurement of egg-induced granulomas, paraffin-embedded liver sections were stained with Haematoxylin and Eosin and

granuloma size was microscopically determined (LEICA compound microscope and imaging software). Only well defined granulomas containing one visible egg were measured.

### *2.2 In vivo antibody, recombinant protein and BrdU treatments*

To label dividing cells *in vivo* mice received an intra-peritoneal (i.p.) injection of 1 mg BrdU (Sigma-Aldrich) in PBS 24-hours prior to autopsy. For BrdU pulse chase experiments mice received an i.p injection of 1 mg BrdU in PBS post *H. polygyrus* infection followed by administration of BrdU in the drinking water at 0.8 mg/ml for a further 3 days thus comprising the pulse. To block ICOS, mice received an i.p injection of 500 μg of anti-ICOS antibody (17G9, Bioxcell) every 3 days starting on day 0 of infection with either *H. polygyrus* or *S. mansoni*. To induce nuocytes mice mice received an i.p. injection of 400 ng of recombinant IL-17E (IL-25) (Peprotech) on day 0, 1 and 2 prior to autopsy on day 4.

### *2.3 Cell purifications and in vitro restimulations*

For *H. polygyrus* infections the MLN and small intestines were isolated. For acute egg-phase *S. mansoni* infections the MLN, spleen and liver were collected. For early *S. mansoni* infections, the lungs, thoracic lymph nodes (tLN) draining the pleural cavity, the inguinal lymph nodes (iLN) draining the abdominal skin and the spleen were collected. For *L. sigmodontis* infections, throacic lymph nodes and the spleen were collected and pleural cavity cells were obtained by lavage. MLN, tLN, iLN and spleens were dissociated to obtain a single cell suspension and washed in RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5% foetal-calf serum (FCS).

To isolate LP mononuclear cells from the small intestine, external adipose tissue and Peyers Patches were removed. The intestine was opened longitudinally, washed in cold

RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 3% FCS and 0.02 M Hepes (Sigma), cut into 1cm pieces, and washed a further three times in Wash Buffer comprising of RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.02 M Hepes and 2 mM EDTA (Invitrogen). The small intestine segments were incubated for 15 mins at 37°C in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 3% FCS, 0.02 M Hepes, 0.16mg/ml DTT (sigma) and 5.5 mM EDTA then washed a further three times in Wash Buffer followed by a second incubation for 30 mins at 37°C in RPMI 1640, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.02M Hepes, non-essential amino acids (NEAA) (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen), 0.5 mM β-Mercaptoethanol, 0.1 mg/ml liberase TL (Roche) and 0.5 mg/ml DNase I from bovine pancreas (Sigma). The digested small intestine was passed through 70 and 40 μm filters (BD Biosciences) to obtain a single cell suspension.

To isolate liver cells, the two large lobes of the liver were resuspended in RPMI 1640 with 5% FCS (Invitrogen), 100 U/mL penicillin- 100 μg/mL streptomycin and 2 mM L-glutamine. Liver lobes were then disrupted and incubated with collagenase D 250 μg/mL (Roche) and DNAse 10 μg/mL (Roche) for 30 min at 37°C before being ground through a 70 μm nylon mesh to prepare a single cell suspension. After being washed twice in RPMI 1640, liver leukocytes were separated on a 40% (vol/vol) percoll gradient (2000 RPM for 20 min). The leukocytes were washed once in MACS buffer before being treated for 5 min with 5 ml red blood cell lysis buffer (Sigma). The leukocytes were washed a further two times in MACS buffer before counting.

To isolate lung lymphocytes, the two lung lobes were resuspended in RPMI 1640 with 5% FCS (Invitrogen), 100 U/mL penicillin- 100 μg/mL streptomycin and 2 mM L-glutamine. The lobes were then mechanically dissociated prior to incubation in collagenase D 250 μg/mL

(Roche) and DNAse 10 μg/mL (Roche) for 30 min at $37^0$C before being ground through a 70 μm nylon mesh to prepare a single cell suspension. After being washed twice in RPMI 1640, lung leukocytes were treated for 5 min with 5 ml red blood cell lysis buffer (Sigma). The leukocytes were washed a further two times in MACS buffer before counting.

For measurement of intracellular cytokines, cells were stimulated for 4 hours with 0.5 μg/ml PMA and 1 μg/ml ionomycin, with 10 μg/ml Brefeldin A added for the final 2 hours (Sigma Aldrich).

### *2.4 CD4 negative enrichment and FACS sorting*

#### *2.4.1 Ly5.1/5.2 Foxp3 GFP RAG$^{-/-}$ transfer experiment*

MLN and spleens were isolated from Ly5.1 or Ly5.2 Foxp3 GFP mice and placed into RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5% FCS. These were then dissociated to obtain a single cell suspension and washed in RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5% FCS. Cells were resuspended in HBSS with 2% FCS and stained with the following Abs to negatively enrich for $CD4^+$ cells: anti-CD8 (53.6.72), anti-B220 (RAB632), anti-CD11b (M1/70) and anti-MHCII (M5/114.152) and incubated with dynal M450 sheep-anti-Rat IgG beads (Invitrogen) at $4^0$C for 20 mins. Bound cells were removed with a magnet and the unbound cells in the flow-through were collected and counted before staining with allophycocyanin-conjugated anti-CD4 (RM4-5, BD Bioscience). Cells were washed three times in HBSS with 2% FCS before FACS sorting for Ly5.2 $GFP^+$ or Ly5.1 $GFP^-$ populations on a FACSAria cell sorter (BD Bioscience). 7.4 x $10^5$ and 3.7 x $10^6$ Ly5.2 $GFP^+$ and Ly5.1 $GFP^-$ cells respectively were transferred into $RAG^{-/-}$ mice via intra-venous (i.v.) injection. Recipient mice were given 6 wks for immune reconstitution before infection with *H. polygyrus*.

### *2.4.2 Reconstitution of ICOS$^{-/-}$ mice with WT Foxp3$^{-}$ effector T cells*

MLN and spleens were isolated from Ly5.1 Foxp3 GFP mice and placed into RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5% FCS. These were then dissociated to obtain a single cell suspension and washed in RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5% FCS. Cells were resuspended in HBSS with 2% FCS and stained with allophycocyanin-conjugated anti-CD4 (RM4-5, BD Bioscience). Cells were washed three times in HBSS with 2% FCS before FACS sorting for CD4$^{+}$ Ly5.1 GFP$^{-}$ cells. 2.4 x $10^{6}$ Ly5.1 GFP$^{-}$ cells were transferred into ICOS$^{-/-}$ mice by i.v. injection. The following day recipient mice were infected with 200 *H. polygyrus* L3 larvae by oral gavage.

### *2.5 Flow cytometry*

The following Abs were used: Alexa Fluor 700-conjugated anti-CD4 (RM4-5, BD Bioscience), allophycocyanin-conjugated anti-Foxp3 (FJK-16s, ebioscience), phycoerythrin-conjugated anti-Helios (22F6, Biolegend), phycoerythrin-conjugated anti-ICOS (7E.17G9, Biolegend), phycoerythrin-conjugated anit-CD25 (PC61 5.3, Invitrogen), phycoerythrin-conjugated anti-IL-4 (11B11), PE-Cy7 conjugated anti-PD-1 (RMP1-30, Biolegend), biotin-conjugated anti-CD103 (M290, BD Bioscience), Alexa Fluor 647-conjugated anti-IL-13 (ebio13A; ebioscience), fluorescein isothiocyanate-conjugated anti-BrdU with DNase (B44, BD Bioscience), allophycocyanin-conjugated Annexin V (BD Bioscience), Pacific Blue-conjugated anti-TCR-β (H57-597, Biolegend), phycoerythrin-conjugated anti-CD127 (A7R34, Biolegend), allophycocyanin-conjugated anti-CD62L (MEL-14, Biolegend), eflour-450-conjugated anti-IL-2 (JES6-5H4, BD Bioscience), phycoerythrin-conjugated anti-IL-17 (TC11-18H10.1, Biolegend), biotinylated anti-CXCR5 (2G8, BD Bioscience), fluorescein isothiocyanate-conjugated anti-CD19 (MB19-1, Biolegend), fluorescein isothiocyanate-conjugated anti-CD3 (17A2, Biolegend), fluorescein

isothiocyanate-conjugated anti-CD8a (5H10-1, Biolegend), phycoerythrin-conjugated anti-c-kit (2B8, Biolegend), PE-Cy7 conjugated anti-Ly-6A/E (D7, Biolegend), allophycocyanin-conjugated anti-neuropilin-1 (R and D systems), phycoerythrin-conjugated anti-Stat5 (47/Stat5(pY694), BD Phosflow, BD Bioscience).

Non-specific binding was blocked with 4 μg of rat IgG per 1 × $10^6$ cells. Intracellular staining for Foxp3 and Helios was performed using a Foxp3-staining buffer kit (Ebioscience). For intracellular cytokine staining, dead cells were excluded using Aqua Dead Cell Stain kit (Molecular probes) and cells were fixed and permeabilized using the BD cytofix/cytoperm kit. Annexin V staining was performed as per the manufacturer's instructions (BD Bioscience). For detection of intranuclear pStat5, 1 ml of dissociated cells were immediately fixed in Foxp3 Fix/perm solution (BD Bioscience) before Ab staining. The remaining 4 ml of cells were used to determine cell number. For co-detection of GFP and Foxp3, cells were first surface stained in the usual manner followed by fixation in 100 μl BD cytofix/cytoperm solution (BD Bioscience) with 0.05% Triton-X 100 (Sigma) for 20 mins at $4^0$C. Cells were washed two times with BD permwash (BD Bioscience) before addition of primary polyclonal anti-GFP Ab (ebioscience) followed by a secondary alexaflour488-conjugated goat anti-rabbit Ab (Invitrogen). Cells were washed three times before fixation with Foxp3 Fix/perm solution (BD Bioscience) for 30 mins at $4^0$C. Cells were washed a further two times before staining for Foxp3 in the usual manner. Flow cytometry was performed using a FACSCanto 2, or an LSR 2 (BD Biosciences), running FACSDiva software (BD Biosciences). Analysis was performed using Flowjo (Tree star).

### *2.6 PCR screening of TIGER x ICOS$^{-/-}$ mice*

To identify homozygous ICOS$^{-/-}$ from heterozygous ICOS$^{+/-}$ two separate PCR reactions were performed for the icos and neomycin genes. ICOS$^{-/-}$ were identified as icos negative, neomycin positive, and ICOS$^{+/-}$ were identified as icos positive and neomycin positive. For a list of the primers see table 2.6. Thermocycling was performed at 94$^{0}$C for 35 seconds (s), 60$^{0}$C for 40 s, 72$^{0}$C for 40s for 34 cycles. The PCR products were identified using a 1 or 2% agar gel.

| Primer | Sequence |
|---|---|
| ICOS 5' – 3' | CAGGAGAAATCAATGGCT |
| ICOS 3' – 5' | TTCATAAATATGCAAATATCCTCC |
| Neomycin 5' – 3' | ATCTCCTGTCATCTCACCTTGC |
| Neomycin 3' – 5' | AGAAGGCGATAGAAGGCGATGC |

**Table 2.6**. Primers used for amplification of icos and neomycin genes.

### *2.7 FACS screening of TIGER x ICOS$^{-/-}$ mice*

To identify mice expressing the tiger locus (eGFP under the control of the IL-10 promoter), tail blood was collected and centrifuged through Lympholyte-M (Cedarlane laboratories) at 1000 XG for 20 mins. Lymphocytes were removed from the fluid interface and washed three times in PBS before resuspension in RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 5% FCS. Lymphocytes were stimulated for 72 hours (h) with 100 ng of CpG before FACS staining for CD19, CD4 and ICOS (See flow cytometry section for Ab details) and flow cytometric analysis for eGFP expression.

### *2.8 Antibody ELISA*

To measure parasite specific Ig levels, sera were collected at day 28 and day 14 of *H. polygyrus* infection. ELISA plates (NUNC) were coated with 5 μg/ml HpAg or 1 μg/ml HES diluted in bicarbonate buffer - 0.45M $NaHCO_3$/0.18M $Na_2CO_3$ (Sigma-Aldrich) and left overnight at $4^0$C. Plates were blocked with 200 μl of 2% bovine serum albumin in bicarbonate buffer and incubated for 2 h at $37^0$C. Blocking solution was aspirated and plates were washed 5 times in TBS/0.05% Tween-20 (Sigma-Aldrich). Mouse serum was serially diluted in bicarbonate buffer with 2% FCS and incubated on coated ELISA plates for 90 mins at $37^0$C. To detect Ab isotypes, 50 μl of HRP-conjugated anti-mouse IgA, IgG1, IgG2a (Southern Biotechnology Associates) in bicarbonate buffer with 2% FCS were added to the wells and incubated for 1 h at $37^0$C. Wells were again washed 5 times with TBS/0.05% Tween-20 before addition of 2,2 –azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) peroxidase substrate (KPL). Reactions were developed in the dark at room temperature for 10-30 mins and absorbance at 405 nm was determined. Total serum IgE Abs were quantified by coating ELISA plates with anti-mouse IgE capture mAb (R35-72, BD Biosciences) in bicarbonate buffer overnight at $4^0$C. A secondary HRP-conjugated IgE detection Ab was added (R35-72, Southern Biotechnology) before addition of peroxidase substrate system (KPL).

### *2.9 Statistics*

Statistical analysis was performed using JMP version 8. The data were first checked for homogeneity of variance and normality. If the raw data failed to meet these requirements for parametric analysis, $\log_{10}$ transformations were applied. Parametric analysis of combined data from multiple repeat experiments, or experiments containing more than two groups, was performed using ANOVA, followed by Tukey HSD post-hoc tests. For non-

parametric data, the unpaired Mann-Whitney U test was used. Figures show means when parametric tests were used, and medians when non-parametric tests were used.

## *Chapter 3. ICOS is required for optimal Foxp3$^+$ Treg responses during helminth infection.*

### *3.0 Introduction*

Treg cells are key immune regulators during helminth infections, and identifying the mechanisms governing their induction is of principal importance for the design of therapies for helminths, allergies, and autoimmunity. Little is yet known regarding the co-stimulatory environment that favours the development of Foxp3$^+$ Treg responses during helminth infections. As recent evidence implicates the co-stimulatory receptor ICOS in promoting Foxp3$^+$ Treg functions, we investigated the role of ICOS in the induction of Foxp3$^+$ Treg responses to helminth infection.

The majority of the classical studies into the effects of ICOS co-stimulation on the immune response to helminth infection focused on its contribution to protective type 2 immunity (201, 203, 211, 218, 241). More recently, evidence has emerged suggesting a role for ICOS in the development and function of Treg. For example, ICOS has been shown to contribute to Treg suppressive capacity in a number of *in vivo* models of autoimmune disease, demonstrating that ICOS is important for the maintenance of immune homeostasis. In a mouse model of diabetes, blockade of ICOS impairs Treg suppression of auto-reactive T cells resulting in exacerbation of diabetes disease symptoms (237). Similarly, treatment with an anti-ICOS antibody during the priming phase of EAE produced marked increases in pathology, indicating a regulatory role for ICOS in autoimmune disease (224). Even in un-manipulated mice, Foxp3$^+$ Treg express ICOS at high levels and in the absence of ICOS$^{-/-}$ peripheral Treg populations are reduced by up to 30% of WT (207).

Whilst these data demonstrate the importance of ICOS for Treg in both homeostatic and autoimmune settings, the role of ICOS in Treg responses to helminth infection has not

been investigated. IL-10 is critical for the control of hepatic egg-induced immunopathology during *S. mansoni* infection (30), and ICOS co-stimulation has been closely associated with T cell IL-10 production (205, 206, 211, 234, 236). Blockade of ICOS during *S. mansoni* infection increased egg induced hepatic granulomas indicating enhanced immunopathology (241), suggesting that ICOS plays a regulatory role in *S. mansoni* infection. In addition, Foxp3$^+$ Treg increase expression of ICOS following infection with the filarial nematode *L. sigmodontis* (15), demonstrating that ICOS is important to Foxp3$^+$ Treg during helminth infection. Therefore, we hypothesised that alongside its more noted role in the development of type 2 effector responses to helminth infection, ICOS is also required for optimal Foxp3$^+$ Treg responses.

A common feature of helminth infection is the induction of Treg cell responses but the mechanisms governing this induction can be diverse and are dependent on the nature of the invading helminth. Therefore, we wanted to ask if ICOS co-stimulation was a common pathway utilised by Foxp3$^+$ Treg for expansion in response to diverse helminth infections, including the strictly enteric nematode *H. polygyrus,* the tissue dwelling filarial nematode *L. sigmodontis* and the blood trematode *S. mansoni*.

Another un-resolved issue in Foxp3$^+$ Treg responses to helminth infection is the importance of natural thymus derived Foxp3$^+$ Treg versus adaptive Foxp3$^+$ Treg in the dampening of host protective type 2 immunity. Evidence exists in support of both natural and adaptive Foxp3$^+$ Treg activity in helminth infections. For example, *H. polygyrus* secretes a TGF-β homologue which induces Foxp3 expression in naïve T cells, and inhibition of the TGF-β signalling pathway during the later stages of infection results in enhanced protective immunity suggesting that adaptive Treg actively control host immunity to intestinal nematode infection (144). However, early depletion of Foxp3$^+$ Treg, which presumably targets pre-existing natural Treg, recovers protective immunity to *Strongyloides ratti* (146), which suggest that natural Treg are also important in intestinal

nematode infections. Similarly, depletion of Treg prior to infection with the filarial nematode *L. sigmodontis* restores host protective type 2 immune responses suggesting that natural Treg dominate immune suppression during the early stages of infection (15). In addition, whilst natural Foxp3$^+$ Treg have been shown to control egg-induced immune responses during *S. mansoni* infection (85), components of the eggs themselves can drive adaptive Foxp3$^+$ Treg (244). Recently the transcription factor Helios has been proposed to define natural thymus derived Foxp3$^+$ Treg from adaptive Foxp3$^+$ Treg (69). Therefore, it is now theoretically possible to investigate the contribution of natural versus adaptive Foxp3$^+$ Treg to the suppression of host immunity during helminth infections based on Foxp3$^+$ Treg expression of Helios.

***Chapter aims***

- Is the increased expression of ICOS by Foxp3$^+$ Tregs a common feature of helminth infection?
- What is the contribution of ICOS co-stimulation to the expansion of Foxp3$^+$ Tregs during helminth infection?
- Are natural Foxp3$^+$ Treg or adaptive Foxp3$^+$ Treg preferentially expanded in response to helminth infection, and does ICOS co-stimulation have the same effect on these subsets?

## *Results*

### *3.1 ICOS up-regulation by Foxp3$^+$ Tregs is a common feature of infection with both nematode and trematode parasites.*

It has previously been shown that CD4$^+$Foxp3$^+$ Treg upregulate expression of ICOS in response to infection with filarial nematodes (15), raising the hypothesis that ICOS plays an important role in the initiation of Foxp3$^+$ Treg responses towards helminth parasites. To assess whether ICOS up-regulation by Foxp3$^+$ Tregs is common to other helminth infections we infected C57BL/6 mice with the gastro-intestinal nematode *H. polygyrus*, or with the blood trematode *S. mansoni*, and measured ICOS expression on Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells in the MLN and spleen respectively. At day (d) 7 of *H. polygyrus* infection there was an initial small non-significant increase in the percentage of Foxp3$^+$ Treg expressing ICOS (Fig. 3.1 A & B). By d 14 of infection, ICOS expression was significantly elevated and remained high until d 28. Similarly, during the acute egg-phase of *S. mansoni* infection, CD4$^+$ Foxp3$^+$ Treg in the spleen showed increased ICOS expression compared to naïve controls (Fig. 3.1 C). ICOS was also significantly upregulated on CD4$^+$Foxp3$^-$ Teff at all time-points of both *H. polygyrus* and *S. mansoni* infections (Fig. 3.1 D & E). Thus, upregulation of ICOS by Foxp3$^+$ Tregs is a common feature of infection with both nematode and trematode parasites.

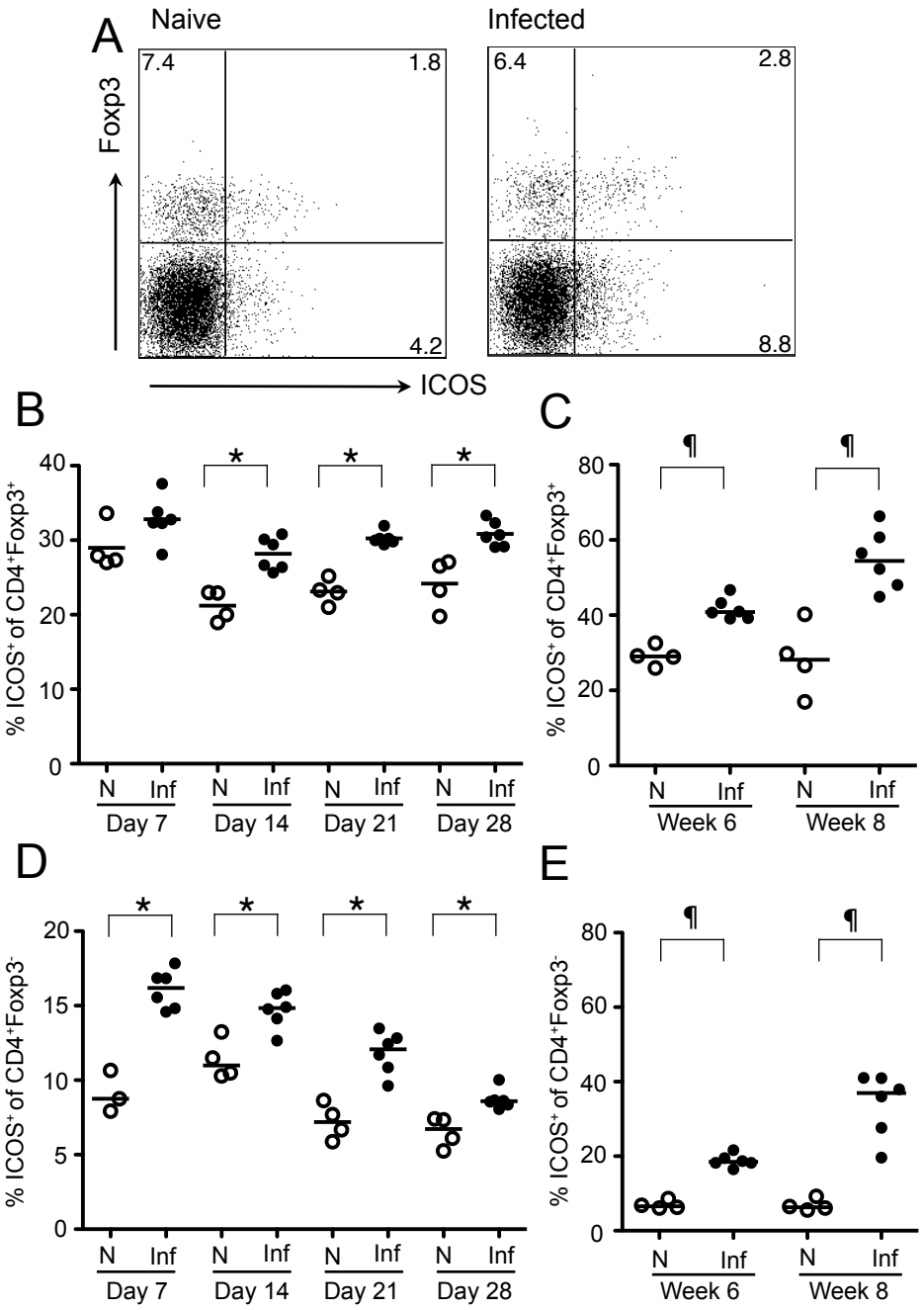



***Figure 3.1 ICOS upregulation by Foxp3$^+$ Tregs is a common feature of infection with both nematode and trematode parasites.*** C57BL/6 mice were infected with *H. polygyrus* (A, B) or *S. mansoni* (C) and the expression of ICOS by CD4$^+$Foxp3$^+$ Tregs assessed over time. (A) Representative staining for Foxp3 vs ICOS on CD4$^+$ T cells isolated from the MLN of naïve and *H. polygyrus* infected mice 14 d pi. (B) Percentage of MLN CD4$^+$Foxp3$^+$ cells expressing ICOS in naïve (open symbols) and *H. polygyrus* infected (closed symbols) mice. (C) Percentage of splenic CD4$^+$Foxp3$^+$ cells expressing ICOS in naïve (open symbols) *S. mansoni* infected (closed symbols) mice. (D) Percentage of MLN CD4$^+$Foxp3$^-$ cells expressing ICOS in naïve (open symbols) and *H. polygyrus* infected (closed symbols) mice. (E) Percentage of splenic CD4$^+$Foxp3$^-$ cells expressing ICOS in naïve (open symbols) *S. mansoni* infected (closed symbols) mice. Results shown are representative of 2 independent experiments, symbols denote individual mice and lines denote means (B) or medians (C). *p = < 0.005 (2-way ANOVA on combined data from two separate experiments). ¶ p = < 0.005 (Mann Whitney U).

### *3.2 ICOS is required for optimal Foxp3$^+$ Treg expansion and maintenance in the local lymph node during* H. polygyrus *infection*

To investigate the role of ICOS in the induction and maintenance of Foxp3$^+$ Treg responses toward the intestinal nematode *H. polygyus,* we infected C56BL/6 ICOS$^{-/-}$ and WT mice with 200 *H. polygyrus* L3 larvae and analysed Foxp3 expression in CD4$^+$ T cells in the draining lymph node at d 7, 14 and 21 of infection, and at d 7 at the site of infection. In line with previous observations (31, 32), there was a significant increase in Foxp3$^+$ Treg numbers in WT mice at d 7 of *H. polygyrus* infection (Fig 3.2 A). Notably, this increase was absent in ICOS deficient mice, suggesting ICOS contributes to the initial expansion of Treg (Fig 3.2 A). Numbers of Treg remained elevated throughout infection in WT mice, and although Treg numbers were increased in ICOS$^{-/-}$ mice at d 14 and d 21 they remained significantly lower than WT (Fig 3.2 A), which would suggest ICOS signalling contributes to the maintenance of the ongoing Foxp3$^+$ Treg response to *H. polygyrus.* Therefore, ICOS co-stimulation augments the primary expansion of Treg following *H. polygyrus* infection and is required for optimal maintenance of the Treg pool as the infection progresses.

In addition to Foxp3$^+$ Tregs, Foxp3$^-$ Teff increased expression of ICOS following helminth infection (Fig. 3.1 D & E). To determine if Foxp3$^-$ Teff showed a similar requirement to Treg for expansion, we measured Foxp3$^-$ Teff numbers during helminth infection in WT and ICOS$^{-/-}$ mice. In contrast to Foxp3$^+$ Tregs, the numbers of CD4$^+$Foxp3$^-$ Teff cells did not increase significantly in WT or ICOS$^{-/-}$ mice until d 14 of *H. polygyrus* infection (Fig 3.2 B), indicating that the CD4$^+$Foxp3$^+$ Tregs expand more rapidly than CD4$^+$Foxp3$^-$ Teff cells. At d 14 post-infection (pi), the ICOS$^{-/-}$ mice had significantly reduced numbers of CD4$^+$Foxp3$^-$ Teff cells than the WT mice (Fig 3.2 B). However, unlike CD4$^+$Foxp3$^+$ Tregs, by d 21 pi the numbers of CD4$^+$Foxp3$^-$ Teff cells in the two strains had equalised (Fig 3.2 B). This data would suggest that the effects of ICOS deficiency on Foxp3$^-$ Teff are only temporary and are confined to the initial stages of intestinal helminth infection.

The transcription factor Helios has been used to denote natural thymus derived Foxp3$^{+}$ Treg (69), whilst adaptive Foxp3$^{+}$ Treg lack expression of Helios (69). It is not known if natural or adaptive Foxp3$^{+}$ Treg preferentially dominate during *H. polygyrus* infection, or if they contribute equally. Further, whether ICOS co-stimulation has different effects on natural or adaptive Treg in the lymph node has not been investigated. Therefore, we measured expression of Helios alongside Foxp3 in the local lymph nodes of *H. polygyrus* infected WT and ICOS$^{-/-}$ mice. In WT mice, in line with the total Foxp3$^{+}$ Treg population, both natural Foxp3$^{+}$ Helios$^{+}$ and adaptive Foxp3$^{+}$ Helios$^{-}$ Treg expanded following *H. polygyrus* infection and their numbers remained elevated throughout infection (Fig 3.2 C & D) In the absence of ICOS the numbers of both natural and adaptive Foxp3$^{+}$ Treg were reduced (Fig 3.2 C & D). Thus, natural and adaptive Treg expand equally in the local lymph node in response to *H. polygyrus* infection, and similar to total Foxp3$^{+}$ Treg, during *H. polygyrus* infection, ICOS contributes to the initial expansion and continued maintenance of natural and adaptive Treg in the MLN.

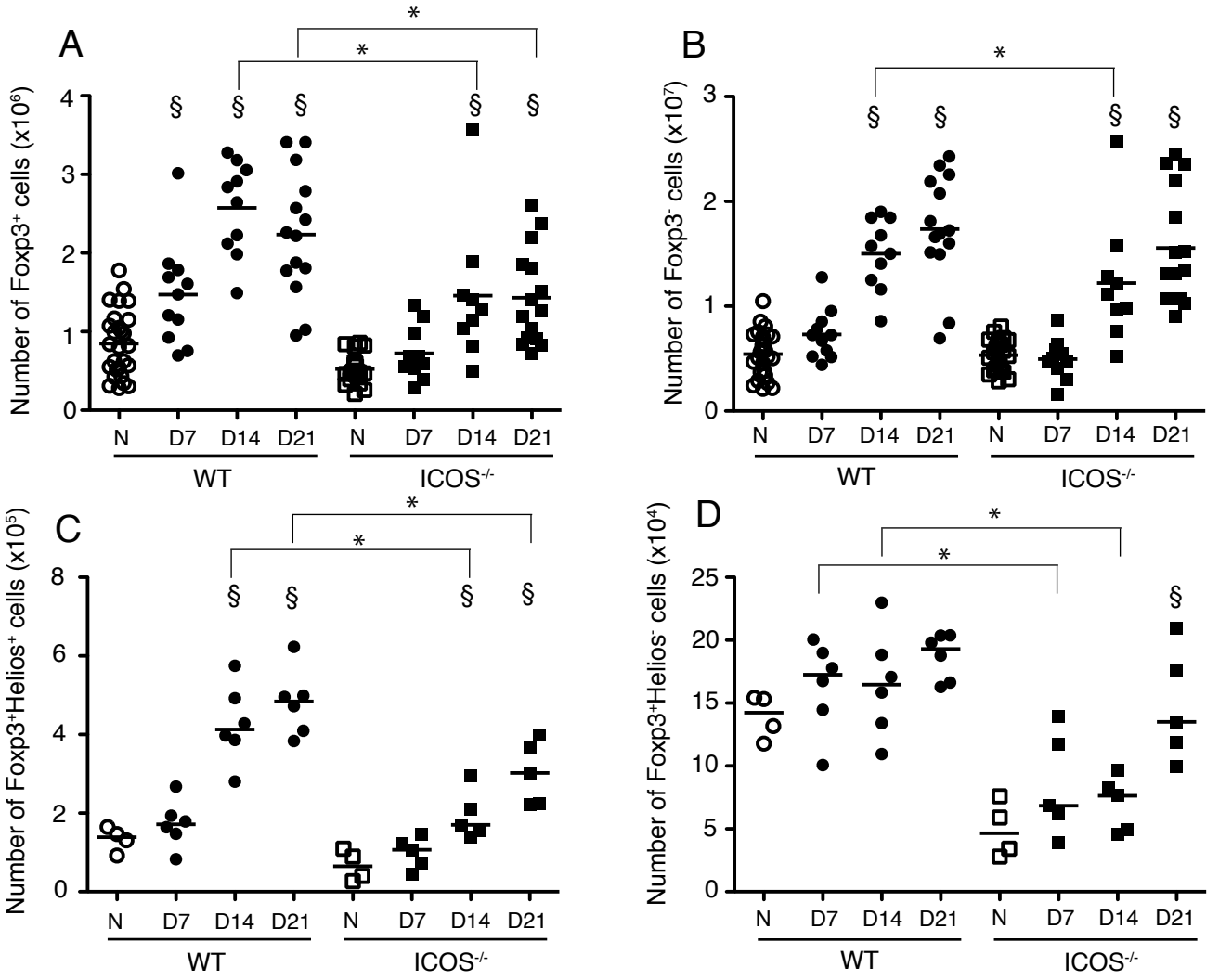



***Figure 3.2 ICOS is required for optimal Foxp3⁺ Treg expansion and maintenance in the local lymph node during H. polygyrus infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and Foxp3$^{+}$ Treg responses in the MLN were measured between d 7-21. (A) Number of CD4$^{+}$Foxp3$^{+}$ Treg cells in the MLN of naïve WT (open circles), *H. polygyrus* infected WT (closed circles), naïve ICOS$^{-/-}$ (open squares) and *H. polygyrus* infected ICOS$^{-/-}$ (closed squares). (B) Number of CD4$^{+}$Foxp3$^{-}$ Teff cells in the MLN of naïve WT (open circles), *H. polygyrus* infected WT (closed circles), naïve ICOS$^{-/-}$ (open squares) and *H. polygyrus* infected ICOS$^{-/-}$ (closed squares). (C) Number of CD4$^{+}$Foxp3$^{+}$ Helios$^{+}$ natural Treg and (D) number of CD4$^{+}$Foxp3$^{+}$ Helios$^{-}$ adaptive Treg in the MLN of naïve WT (open circles), *H. polygyrus* infected WT (closed circles), naïve ICOS$^{-/-}$ (open squares) and *H. polygyrus* infected ICOS$^{-/-}$ (closed squares). Results shown are representative of 2 independent experiments, symbols denote individual mice and lines denote means (A & B) or medians (C & D). § = significant increase on infection. § p = < 0.05. *p = < 0.005 (2-way ANOVA on combined data from two separate experiments). .

### *3.3 Following* H. polygyrus *infection, at the intestinal infection site Helios$^-$ Foxp3$^+$ adaptive Treg preferentially expand in a manner dependent on ICOS.*

Previous studies have demonstrated that ICOS deficiency results in a reduction in peripheral lymphoid Foxp3$^+$ Treg percentages under homeostatic conditions (207), and our own observations confirmed this (data not shown). However, it is not known if ICOS has a similar role for Foxp3$^+$ Treg at tissue sites. Evidence suggests that other co-stimulators, for example OX-40, can have different effects depending on the tissue location (245). Therefore, we hypothesised that ICOS could have different effects on Foxp3$^+$ Treg at the tissue site of infection, the small intestine LP. To test this we analysed Treg responses in the small intestine LP, in both naïve and *H. polygyrus* infected WT and ICOS$^{-/-}$ mice. Notably, in stark contrast to the MLN, in the LP of naive ICOS$^{-/-}$ mice, the basal Foxp3$^+$ Treg population was greater than that of WT (Fig. 3.3 A & B). Analysis of the transcription factor Helios showed that the elevated basal level of LP Foxp3$^+$ Tregs in naïve ICOS$^{-/-}$ mice solely comprised Helios$^+$Foxp3$^+$ Tregs indicating expansion of natural Foxp3$^+$ Tregs (Fig. 3.3 A, C & D). In addition, ICOS$^{-/-}$ Foxp3$^+$ Treg showed an increase in their activation status, marked by increase expression of CD103 and CD25, and decreased expression of the inhibitory co-stimulator PD-1 (Fig. 3.3 E – G). Therefore, under steady state conditions, ICOS co-stimulation has different effects on Treg depending on tissue location, being required for maintenance of the Treg population in the peripheral lymphoid organs, but acting to down-regulate Treg in the intestine tissues.

In line with previous studies (32), in WT mice we noted an expansion in LP Foxp3$^+$ Treg following *H. polygyrus* infection but it is not known if natural or adaptive Foxp3$^+$ Treg preferentially expand at this site. In the intestine, the environment favours the generation of adaptive Foxp3$^+$ Treg responses (70), and *H. polygyrus* secretes a TGF-β homologue that promotes the conversion of naïve T cells towards an adaptive Foxp3$^+$ Treg phenotype (144). In support of this, following *H. polygyrus* infection the LP Foxp3$^+$ Treg response

elicited in WT mice was entirely Helios$^{-}$ in nature (Fig. 3.3 A & D), suggesting the preferential expansion of adaptive Foxp3$^{+}$ Tregs. Interestingly, this expansion of adaptive Helios$^{-}$Foxp3$^{+}$ Tregs by *H. polygyrus* was absent in the ICOS$^{-/-}$ mice, indicating that despite their increased natural Helios$^{+}$Foxp3$^{+}$ Treg, ICOS$^{-/-}$ mice fail to mount an adaptive Helios$^{-}$ Foxp3$^{+}$ Treg response towards *H. polygyrus* (Fig. 3.3 A & D). Thus, upon infection, *H. polygyrus* preferentially expands LP Helios$^{-}$Foxp3$^{+}$ adaptive Tregs in an ICOS dependent manner.

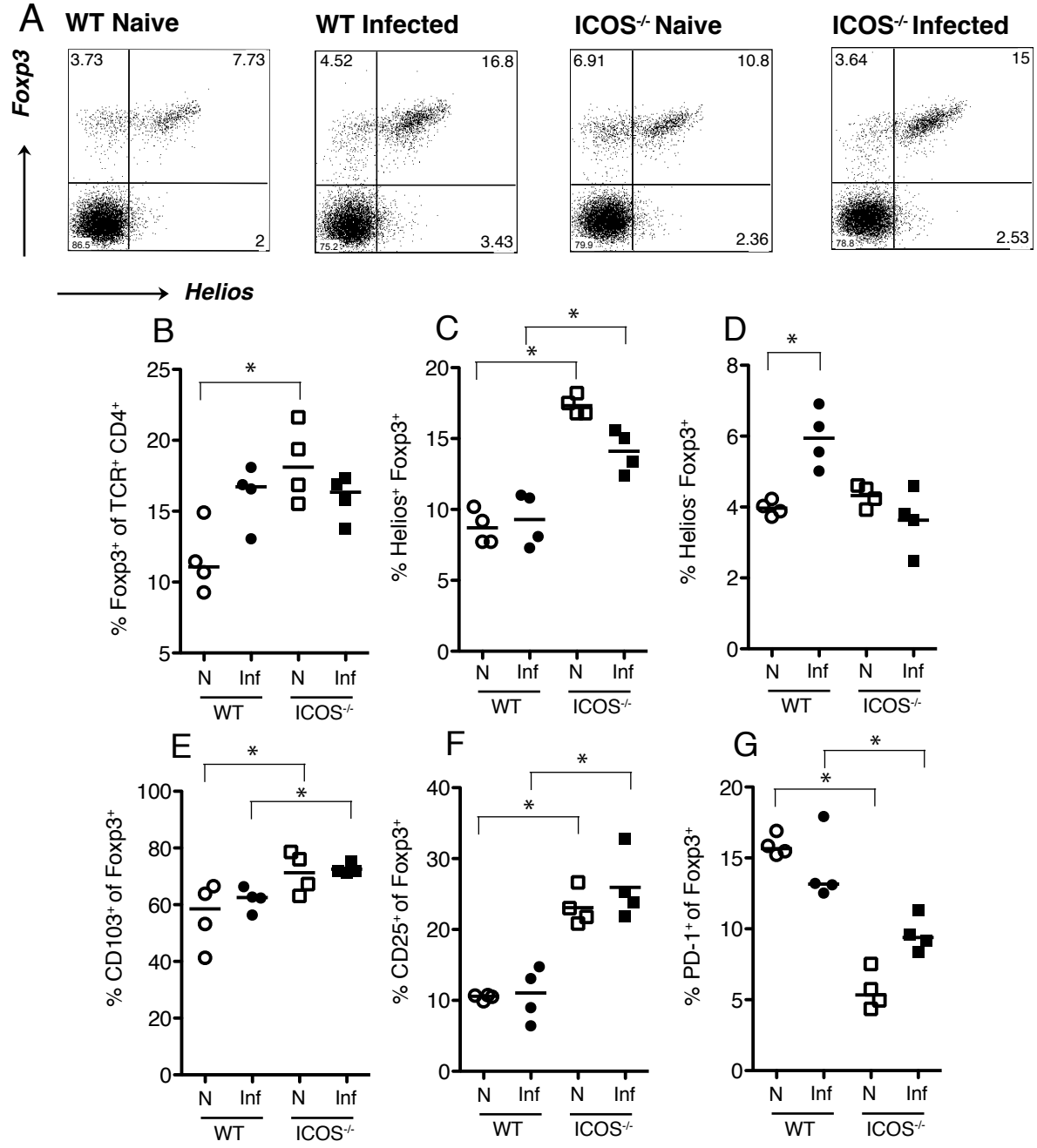



***Figure 3.3 Following* H. polygyrus *infection, at the intestinal infection site Helios$^-$ Foxp3$^+$ adaptive Treg preferentially expand in a manner dependent on ICOS.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and Foxp3$^+$ Treg responses in the small intestine lamina propria (LP) at d 7 pi were measured. (A) Representative staining for Foxp3 vs Helios gated on CD4$^+$ T cells isolated from the LP of naïve and *H. polygyrus* infected WT and ICOS$^{-/-}$ mice 7 d pi (B) Percentage of CD4$^+$Foxp3$^+$ Treg cells in the LP 7 d pi (C) Percentage of Foxp3$^+$ Helios$^+$ cells, and (C) Foxp3$^+$ Helios$^-$ cells within the LP CD4$^+$TCR$^+$ population 7 d pi (E - G) Percentage of LP CD4$^+$Foxp3$^+$ T cells expressing CD103, CD25 and PD-1 at d 7 pi. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Data are representative of three independent experiments. * p = < 0.05, two way ANOVA followed by Tukeys post hoc test.

### *3.4 ICOS contributes to the maintenance of Foxp3$^+$ Treg in the draining lymphoid tissue during the egg-phase of* S. mansoni *infection.*

Foxp3$^+$ Treg are an integral part of host immune regulation during the egg-phase of *S. mansoni* infection suppressing damaging immunopathology (16, 85, 115). Foxp3$^+$ Tregs increased expression of ICOS during *S. mansoni* infection, and ICOS was required for optimal Foxp3$^+$ Treg responses to intestinal nematode infection. Therefore, we reasoned that Foxp3$^+$ Treg may show a similar dependency on ICOS during infection with the blood trematode helminth *S. mansoni*. Previous work has shown that during the acute egg-phase of *S. mansoni* infection the proportion of Foxp3$^+$ Treg remains stable, but their numbers and activation status are increased (85). Our own observations confirmed an increase in number of Foxp3$^+$ Treg in the spleen and MLN during the acute egg-phase of *S. mansoni* infection in WT mice (Fig. 3.4 A & B). However, similar to *H. polygyrus* infection, in the ICOS$^{-/-}$ mice the number of splenic and local lymph node Foxp3$^+$ Treg induced in response to *S. mansoni* infection was significantly lower than that of WT (Fig 3.4 A & B). These data suggest that ICOS is required for the maintenance of Foxp3$^+$ Treg populations in the lymphoid organs during blood trematode infection.

During *H. polygyrus* infection, optimal expansion of both Foxp3$^+$ Treg and Foxp3$^-$ Teff showed a requirement for ICOS, albeit with different kinetics. To test if ICOS signalling was similarly required for Teff during *S. mansoni* infection, we measured Foxp3$^-$ Teff responses in WT and ICOS$^{-/-}$ mice at wk 8 of *S. mansoni* infection. The numbers of Foxp3$^-$ CD4$^+$ Teff in the spleen of WT mice increased upon infection but similar to *H. polygyrus* infection, the increase was of smaller magnitude in ICOS$^{-/-}$ mice, such that the number of Foxp3$^-$CD4$^+$ Teff was significantly lower than WT mice (Fig 3.4 C). This would suggest that both Foxp3$^+$ Treg and Foxp3$^-$ Teff show a requirement for ICOS mediated maintenance during *S. mansoni* infection. In contrast to the spleen, however, the numbers of Foxp3$^-$ CD4$^+$ Teff in the MLN increased in response to infection in both WT and ICOS$^{-/-}$ mice (Fig

3.4 D), and there was no difference between strains, suggesting that ICOS is not required for the expansion of Foxp3$^-$ Teff in the MLN.

During the egg-phase of *S. mansoni* infection, evidence exists for the activity of both natural and adaptive Foxp3$^+$ Treg subsets. For example, Foxp3$^+$ natural Treg have been shown to control egg-induced pathology during *S. mansoni* infection (115), whilst certain components of *S. mansoni* eggs can induce Foxp3 expression in T cells suggesting adaptive Treg are generated during the egg-phase (244). However, whether natural or adaptive Foxp3$^+$ Treg contribute equally to the control of egg-induced immune responses, or if one subset is dominant in this governance, has yet to be determined. Therefore, we measured Helios expression in conjunction with Foxp3 in the spleens of *S. mansoni* infected WT and ICOS$^{-/-}$ mice. At wk 8 of infection, both Foxp3$^+$Helios$^+$ and Foxp3$^+$Helios$^-$ CD4$^+$ T cells increased in number in WT mice demonstrating an equal expansion of natural and adaptive Treg (Fig 3.4 E & F). In line with the total Foxp3$^+$ population, in ICOS$^{-/-}$ mice, the expansion of both natural and adaptive Treg was lower than that of WT (Fig 3.4 E & F). Thus, similar to *H. polygyrus* infection, both natural and adaptive Treg subsets expand during *S. mansoni* infection and both require ICOS co-stimulation for optimal expansion.

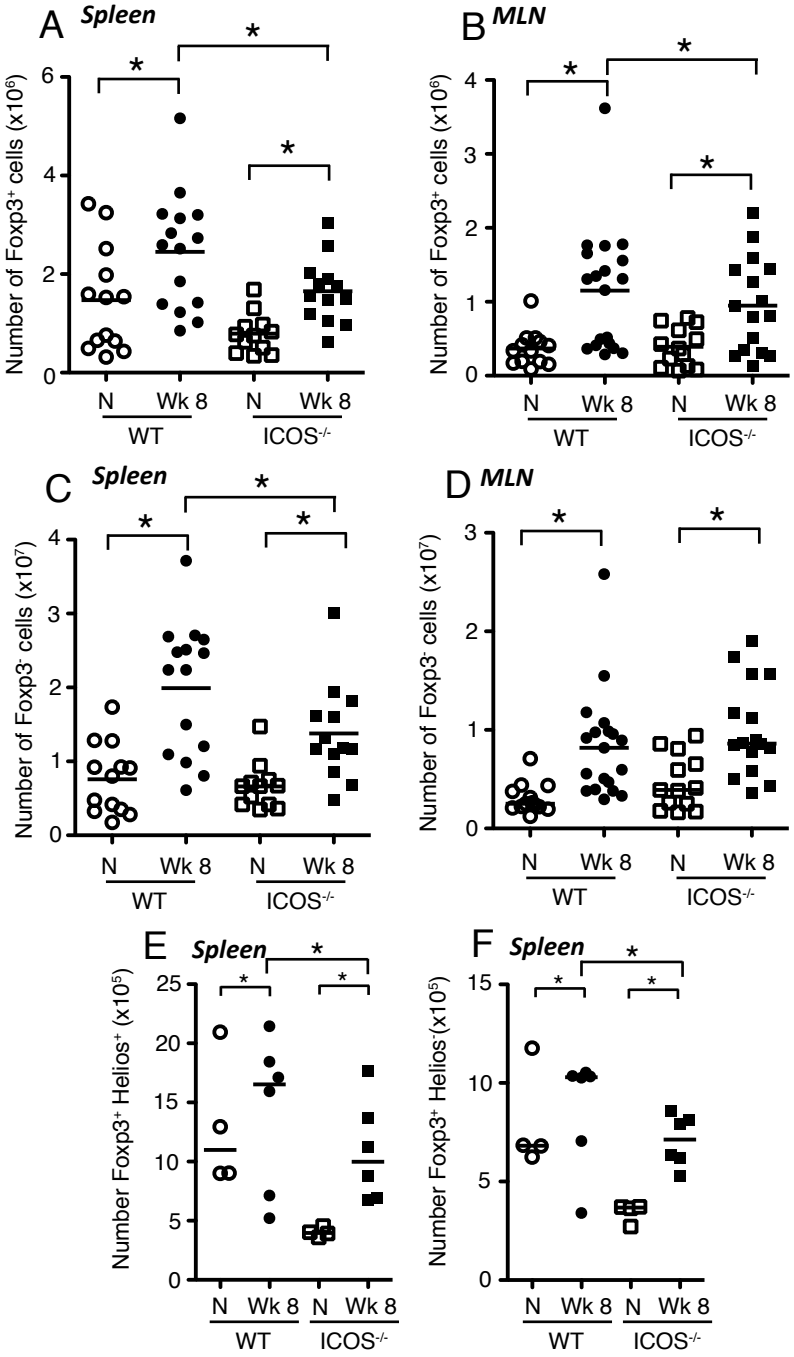



***Figure 3.4 ICOS contributes to the maintenance of Foxp3⁺ Treg in the draining lymphoid tissue during the egg-phase of* S. mansoni *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and Foxp3$^{+}$ Treg responses in the spleen and MLN at wk 8 pi were measured. Number of CD4$^{+}$Foxp3$^{+}$ Treg cells in the spleen (A) and MLN (B) of naïve and *S. mansoni* infected WT and ICOS$^{-/-}$ mice at wk 8 pi (C) Number of CD4$^{+}$Foxp3$^{-}$ Teff cells in the spleen of naïve and *S. mansoni* infected WT and ICOS$^{-/-}$ mice at wk 8 pi (D) Number of CD4$^{+}$Foxp3$^{+}$ Helios$^{+}$ natural Treg and (E) number of CD4$^{+}$Foxp3$^{+}$ Helios$^{-}$ adaptive Treg in the spleen of naïve and *S. mansoni* infected WT and ICOS$^{-/-}$ mice at wk 8 pi Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Data are representative of three independent experiments. * p = < 0.05, two way ANOVA followed by Tukeys post hoc test.

### *3.5 ICOS mediates expansion of hepatic Foxp3$^+$ Treg during the egg-phase of* S. mansoni *infection.*

During the acute phase of *S. mansoni* the liver is the site of intense egg-induced immune inflammation which is kept in check by Foxp3$^+$ Treg (85, 115). ICOS had different effects on Foxp3$^+$ Treg depending on tissue location following *H. polygyrus* infection, leading us to the hypothesis that ICOS would have different effects on Foxp3$^+$ Treg in the liver compared to the spleen and MLN during *S. mansoni* infection. To determine if the ICOS pathway was important for hepatic Treg during *S. mansoni* infection, we measured Foxp3 expression in hepatic lymphocytes from wk 8 of *S. mansoni* infected WT and ICOS$^{-/-}$ mice. Similar to the LP, in the liver of naive ICOS$^{-/-}$ mice the proportion of total Foxp3$^+$ Treg was raised when compared to WT mice (Fig. 3.5 A) due to an increase in the Helios$^+$Foxp3$^+$ natural Treg population (Fig. 3.5 B & C). Following *S. mansoni* infection, total Foxp3$^+$ Treg increased only in WT mice, and analysis of Helios showed that this expansion entirely comprised Helios$^+$ Foxp3$^+$ natural Treg (Fig 3.5 B & C). This would suggest that natural Foxp3$^+$ Treg are more important than adaptive Foxp3$^+$ Treg for control of egg-induced immune responses in the liver. In ICOS deficient mice, the increase in Helios$^+$Foxp3$^+$ natural Treg seen in WT mice was absent (Fig 3.5 B & C), suggesting that, in contrast to its role in driving adaptive Foxp3$^+$ Treg in the LP during *H. polgyrus* infection, ICOS is required for the *S. mansoni* infection induced expansion of hepatic Helios$^+$Foxp3$^+$ natural Treg, although further experiments are required to confirm this preliminary data.

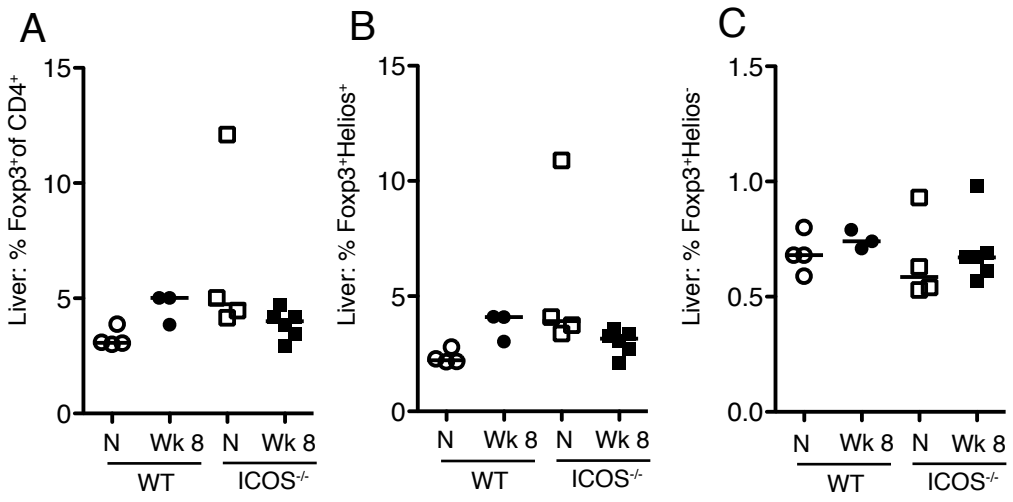



***Figure 3.5 ICOS mediates expansion of hepatic Foxp3+ Treg during the egg-phase of* S. mansoni *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and Foxp3$^{+}$ Treg responses in the liver at wk 9 p.i were measured. (A) Percentage of CD4$^{+}$Foxp3$^{+}$ Treg cells in the liver at wk 9 pi (B) Percentage of Foxp3$^{+}$ Helios$^{+}$ cells, and (C) Foxp3$^{+}$Helios$^{-}$ cells within the liver CD4$^{+}$ TCR$^{+}$ population 9 wks pi Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Results shown are representative of 1 experiment, symbols denote individual mice and lines denote medians.

### *3.6 The expansion of Foxp3+ Treg in the local lymph node and site of infection with the filarial nematode* Litomosoides sigmodontis *requires ICOS co-stimulation.*

Our data suggest that during intestinal nematode and blood trematode infections optimal Foxp3$^{+}$ Treg responses are dependent on the ICOS pathway. Infection with the filarial nematode *L. sigmodontis* has been shown to induce an early expansion of Foxp3$^{+}$ Teg and an increase in the percentage of Treg expressing ICOS (15). Unlike the intestinal nematode *H. polygyrus* that primarily inhabits the intestinal lumen, *L. sigmodontis* is

entirely tissue resident and the immune effector responses directed against it are distinct from that of *H. polygyrus.* We supposed that ICOS signalling represents a common pathway for Treg activation during helminth infection. To test if ICOS was also required for Treg responses to a tissue resident filarial nematode infection, we infected the susceptible BALB/c strain with *L. sigmodontis* and treated them with an anti-ICOS antibody (Ab), because the $ICOS^{-/-}$ mice were on the C57BL/6 *L. sigmodontis* resistant background. Mice were autopsied on d 12 of infection to measure early Foxp3 Treg responses in the spleen, and draining lymph nodes (thoracic lymph nodes - tLN). As previously reported (15), there were only minor increases in the number of $Foxp3^+$ Treg in the spleen (Fig. 3.6 A). In the tLN, $Foxp3^+$ Treg expanded significantly in infected mice treated with control antibody (Fig. 3.6 B). In infected mice treated with anti-ICOS Ab, there was no increase in $Foxp3^+$ Treg within the tLN. Therefore, similar to *H. polygyrus* and *S. mansoni* infection, initial data suggests that ICOS signalling augments expansion of $Foxp3^+$ Treg during *L. sigmodontis* infection.

*L. sigmodontis* parasites migrate through the skin and ultimately inhabit the pleural cavity where they rapidly induce a strong regulatory response (15). Therefore, to assess the role of ICOS in $Foxp3^+$ Treg responses at the site of *L. sigmodontis* infection we measured Foxp3 expression within $CD4^+$ T cells in the pleural cavity of *L. sigmodontis* infected BALB/c mice treated with IgG control or anti-ICOS antibody. In confirmation of previous data, at d 12 of *L. sigmodontis* infection, the number of $Foxp3^+$ Treg in the pleural cavity increased in mice treated with control antibody (Fig 3.6 E). However, following treatment with anti-ICOS antibody, this increase in Treg in the pleural cavity was lost (Fig 3.6 E). Alongside Treg, $Foxp3^-$ Teff also showed an infection induced increase that was dependent on ICOS co-stimulation (Fig 3.6 F). Taken together these data suggest that at the site of filarial nematode infection ICOS is required for the expansion of both $Foxp3^+$ Treg and $Foxp3^-$ Teff cells.

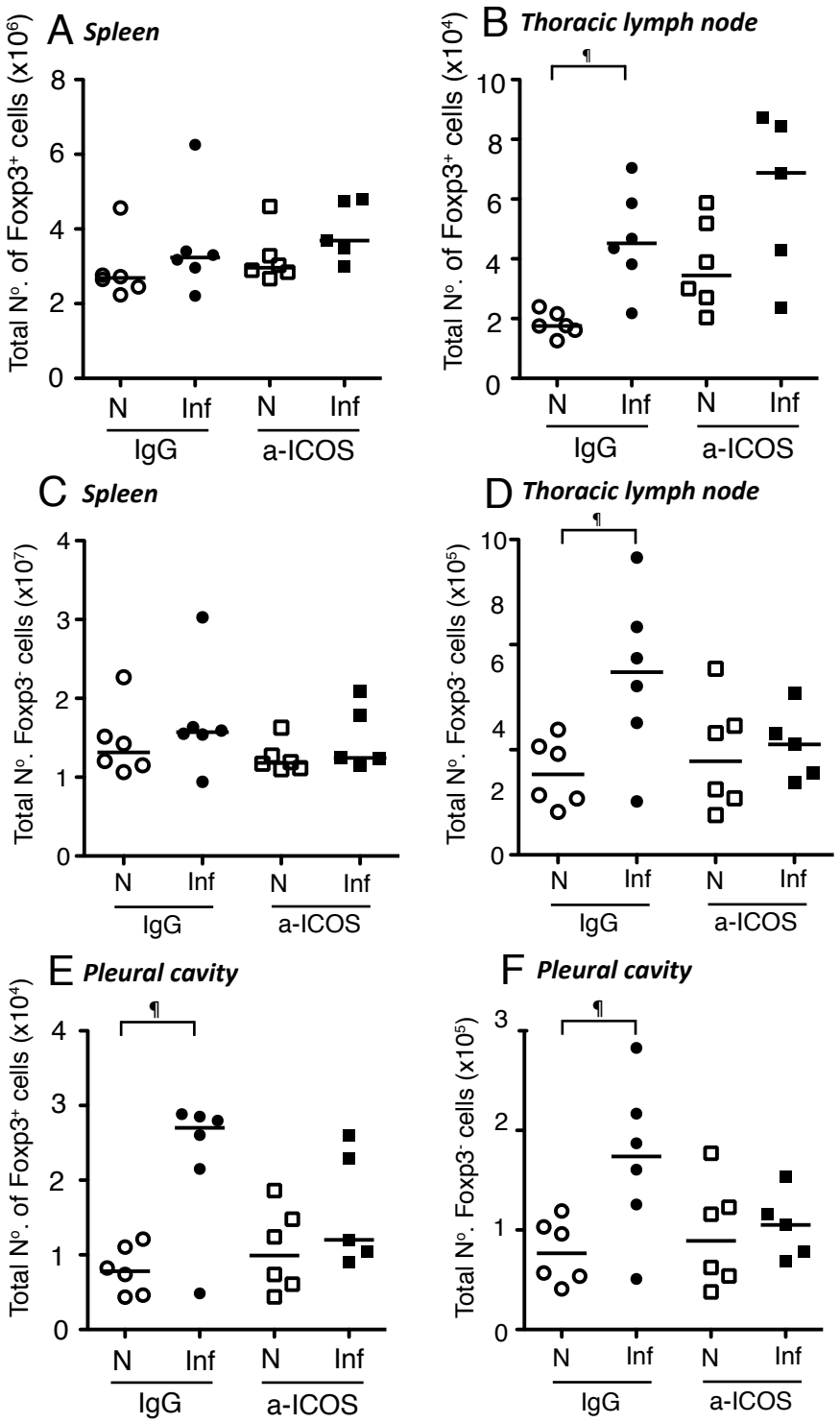



***Figure 3.6 The expansion of Foxp3⁺ Treg in the local lymph node and site of infection with the filarial nematode* L. sigmodontis *requires ICOS co-stimualtion.*** BALB/c mice were infected with *L. sigmodontis* and treated with anti-ICOS or isotype control antibody and $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cell responses were measured in the spleen, tLN and PC at d 12 pi. The number of $CD4^+Foxp3^+$ Treg cells in the PC (A), the spleen (B) and tLN (D) at d 12 pi. The number of $CD4^+Foxp3^-$ Teff cells in the PC (D), the spleen (E) and tLN (F) at d 12 pi. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve $ICOS^{-/-}$ mice and closed squares represent infected $ICOS^{-/-}$ mice. Results shown are representative of 1 experiment, symbols denote individual mice and lines denote medians. ¶ $p = < 0.005$ (Mann Whitney U).

### *3.7 Development of a novel system to measure natural versus adaptive Treg induced by intestinal helminth infection.*

An important unresolved issue in the field of helminth induced Treg responses, is whether natural or adaptive Foxp3$^+$ Treg dominate or contribute equally to the suppression of protective effector immune responses. The use of Helios as a marker for natural Foxp3$^+$ Treg has been of value in this study. However, under specific immune conditions it has been suggested that Helios expression may not be restricted to natural Foxp3$^+$ Treg (246). Therefore, to test if the expansion of Foxp3$^+$ Helios$^-$ Treg seen in the LP of *H. polygyrus* infected WT mice truly represented an induction of adaptive Foxp3$^+$ Treg populations, we developed an experimental system with the aim of tracking the conversion of naïve CD4$^+$Foxp3$^-$ T cells into CD4$^+$Foxp3$^+$ Treg during helminth infection to measure adaptive and natural Foxp3$^+$ Treg in this setting.

With this aim, we reconstituted recombination activating gene deficient (RAG$^{-/-}$) mice with congenically marked Ly5.1$^+$Foxp3$^-$CD4$^+$ T cells, and Ly5.2$^+$Foxp3$^+$CD4$^+$ natural Treg from mice expressing GFP under the control of the Foxp3 promoter. Thus, FACS sorted Ly5.1$^+$ Foxp3$^-$ and Ly5.2$^+$Foxp3$^+$ cells from Ly5.1 and Ly5.2 Foxp3$^{GFP}$ mice respectively were injected i.v. into RAG$^{-/-}$ hosts at a ratio of 5:1 respectively. Therefore, induction of Foxp3 in the previously Foxp3$^-$ Ly5.1 population would identify adaptive Foxp3$^+$ Treg, whilst Ly5.2 marks the natural Foxp3$^+$ Treg population.

RAG$^{-/-}$ recipient mice were left for four wks to allow the transferred CD4$^+$ T cells to expand and re-populate. Three wks into this period, tail blood was collected from recipient mice and PBMC were subject to flow cytometric analysis to check engraftment of the transferred T cells. This demonstrated a large population of CD4$^+$ Ly5.1 and Ly5.2 T cells present in RAG$^{-/-}$ recipient mice demonstrating successful T cell engraftment (data not shown). At four wks post transfer, recipient RAG$^{-/-}$ mice were infected with *H. polygyrus* and the

percentage of adaptive GFP$^+$Ly5.1$^+$ and natural GFP$^+$Ly5.2$^+$ Treg in the MLN and LP were measured at d 7 and compared to naive controls. Significant homeostatic conversion from naïve Foxp3$^-$ T cells to adaptive Foxp3$^+$ Treg took place prior to *H. polygyrus* infection, evident from the high percentage of GFP$^+$Ly5.1$^+$ cells in the MLN and LP of un-infected RAG$^{-/-}$ recipient mice (Fig 3.7 A, B & D).

Following *H. polygyrus* infection, there was no increase in the proportion of adaptive Ly5.1$^+$GFP$^+$ Treg over naïve controls in the MLN (Fig 3.7 B). Infection also had no impact on the proportion of natural Ly5.2$^+$GFP$^+$ cells in the MLN (Fig 3.7 C). Similarly, there was no expansion of Ly5.1$^+$GFP$^+$ adaptive or Ly5.2$^+$GFP$^+$ natural Treg in the LP (Fig 3.7 D & E) in response to *H. polygyrus* infection, which contrasts with the expansion of LP Foxp3$^+$ Helios$^-$ adaptive Treg seen in WT mice (Fig 3.3 D). That neither population underwent an expansion suggests that this system failed to recapitulate the natural immune response, so in this instance it was not possible to determine if natural or adaptive Treg are dominant during *H. polygyrus* infection.

It has been suggested that expression of Nrp-1, a receptor for semaphorins and a co-receptor for vascular endothelial growth factor, is principally restricted to natural Foxp3$^+$ Treg (Dr Jeff Bluestone, Keystone symposium, Breckenridge CO 2011). Therefore, as an additional marker to define natural Foxp3$^+$ Treg, we measured expression of Nrp-1 on adaptive GFP$^+$Ly5.1$^+$ and natural GFP$^+$Ly5.2$^+$ Treg in the MLN and LP at d 7 pi. Only adaptive GFP$^+$Ly5.1$^+$ cells in the MLN (Fig 3.7 F & G), and to a lesser extent in the LP (Fig 3.7 H & I), down regulated expression of Nrp-1. This could give some suggestion that adaptive Foxp3$^+$ Treg, but not natural Foxp3$^+$ Treg, are activated during *H.polygrus* infection, because Nrp-1 has also been demonstrated to stabilise DC-Treg interactions (113), and those Foxp3$^+$ Treg that have down-regulated Nrp-1 may represent Foxp3$^+$ Treg activated by antigen in the context of MHCII.

It must be noted that this preliminary experiment was the first step in the development of a system to track adaptive Foxp3$^{+}$ Treg in helminth infection and further work is required for its refinement. For example, the absence of B cells in RAG$^{-/-}$ recipient mice may adversely impact Foxp3$^{+}$ Treg populations as B cell depletion can result in diminished Treg (247). Future studies aim to use T cell specific deficient (CD3ε$^{-/-}$ mice) recipient mice to recover the B cell response and more accurately reflect the immune environment of WT lymphocyte-replete mice. In summary, although it requires further refinement, this experimental system represents an important tool for the study of the importance of natural versus adaptive Foxp3$^{+}$ Treg responses during helminth infection, and the kinetics by which they are generated.

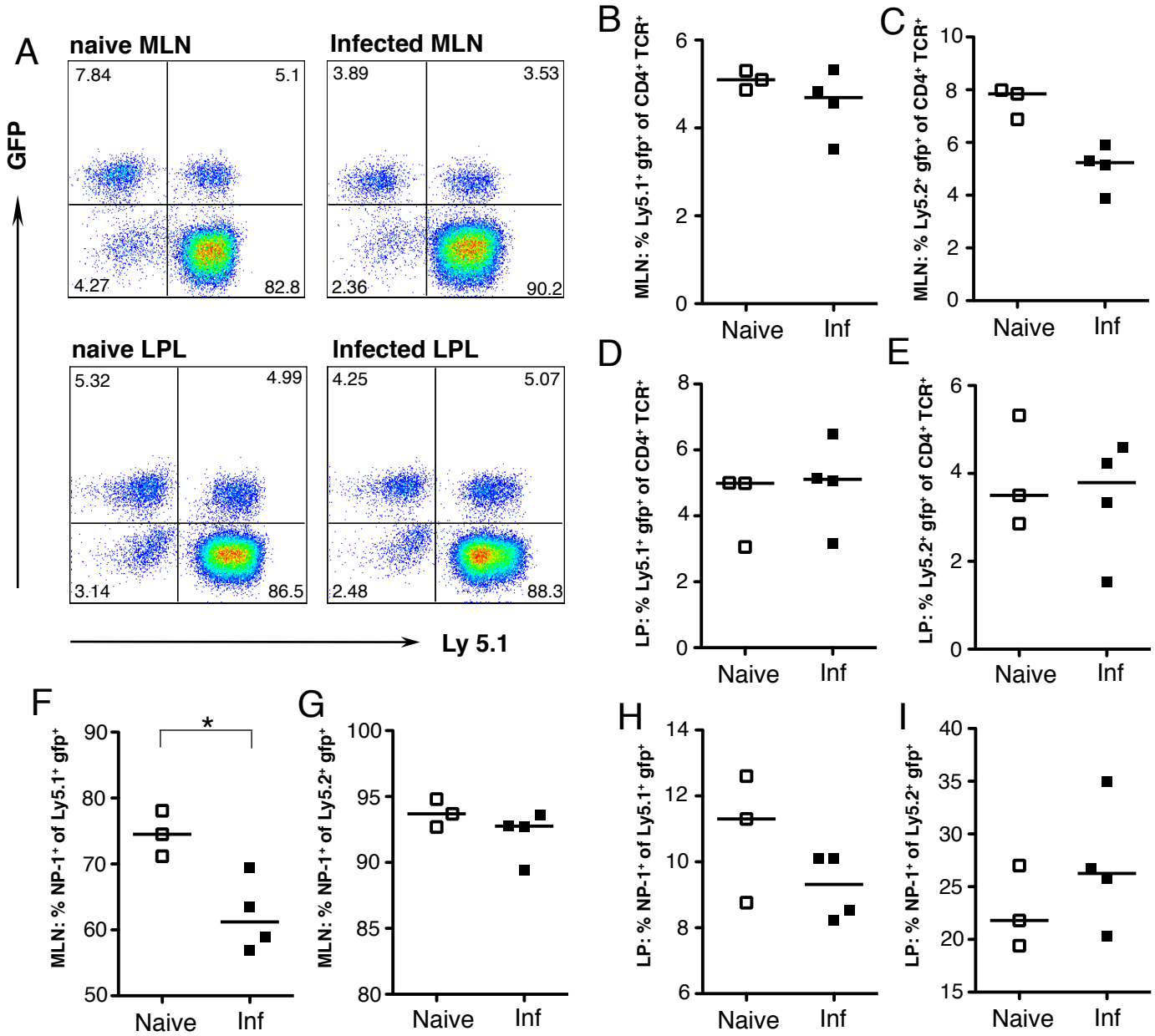



***Figure 3.7 Development of a novel system to measure natural versus adaptive Treg induced by intestinal helminth infection.*** GFP$^-$Ly5.1$^+$ and GFP$^+$Ly5.2$^+$ from Foxp3$^{GFP}$ mice were FACS sorted and transferred into RAG$^{-/-}$ recipients at a ratio of 5:1. At 4 wks post transfer recipient mice were infected with 200 *H. polygyrus* L3 larvae and GFP expression was measured within the CD4$^+$ Ly5.1 and Ly5.2 populations at d 7 pi (A) Representative FACS plots showing GFP (Foxp3) versus Ly5.1 in the LPL and MLN of naïve and d 7 *H. polygyrus* infected recipient mice. Graphs showing percentage of Ly5.1$^+$ (B) and Ly5.2$^+$ (C) cells expressing GFP in the LP of naïve and *H. polygyrus* infected recipient mice. Graphs showing percentage of Ly5.1$^+$ (D) and Ly5.2$^+$ (E) cells expressing GFP in the MLN of naïve and *H. polygyrus* infected recipient mice. (F) Percentage of Ly5.1$^+$GFP$^+$ cells expressing Nrp-1 and (G) percentage of GFP$^+$Ly5.2$^+$cells expressing Nrp-1 in the LP of naïve and d 7 *H. polygyrus* infected recipient mice. (H) Percentage of GFP$^+$Ly5.1$^+$ cells expressing Nrp-1 and (I) percentage of GFP$^+$Ly5.2$^+$ cells expressing Nrp-1 in the MLN of naïve and d 7 *H. polygyrus* infected recipient mice. Open squares represent naïve mice and closed squares represent infected mice. * p = < 0.05 Mann Whitney U.

### *3.8 Discussion*

Following infection with parasitic helminths, Foxp3$^+$ Treg exhibit an activated phenotype as evidenced by up-regulation of surface markers (15, 31, 85). More specifically, it has been shown that ICOS is up-regulated on Treg during infection with the filarial nematode *L. sigmodontis* (15). Here, we have shown that Foxp3$^+$ Tregs increase ICOS expression following infection with the intestinal nematode *H. polygyrus*, and with the blood trematode *S. mansoni*. Thus, the upregulation of ICOS on Foxp3$^+$ Tregs is common to both nematode and trematode helminth infections. *In vitro*, the increase in ICOS expression on T cells has been shown to be dependent on TCR engagement, and this is augmented with concomitant CD28 stimulation (189). Therefore, it is possible that those Foxp3$^+$ Tregs that upregulate ICOS during helminth infection represent parasite specific Treg that have been activated by antigen in the context of MHC, alongside CD80/86 costimulation. Alternatively, these ICOS expressing Treg could have become activated in response to the expansion of infection induced anti-parasite Foxp3$^-$ Teff cells. Indeed evidence suggests Foxp3$^+$ Treg activation is increased in the presence of Foxp3$^-$ Teff, demonstrated by the upregulation in the production of inhibitory cytokines when Foxp3$^+$ Treg are co-cultured with Teff (98). A third possibility is that ICOS expression is increased in response to tissue damage incurred as the parasites migrate. For example, heat shock proteins (HSP) are ubiquitous cellular proteins induced in response to environmental and chemical stress such as those experienced during infection (248). HSP exhibit immunoregulatory properties (249-251), and the human HSP60 has been shown to enhance the suppressive capacity of Treg suggesting an activated phenotype (252, 253). Thus, increased expression of ICOS on Foxp3$^+$ Treg could reflect activation of Foxp3$^+$ Treg responding to HSP released by damaged tissue cells following parasite invasion.

Interestingly, even in the steady state ICOS is expressed at high levels on Foxp3$^+$ Treg when compared to Foxp3$^-$ Teff (207), which could reflect low level activation of Treg specific for self antigen. The increment in ICOS expression on Foxp3$^+$ Treg is not unique to parasite infection; for example, Foxp3$^+$ Treg increase ICOS expression in response to *Mycobacterium tuberculosis* infection, both in the draining lymph node and at the site of infection, with the latter site showing the greatest increase (254). Treg have also been shown to increase expression of ICOS in a number of autoimmune disease models; in a mouse model of diabetes the majority of CD25$^+$ Treg in the pancreatic lesion showed high ICOS expression (237). In addition, Foxp3$^+$ Treg induced during contact hypersensitivity show increased expression of ICOS (255). These findings suggest that one of the hallmarks of Treg activation, whether in response to infection or tissue self-antigen, is increased expression of ICOS.

That Treg increase ICOS expression during helminth infection suggests that ICOS is important for the Treg response. Although it is well known that ICOS contributes to Th2 responses induced by helminths (201, 203, 218), no previous studies have described a role for ICOS in regulatory responses to helminth infection. Here we have shown that ICOS is important for the early expansion of Treg during *H. polygyrus* infection. Further, ICOS signalling was required for the maintenance of Treg during chronic helminth infection, evidenced by the reduced Treg response throughout *H. polygyrus* infection, and during the egg-phase of *S. mansoni* infection. The continued requirement of Foxp3$^+$ Treg for ICOS co-stimulation contrasts with the temporary defects seen in Foxp3$^-$ Th2 effector cells in the lymph node of helminth infected ICOS$^{-/-}$ mice (201, 203, 218). In keeping with this, the initial reduction in Foxp3$^-$ Teff seen in ICOS$^{-/-}$ mice recovered toward the late stage of *H. polygyrus* infection. During the acute egg-stage of *S. mansoni* infection, Foxp3$^-$ Teff remained reduced in ICOS$^{-/-}$ mice suggesting a continued dependence on ICOS signalling. However it is possible that Teff numbers showed a recovery as the egg-stage of

infection entered the chronic phase and future work should aim to determine this. Taken together, these data suggest that although ICOS is required to optimally initiate both type 2 and Foxp3$^+$ Treg responses to helminths, it has a further role in sustaining Foxp3$^+$ Tregs during *H. polygyrus* and *S. mansoni* infection.

Under homeostatic conditions the impact of ICOS deficiency on Foxp3$^+$ Tregs differed depending on immune location. Whilst the proportion of Foxp3$^+$ Tregs was reduced within the lymph node (LN) of naïve ICOS$^{-/-}$ mice in agreement with previous studies (207, 256), the percentage of CD4$^+$ T cells expressing Foxp3 within the LP was increased. This was associated with elevated expression of CD103 and CD25, and reduced expression of PD-1, indicating that the Foxp3$^+$ Tregs were in a heightened state of activation. Analysis of Helios expression showed that the expanded proportion of Treg in ICOS$^{-/-}$ mice was due to increased Helios$^+$ natural Treg. Thus, in the intestinal tissues, ICOS acts to downregulate Treg but in contrast, ICOS promotes Treg responses in the lymphoid organs. Further evidence of tissue specific effects of co-stimulators on Treg has come from analysis of OX-40 deficient mice, which show a colon specific impairment in Foxp3$^+$ Treg (245). The different effects of ICOS in the intestine versus the lymph nodes could result from differences in the local immune environment. The immunological milieu of the intestine is markedly different from that of the lymph node, and here other factors/cytokines such as TSLP may act as growth factors to promote Treg survival (257). For example, in the steady state TSLP is produced by intestinal epithelial cells (258-260), and in response to TLR signalling, intestinal CD103$^+$ DCs produce TSLP that supports the generation of Foxp3$^+$ Treg (257). Because intestinal DC are stimulated through TLRs (257), gut resident commensal bacteria could drive DC TSLP under homeostatic conditions to support Foxp3$^+$ Treg.

Despite the elevated natural Foxp3$^+$ Treg activity in the naïve setting, upon *H. polygyrus* infection ICOS$^{-/-}$ mice failed to mount an adaptive Foxp3$^+$ Treg response within the LP. The mucosal environment has a propensity for the generation of adaptive Foxp3$^+$ Tregs (70, 261), and *H. polygyrus* secretes a TGF-β mimic capable of inducing Foxp3 expression in naïve T cells (144). Thus, based on expression of the natural Foxp3$^+$ Treg marker Helios (69), it was not unexpected that the LP Foxp3$^+$ Tregs recruited in response to *H. polygyrus* infection of WT mice were all Helios$^-$. This indicates that *H. polygyrus* primarily induces an adaptive Foxp3$^+$ Treg response at the infection site, albeit with the caveat that the use of Helios as a natural Foxp3$^+$ Treg marker is controversial and may not be accurate in all immune contexts (246). Although studies indicate that ICOS is not required for the induction of adaptive Foxp3$^+$ Tregs from CD4$^+$CD25$^-$ precursors in naïve mice (256), the LP Helios$^-$Foxp3$^+$ Tregs population failed to expand in response to *H. polygyrus* infection suggesting that ICOS is required for intestinal adaptive Foxp3$^+$ Treg responses during helminth infection.

Adaptive Foxp3$^+$ Helios$^-$ Treg were preferentially induced in the intestine following *H. polygyrus* infection, but it is not known whether natural or adaptive Foxp3$^+$ Treg in the lymph node are selectively expanded in response to *H. polygyrus* infection. In contrast to the intestine, in the MLN, numbers of both natural Foxp3$^+$ Helios$^+$ and adaptive Foxp3$^+$ Helios$^-$ cells expanded in response to infection. Further, natural and adaptive Foxp3$^+$ Tregs were reduced in ICOS deficient mice suggesting that ICOS maintains both populations in the local lymph node during *H. polygyrus* infection. During egg-phase *S. mansoni* infection, natural Foxp3$^+$ Treg have been shown to control the magnitude of the type 2 response to the eggs (85, 115), and *in vitro*, Foxp3 expression can be induced in T cells exposed to *S. mansoni* egg antigens (244) suggesting that both natural and adaptive Foxp3$^+$ Treg are active during the egg-phase. In keeping with these observations and similar to *H. polygyrus* infection, during *S. mansoni* infection optimal expansion of both

natural and adaptive Foxp3$^+$ Treg required ICOS. Thus, ICOS contributes to the maintenance of natural and adaptive Treg during both intestinal nematode and blood trematode infection.

In some immune settings the use of the transcription factor Helios to distinguish natural Treg from adaptive Treg may not be valid. For example, Foxp3$^+$ Treg induced from Foxp3$^-$ T cells *in vivo* following recognition of cognate antigen were Helios$^+$, demonstrating that adaptive Foxp3$^+$ Treg can express Helios (246). In addition, Helios expression can be induced in Foxp3$^-$ effector T cells following *in vitro* TCR stimulation (262) and Helios has been associated with Foxp3$^-$ Th2 and Tfh cells in an alum-OVA model of Th2 immunity (263). Therefore, Helios may not exclusively mark and accurately distinguish natural Foxp3$^+$ Treg from Foxp3$^+$ adaptive Treg. To test if the expansion of putative Helios$^-$ Foxp3$^+$ Treg seen in the LP of *H. polygyrus* infected mice accurately reflected expansion of adaptive Treg we set up a novel system to measure *in vivo* conversion of Foxp3$^-$ naïve T cells to Foxp3$^+$ Treg during helminth infection. RAG$^{-/-}$ mice were reconstituted with GFP$^-$ and GFP$^+$ T cells from congenically distinct Foxp3$^{GFP}$ mice, allowing us to track GFP$^-$ cells that switch on Foxp3 to become GFP$^+$. In naïve recipient mice, a proportion of Foxp3$^+$ (GFP$^+$) cells expressed Ly5.1 suggesting homeostatic conversion of Ly5.1 Foxp3$^-$ to Ly5.1 Foxp3$^+$ T cells in recipient mice. The GALT are well known for the conversion of naïve T cells to adaptive Foxp3$^+$ Treg through TGF-β and retinoic acid signalling (70, 264), and gut resident microbiota have been shown to induce Foxp3 expression in intestinal tissues (265, 266). Therefore, the presence of GFP$^+$Ly5.1$^+$ cells in the MLN and LP of naive mice could represent those naive T cells activated by gut commensals in the presence of TGF-β and retinoic acid to become adaptive Foxp3$^+$ Treg.

Following *H. polygyrus* infection, there was no expansion of either natural GFP$^+$Ly5.2$^+$ or adaptive GFP$^+$Ly5.1$^+$ Treg in the MLN or the LP. This was in contrast with Helios and Foxp3 flow staining, which showed expansion of Foxp3$^+$Helios$^-$ adaptive Treg in the LP of

*H. polygyrus* infected WT mice. That we did not see an expansion of either population in the GFP-tagged Treg tracking system could result from the inadequate immune environment of $RAG^{-/-}$ recipient mice. For example, the absence of B cells in $RAG^{-/-}$ mice could impede expansion of $Foxp3^+$ Treg, as B cell depletion has been shown to reduce Treg numbers and impair their suppressive capacity resulting in enhanced autoimmunity (247). Critically, B cells are also important for the expansion of Treg during helminth infection, demonstrated by the failure of $Foxp3^+$ Treg to expand in *H. polygyrus* infected μMT mice (Dr Katherine Smith, Dr Rick Maizels, personal communication). Therefore, the lack of a $Foxp3^+$ Treg response in our $RAG^{-/-}$ recipient mice could result from the absence of B cells and future experiments aim to re-populate $CD3\varepsilon^{-/-}$ mice, which have the propensity to develop B cell responses once reconstituted with T cells, to circumvent this problem. Despite these issues, following *H. polygyrus* infection only adaptive $Foxp3^+$ Treg showed reduced expression of Nrp-1, which could indicate activation of this population (113). This would be in keeping with the accumulating evidence suggesting preferential induction of adaptive Treg by *H. polygyrus,* such as induction by HES (144), and in addition, live *H. polygyrus* infection has been shown to enhance Foxp3 induction in DO11.10 cells in a model of OVA oral antigen exposure (144) demonstrating *in vivo* stimulation of adaptive Treg by *H. polygyrus.* Interestingly, activation of $Foxp3^+$ Treg during helminth infection may be more important for the suppression of host protective immune responses than expansion of the population as a whole, as the increased activation phenotype of $Foxp3^+$ Treg denotes cells with enhanced suppressive capacity (31, 32). Alongside adaptive $Foxp3^+$ Treg, a role for natural $Foxp3^+$ Treg has been described during infection with the intestinal nematode *S. ratti* (146). Therefore, it is most likely that both adaptive and natural $Foxp3^+$ Treg are active during *H. polygyrus* infection and further refinement of the GFP tagged $Foxp3^+$ Treg tracking system described here will

allow the future study of the importance of these populations in the suppression of host protective immunity to helminth infection.

***Summary***

- Increased expression of ICOS is a hallmark of $Foxp3^+$ Treg activation during infection with diverse helminth species.
- $Foxp3^+$ Treg require ICOS co-stimulation for optimal expansion during intestinal and filarial nematode infections, and blood trematode infection.
- On the basis of Helios expression, both natural and adaptive $Foxp3^+$ Treg depend on ICOS for optimal expansion in the reactive lymphoid organs during *H. polygyrus* and *S. mansoni* infections.
- The effects of ICOS co-stimulation on $Foxp3^+$ Treg responses depend on the tissue location; under homeostatic conditions, ICOS promotes $Foxp3^+$ Treg responses in the lymphoid tissues, but in the intestinal tissue ICOS deficiency leads to an increase in the proportion of natural $Helios^+Foxp3^+$ Treg.
- At the tissue site of *H. polygyrus* infection, $Helios^-Foxp3^+$ adaptive Treg preferentially expand in a manner dependent on ICOS.

## *Chapter 4. Mechanisms of Foxp3$^+$ Treg deficiency in helminth infected ICOS$^{-/-}$ mice.*

### *4.0 Introduction*

In chapter 3 we showed that ICOS co-stimulation is required for optimal Foxp3$^+$ Treg responses to both nematode and trematode helminth infections. For a complete regulatory response, Foxp3$^+$ Treg must first be primed by antigen in the presence of co-stimulatory signals to become activated (157). Following activation, co-stimulatory signals promote Foxp3$^+$ Treg proliferation and expansion (154), and they play a further role in maintaining the Foxp3$^+$ Treg population and driving regulatory function such as secretion of inhibitory cytokines (154). The reduced Foxp3$^+$ Treg response seen in helminth infected ICOS$^{-/-}$ mice could result from a failure in any one of these processes that together constitute a complete Treg response. We hypothesised, therefore, that ICOS signalling is utilised by Foxp3$^+$ Treg during helminth infection to promote one or more of the following: i) priming and activation, ii) proliferation, iii) survival, iv) cytokine secretion.

To generate an immune response, naïve T cells require priming through cognate antigen specific and non-cognate co-stimulatory signals before subsequent proliferation and expansion. Following antigen priming, T cells begin to express activation markers associated with cytokine signalling, co-stimulation and tissue trafficking (267, 268), and measurement of these markers can be used to give an indication of recently primed T cells. In response to helminth infection, Foxp3$^+$ Treg increase expression of activation markers (15, 31, 85), and this has been associated with an enhanced ability to proliferate and suppress effector immunity (31, 32). ICOS is itself used as a marker for Treg activation (269), and Foxp3$^+$ Treg upregulate ICOS during helminth infection (15) (Chapter 3, Fig. 3.1 A-C). *In vitro* evidence suggests that ICOS co-stimulation augments expression of the T cell activation markers CD40L, CD69 and CD25 (187, 199), but it is not known if ICOS signalling contributes to the priming and activation of Foxp3$^+$ Treg during helminth

infection. We hypothesised that the failed Foxp3$^+$ Treg response seen in helminth infected ICOS$^{-/-}$ mice could be due to impaired priming in the absence of ICOS co-stimulatory signals.

A second possibility is that ICOS acts down-stream of immune priming to drive Foxp3$^+$ Treg proliferation. In support of this, early work to elucidate the effects of ICOS co-stimulation demonstrated that stimulation of ICOS *in vitro* enhances CD4$^+$ T cell proliferation (189), and that ICOS$^{-/-}$ CD4$^+$ T cells have a defective proliferative capacity following *in vitro* stimulation (197, 199). These early *in vitro* studies were expanded upon to show that ICOS promotes T cell proliferation in a number of *in vivo* models of autoimmunity and infection. For example, blockade of ICOS following the establishment of EAE reduced antigen specific splenocyte proliferation (224) suggesting that ICOS co-stimulation drives auto-reactive effector T cell responses. In addition, ICOS has been shown to contribute to CD4$^+$ Teff cell proliferation during both protozoan and helminth parasite infections. During *T. muris* infection of ICOS$^{-/-}$ mice, BrdU incorporation analysis demonstrated a four-fold reduction in the proportion of proliferating ICOS$^{-/-}$ CD4$^+$ Th2 effector cells compared to WT Th2 cells, and similarly, ICOS$^{-/-}$ CD4$^+$ Th1 effector cells exhibited a reduced proliferative response following infection with the protozoan parasite *T. gondii* (203). In another model of Th2 infection, blockade of ICOS in mice infected with *T. spiralis* dramatically reduced antigen specific proliferation in local lymph node cells (218). Thus, ICOS has been shown to contribute to Foxp3$^-$ Teff proliferation during helminth infection. Alongside Foxp3$^-$ Teff, the rate of proliferation of Foxp3$^+$ Treg rapidly increases in response to helminth infection (15). Whether the ICOS pathway has a further role in driving Foxp3$^+$ Treg proliferation during helminth infection has not been investigated.

An alternate explanation for the reduced expansion of Foxp3$^+$ Treg seen in helminth infected ICOS deficient mice is that ICOS$^{-/-}$ Foxp3$^+$ Treg fail to survive, resulting in a

reduction in their numbers. Previous work has shown that ICOS aids the survival of T cells in a model of transfer colitis (270), and following T cell cognate antigen activation (207). ICOS is also essential for the survival of NKT cells *in vivo* (271). The observation that $Foxp3^+$ Treg are intact in the thymus of $ICOS^{-/-}$ mice, but are reduced in the periphery (207) could suggest that ICOS contributes to peripheral $Foxp3^+$ Treg survival. We hypothesised, therefore, that ICOS has a further role in sustaining the survival of $Foxp3^+$ Treg, and in doing so contributes to their expansion and maintenance during helminth infection.

In addition to elucidating the mechanisms underlying the ICOS mediated expansion and maintenance of $Foxp3^+$ Treg during helminth infection, we asked if ICOS was also required for $Foxp3^+$ Treg function during helminth infection. Multiple mechanisms are used by $Foxp3^+$ Treg to suppress effector immune responses (149), and amongst these mechanisms production of the suppressive cytokine IL-10 is key to $Foxp3^+$ Treg function (80, 81, 272). ICOS expression has been associated with $CD4^+$ T cell IL-10 production both *in vitro* and *in vivo* (205, 206, 208, 211), and ICOS expression defines human $Foxp3^+$ Treg with the ability to produce IL-10 (234). However, a firm functional link between ICOS and $Foxp3^+$ Treg IL-10 has not been established, and it is not known if ICOS is required for $Foxp3^+$ Treg IL-10 during helminth infection.

***Chapter Aims***

Is the impaired Foxp3$^{+}$ Treg response seen in helminth infected ICOS$^{-/-}$ mice due to a failure in one or more of the following functional aspects of a Foxp3$^{+}$ Treg response:

- Improper priming, measured by expression of activation markers?
- A reduction in the rate of Treg proliferation?
- Failure of Treg survival?

Furthermore, is ICOS required for induction of Foxp3$^{+}$ Treg IL-10 during helminth infection?

***Results***

### *4.1 Priming and activation of Foxp3$^+$ Treg is normal in* H. polygyrus *infected ICOS$^{-/-}$ mice.*

Following immune priming, T cells begin to increase expression of cell surface proteins associated with activation (267, 268). To test if ICOS co-stimulation is required for priming and activation of Foxp3$^+$ Treg during helminth infection, we measured the expression of a number of Foxp3$^+$ Treg activation markers at d 7 of *H. polygrus* infection in the MLN of WT and ICOS$^{-/-}$ mice. Increases in the expression of GITR, CD25 and CD103 are indicative of Foxp3$^+$ Treg activation. At d 7 of *H. polygyrus* infection, there was a significant increase in the expression of GITR on Foxp3$^+$ Treg in both WT and ICOS$^{-/-}$ mice (Fig 4.1 A). In addition, the level of CD25 on Foxp3$^+$ Treg was significantly increased in ICOS$^{-/-}$ mice, with WT Foxp3$^+$ Treg exhibiting a trend for increased CD25 expression (Fig 4.1 C). There was also a trend for increased CD103 expression on Foxp3$^+$ Treg in both strains (Fig 4.1 D).

Conversely, downregulation in the expression of CD62L and PD-1 also indicates Foxp3$^+$ Treg activation. Following infection, the expression of CD62L on Foxp3$^+$ Treg significantly decreased in both strains of mice (Fig 4.1 B), and the expression of the inhibitory co-stimulator PD-1 showed a trend toward down-regulation on Foxp3$^+$ Treg in both strains (Fig 4.1 E). Notably, whereas WT Foxp3$^+$ Treg showed a decrease in the expression of CD127 at d 7 of *H. polygyrus* infection, the level of CD127 expression on ICOS$^{-/-}$ Foxp3$^+$ Treg did not change (Fig. 4.1 F) which could indicate that ICOS deficient Treg remain dependent on IL-7 signalling for homeostasis during *H. polygyrus* infection. Taken together, these data suggest that ICOS co-stimulation is dispensable for the activation of Foxp3$^+$ Treg during *H. polygyrus* infection. This would imply that the reduced expansion of Foxp3$^+$ Treg seen in helminth infected ICOS$^{-/-}$ mice is not due to impaired priming and

activation of $Foxp3^+$ Treg.

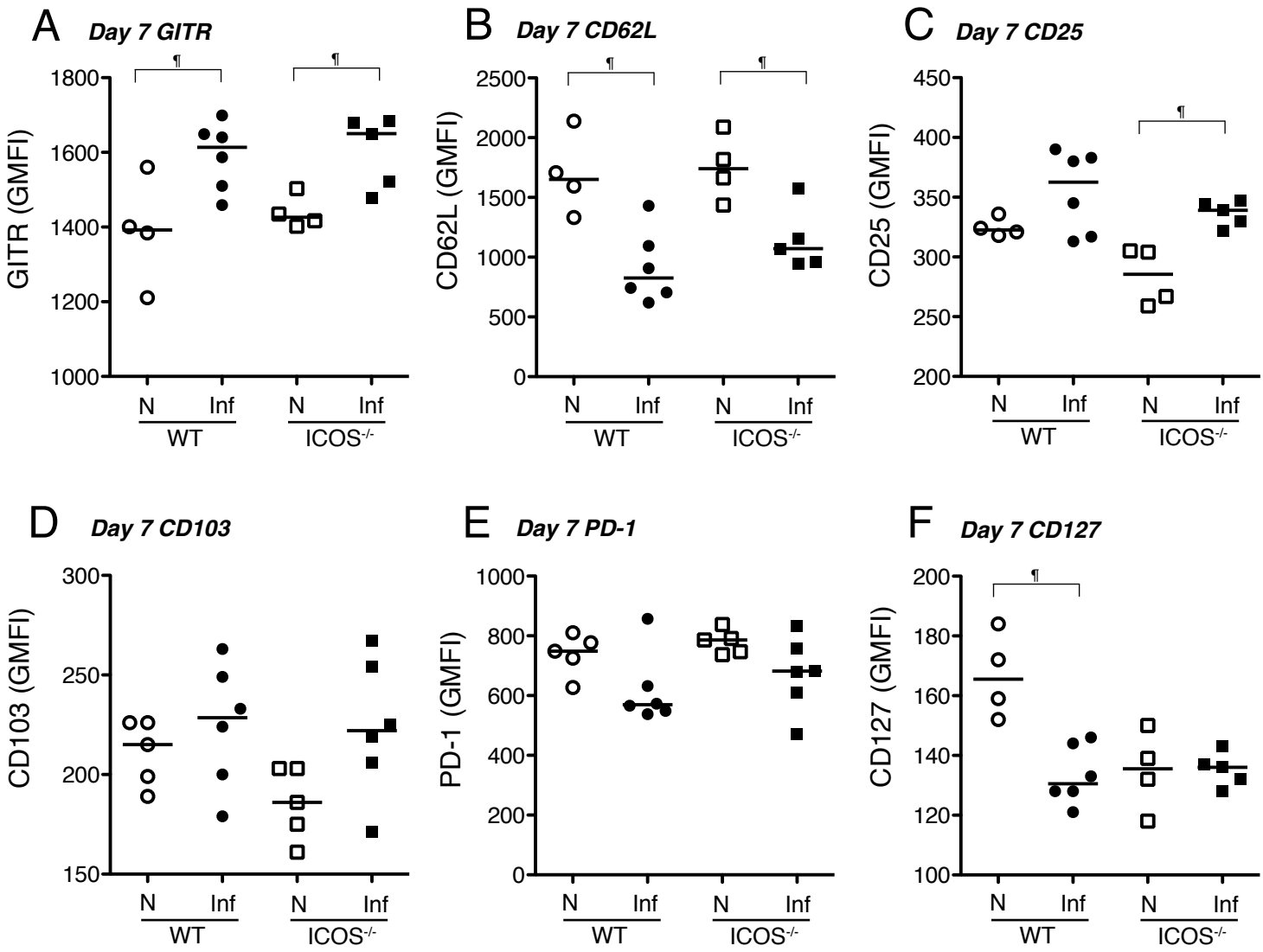



***Figure 4.1 ICOS is dispensable for Foxp3+ Treg activation following* H. polygyrus *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the expression of a number of surface markers known to denote Foxp3$^{+}$ Treg activation were measured in the MLN at d 7 pi. (A-F) Geometric mean of fluorescence intensity for GITR, CD62L, CD25, CD103, PD-1 and CD127 on Foxp3$^{+}$ Treg in the MLN at d 7 pi. (G-L) Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Symbols denote individual mice and lines denote median values. Data are representative of two separate experiments. ¶ denotes non-parametric significance tests $p = < 0.05$, Mann Whitney U.

### *4.2 Activation of Foxp3$^+$ Treg during egg-phase* **S. mansoni** *infection does not require ICOS.*

To test if ICOS was also required for Foxp3$^+$ Treg activation during the egg-phase of *S. mansoni* infection, we measured the expression of CD25 and CD103 on Foxp3$^+$ Treg in the spleen and MLN of WT and ICOS$^{-/-}$ mice at wk 8 of *S. mansoni* infection. In keeping with previous observations, Foxp3$^+$ Tregs in the spleen and MLN of WT mice increased expression of CD25 and CD103 upon *S. mansoni* infection (Fig 4.2 A-D). Alongside WT Foxp3$^+$ Treg, ICOS$^{-/-}$ Foxp3$^+$ Treg showed a similar increase in CD25 and CD103 expression following infection (Fig. 4.2 A-D). Therefore, in terms of CD25 and CD103 expression, this data suggests that similar to *H. polygyrus* infection, ICOS signalling does not affect activation of Foxp3$^+$ Treg during *S. mansoni* infection.

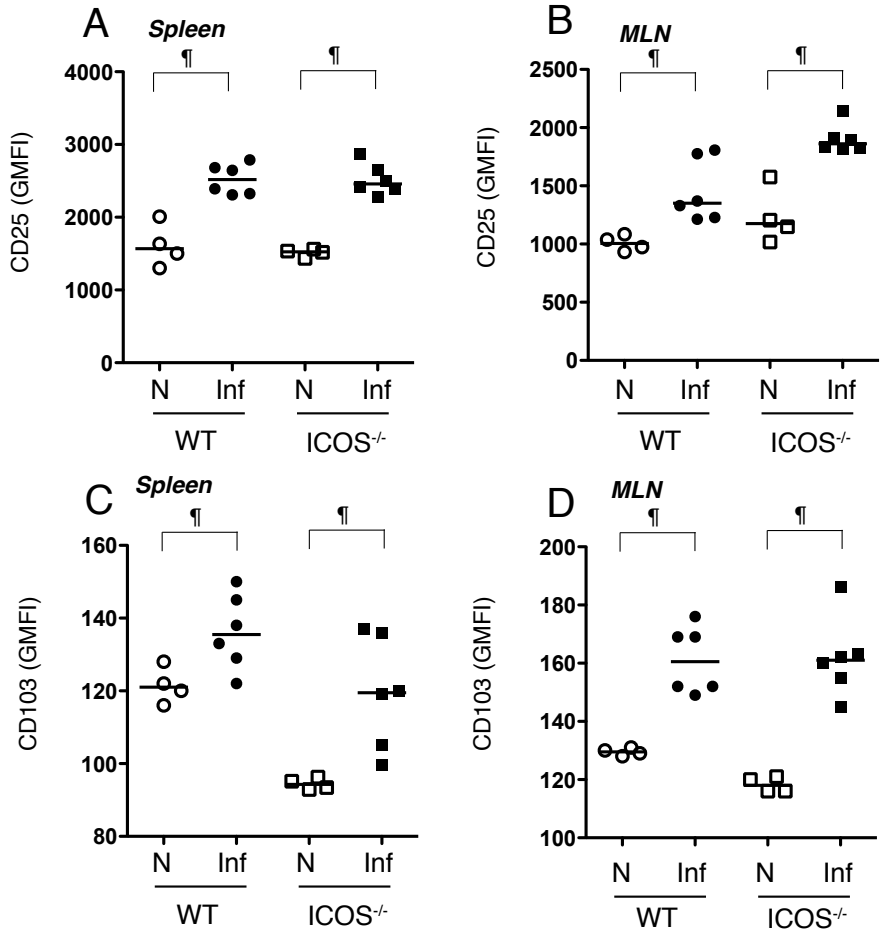



***Figure 4.2 During* S. mansoni *infection Foxp3$^+$ Treg activation is independent of ICOS.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *S. mansoni* and the expression of CD25 and CD103 on Foxp3$^+$ Treg were measured in the spleen and MLN at wk 8 pi. (A & B) Geometric mean of fluorescence intensity for CD25 on CD4$^+$Foxp3$^+$ Treg in the spleen and MLN respectively at wk 8 pi. (C & D) Geometric mean of fluorescence intensity for CD103 on CD4$^+$Foxp3$^+$ Treg in the spleen and MLN respectively at wk 8 pi. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Symbols denote individual mice and lines denote median values. Data are representative of two separate experiments. ¶ denotes non-parametric significance tests $p = < 0.05$, Mann Whitney U.

### *4.3 ICOS is dispensable for activation of Treg during infection with the filarial nematode* L. sigmodontis*.*

Infection with the filarial nematode *L. sigmodontis* induces increases in Foxp3$^+$ Treg expression of ICOS, PD-1 and GITR (15). To ask if Foxp3$^+$ Treg activation during filarial nematode infection showed a requirement for ICOS co-stimulation, we measured the expression of GITR and PD-1 on Foxp3$^+$ Treg in the pleural cavity (PC), spleen and tLN of BALB/*c L. sigmodontis* infected mice treated with anti-ICOS Ab or IgG control Ab. In line with our previous observations (15), *L. sigmodontis* infection induced an increase in GITR expression on Foxp3$^+$ Treg in the PC and spleen (Fig. 4.3 A & B). However, due to variation, this did not reach statistical significance in the tLN (Fig. 4.3 C). There was no difference in the intensity of Foxp3$^+$ Treg GITR expression between IgG control and anti-ICOS Ab treated groups suggesting that ICOS does not affect Foxp3$^+$ Treg GITR expression during *L. sigmodontis* infection (Fig. 4.3 A & B). Similarly, in the PC and spleen, Foxp3$^+$ Treg increased expression of PD-1 in response to infection (Fig. 4.3 D & E). In the tLN there was no increase in Foxp3$^+$ Treg PD-1 expression in control mice, but in the anti-ICOS Ab treated mice the intensity of PD-1 expression on tLN Foxp3$^+$ Treg showed a significant decrease (Fig. 4.3 F). In the PC, the infection-induced increase in PD-1 expression by Foxp3$^+$ Treg was unaffected by anti-ICOS treatment, further suggesting that Foxp3$^+$ Treg activation is independent of ICOS (Fig. 4.3 D). In the spleen, however, anti-ICOS Ab treatment boosted both the basal and infected levels of Foxp3$^+$ Treg PD-1 to a level significantly greater than in control Ab treated mice (Fig. 4.3 E). This could indicate that ICOS down-regulates Foxp3$^+$ Treg PD-1 expression in the spleen, or it is possible that Foxp3$^+$ Tregs are primed and activated as part of an immune response to the anti-ICOS antibody. With the exception of PD-1 in the spleen, these data suggest, that in keeping with *H. polygyrus* and *S. mansoni* infection, activation of Foxp3$^+$ Treg does not require ICOS co-stimulation during *L. sigmodontis* infection.

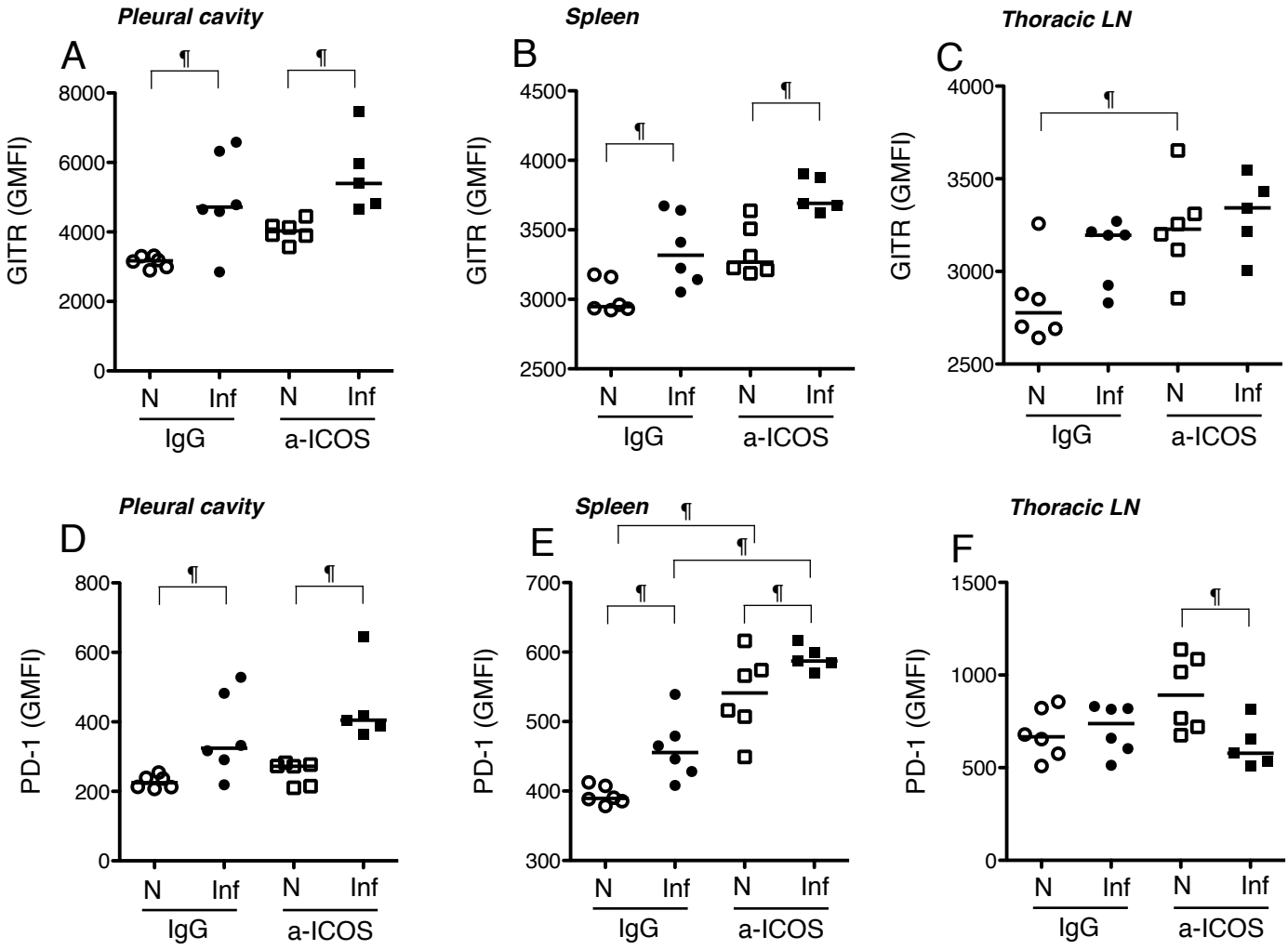



***Figure 4.3 During* L. sigmodontis *infection Foxp3⁺ Treg activation is independent of ICOS.*** BALB/c mice were infected with *L. sigmodontis* and treated with either anti-ICOS or an irrelevant isotype control mAb. Mice were autopsied on d 12 pi and the expression of GITR and PD-1 on Foxp3$^+$ Treg were measured in the PC, spleen and tLN. (A-C) Geometric mean of fluorescence intensity for GITR on CD4$^+$Foxp3$^+$ Treg in the PC, spleen and tLN respectively at day 12 of *L. sigmodontis* infection. (D-F) Geometric mean of fluorescence intensity for PD-1 on CD4$^+$Foxp3$^+$ Treg in the PC, spleen and tLN respectively at day 12 of *L. sigmodontis* infection. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice . Symbols denote individual mice and lines denote median values. ¶ denotes non-parametric significance tests p = < 0.05, Mann Whitney U.

### *4.4 ICOS is dispensable for the proliferation of Foxp3$^+$ Tregs during helminth infection*

That activation of Foxp3$^+$ Treg took place in the absence of ICOS implies that priming of Foxp3$^+$ Treg is unaffected by ICOS. This could suggest that ICOS acts downstream of immune priming and drives the proliferation of Foxp3$^+$ Tregs during helminth infection. To test if ICOS was required for proliferation of Foxp3$^+$ Tregs we labelled dividing cells *in vivo* by administration of BrdU to *H. polygyrus* and *S. mansoni* infected WT and ICOS$^{-/-}$ mice 1 d prior to autopsy. Similarly, BALB/c mice infected with *L. sigmodontis* and treated with an anti-ICOS antibody were given BrdU 1 d prior to autopsy.

Following *H. polygyrus* infection, the percentage of BrdU$^+$Foxp3$^+$ cells in the MLN increased significantly in both strains of mice demonstrating that Foxp3$^+$ Tregs proliferate in response to *H. polygyrus* infection (Fig. 4.4 A). There was no difference in the percentage of BrdU$^+$Foxp3$^+$ Tregs between infected ICOS$^{-/-}$ and WT mice. This would suggest that ICOS is not required for Foxp3$^+$ Treg proliferation during *H. polygyrus* infection.

In contrast to *H. polygyrus* infection, infection with *S. mansoni* had no marked effect on Foxp3$^+$ Treg proliferation. At both wk 6 and wk 8 of *S. mansoni* infection there was no change in the percentage of proliferating Foxp3$^+$ Tregs in the spleen (Fig 4.4 B & C). Only in the MLN at wk 8 of infection was there a small non-significant increase in the percentage of proliferating Foxp3$^+$ Treg in both WT and ICOS$^{-/-}$ mice (Fig. 4.4 D). Therefore, although unchanged by infection, there was no difference in the percentage of BrdU$^+$Foxp3$^+$ Tregs between WT and ICOS$^{-/-}$ mice, further demonstrating that ICOS is dispensable for Foxp3$^+$ Treg proliferation.

In keeping with previous studies, *L. sigmodontis* induced an increase in the proportion of BrdU$^+$Foxp3$^+$ Treg in the PC indicating an increase in the rate of Foxp3$^+$ Treg proliferation

at the site of infection (Fig. 4.4 E). In the spleen and draining lymph node, however, preliminary data showed there were only trends for an increase in the proportion of $BrdU^+$ $Foxp3^+$ Treg following infection (Fig. 4.4 F & G). In the PC, ICOS blockade had no effect on the percentage of $BrdU^+Foxp3^+$ Treg (Fig. 4.4 E), suggesting that similar to *H. polygyrus* and *S. mansoni* infection, *L. sigmodontis* induced $Foxp3^+$ Treg proliferation is independent of ICOS signalling. Taken together, these data suggest that the proliferative capacity of $Foxp3^+$ Treg is unchanged by ICOS co-stimulation during helminth infection. This suggests that the failed expansion of $Foxp3^+$ Treg seen in helminth infected $ICOS^{-/-}$ mice is not due to reduced $Foxp3^+$ Treg division.

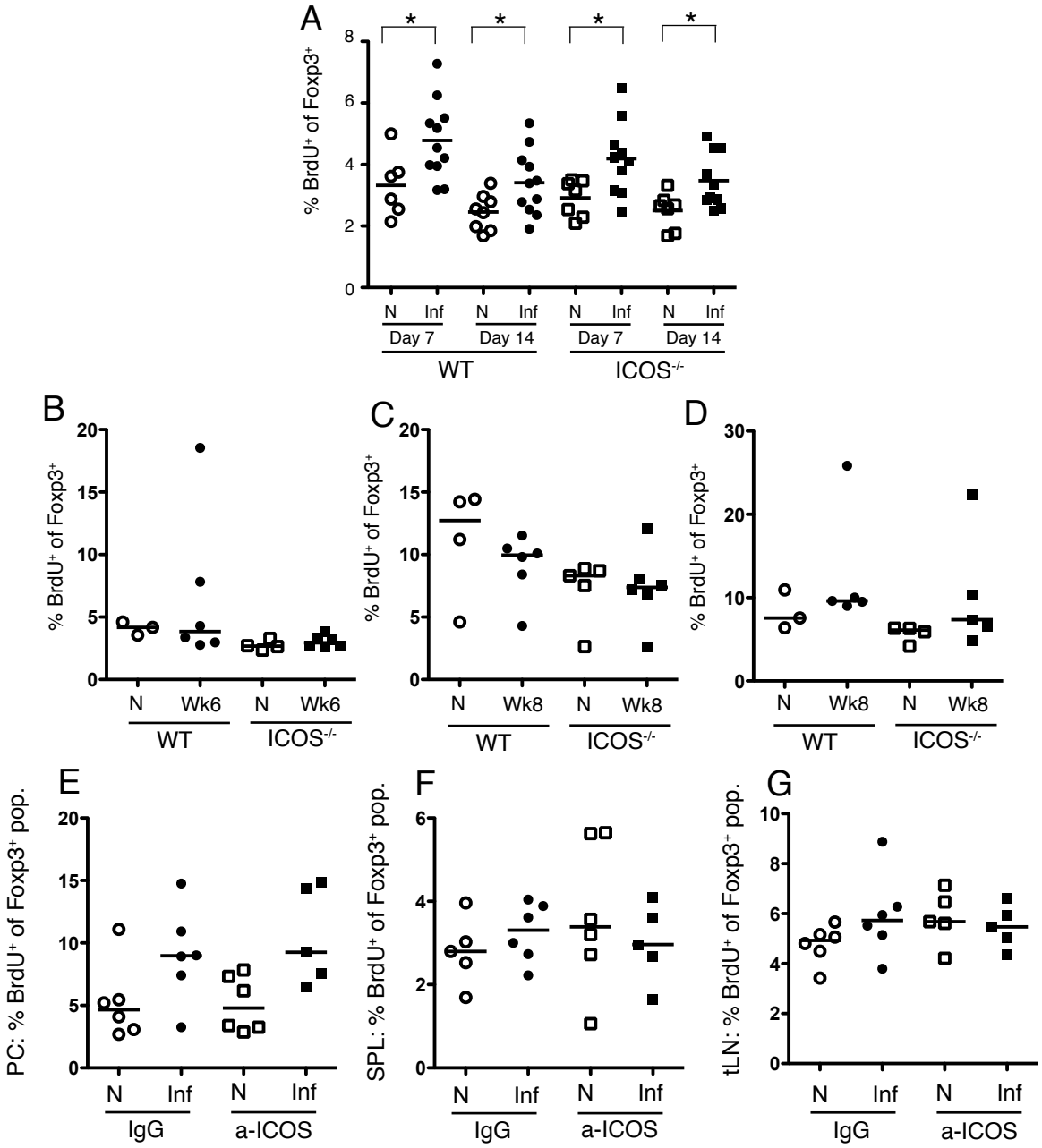



***Figure 4.4. ICOS is dispensable for the proliferation of Foxp3+ Tregs during infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* (A), *S. mansoni* (B-D) or *L. sigmodontis* (E-G) and given 1mg of BrdU i.p. 1 d prior to autopsy to label dividing cells. (A) Percentage of CD4$^+$ Foxp3$^+$ cells expressing BrdU in the MLN at d 7 and d 14 of *H. polygyrus* infection. (B & C) Percentage of CD4$^+$ Foxp3$^+$ cells expressing BrdU in the spleen at wk 6 and wk 8 of *S. mansoni* infection respectively. (D) Percentage of CD4$^+$ Foxp3$^+$ cells expressing BrdU in the MLN at wk 8 of *S. mansoni* infection. (E-G) Percentage of CD4$^+$ Foxp3$^+$ cells expressing BrdU in the PC, spleen and tLN respectively at d 12 of *L. sigmodontis* infection. Symbols denote individual mice and lines denote means. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. For (A), data points show individual mice from two separate experiments. * p = < 0.05, two way ANOVA followed by Tukeys post hoc test. For (B-G), data points show individual mice from one experiment.

### *4.5 In contrast to Foxp3$^+$ Treg, Foxp3$^-$ Teff require ICOS co-stimulation for optimal proliferation during helminth infection.*

Whilst the proliferation of Foxp3$^+$ Treg was independent of ICOS, the rate of proliferation of Foxp3$^-$ Teff showed a marked dependency on ICOS during helminth infection. At d 7 of *H. polygyrus* infection, there was a significant increase in the percentage of BrdU$^+$Foxp3$^-$ Teff in WT mice (Fig. 4.5 A). Although the percentage of BrdU$^+$Foxp3$^-$ Teff also increased in ICOS deficient mice, the increase was of smaller magnitude such that the rate of proliferating Foxp3$^-$ Teff was significantly lower than that of WT mice. At d 14 of infection the proportion of proliferating Foxp3$^-$ Teff remained increased, although this did not reach statistical significance, and was at a similar level in both WT and ICOS$^{-/-}$ mice (Fig. 4.5 A). This was in contrast with the proportion of proliferating Foxp3$^+$ Treg, which remained significantly elevated at d 14 of infection, indicating a stronger Foxp3$^+$ Treg response. Thus, unlike Foxp3$^+$ Treg, Foxp3$^-$ Teff require ICOS co-stimulation for optimal proliferation during early *H. polygyrus* infection.

Foxp3$^-$ Teff proliferation showed a similar requirement for ICOS during *S. mansoni* infection. At wk 8 of *S. mansoni* infection, both WT and ICOS$^{-/-}$ mice showed an increase in the percentage of BrdU$^+$ Foxp3$^-$ Teff in the spleen (Fig. 4.5 B), but in the ICOS$^{-/-}$ mice the percentage of BrdU$^+$ Foxp3$^-$ Teff was significantly lower than WT mice, suggesting that ICOS signalling contributes to Foxp3$^-$ Teff proliferation during *S. mansoni* infection. In the MLN there was a similar increase in the percentage of proliferating Foxp3$^-$ Teff in both WT mice and ICOS$^{-/-}$ mice, but again this trended toward a lower level in the ICOS$^{-/-}$ mice (Fig. 4.5 C). In the spleen at wk 6 of infection, in WT mice the increase in the percentage of BrdU$^+$ Teff was lower than that of wk 8 and did not reach statistical significance, but mirroring the trends of wk 8, this was absent in ICOS$^{-/-}$ mice (Fig. 4.5 D). Due to cell death we were unable to measure the proportion of BrdU$^+$ Teff in the MLN at wk 6 of infection (data not shown). These data suggest that ICOS promotes Foxp3$^-$ Teff proliferation during

the early egg-phase of *S. mansoni* infection.

Blockade of ICOS with an antibody had a different effect on Teff proliferation to germline ICOS deficiency. During *L. sigmodontis* infection, the percentage of proliferating $BrdU^+$ $Foxp3^-$ Teff increased in both the spleen and PC and this was unaffected by treatment with anti-ICOS Ab, although further experiments are required to confirm this (Fig. 4.5 E & F respectively). Taken together, these data suggest that ICOS has different effects on $Foxp3^+$ Treg and $Foxp3^-$ Teff proliferation. ICOS was dispensable for the proliferation of $Foxp3^+$ Treg, but $Foxp3^-$ Teff were dependent on ICOS optimal proliferation during the early stages of *H. polygyrus* infection and egg-phase of *S. mansoni* infection.

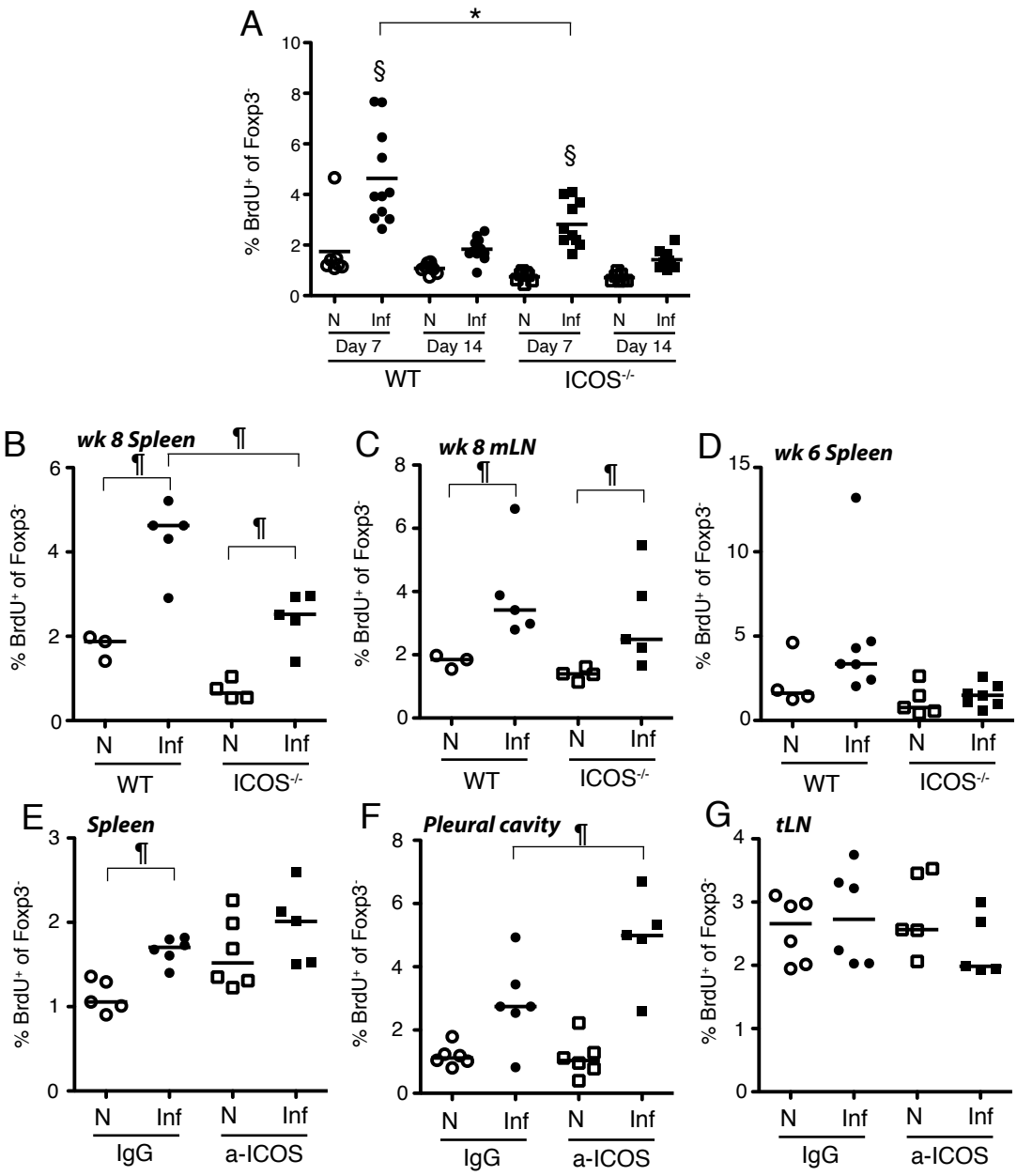



***Figure 4.5. In contrast to Foxp3+ Treg, Foxp3- Teff require ICOS for maximum proliferation during helminth infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* (A), *S. mansoni* (B-D) or *L. sigmodontis* (E-G) and given 1mg of BrdU i.p. 1 d prior to autopsy to label dividing cells. (A) Percentage of CD4$^{+}$Foxp3$^{-}$ cells expressing BrdU at d 7 and d 14 of *H. polygyrus* infection. (B & C) Percentage of CD4$^{+}$Foxp3$^{-}$ cells expressing BrdU in the spleen and MLN and wk 8 of *S. mansoni* infection respectively. (D) Percentage of CD4$^{+}$ Foxp3$^{-}$ cells expressing BrdU in the spleen at wk 6 of *S. mansoni* infection. (E-G) Percentage of CD4$^{+}$Foxp3$^{-}$ cells expressing BrdU in the PC, spleen and tLN respectively at day 12 of *L. sigmodontis* infection. Symbols denote individual mice and lines denote means. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. For (A), data points represent individual mice from two separate experiments. § denotes significant increase due to infection. * $p = < 0.05$, two way ANOVA followed by Tukeys post hoc test. ¶ denotes non-parametric significance tests $p = < 0.05$, Mann Whitney U.

### *4.6 ICOS deficiency leads to an increase in the rate of Treg apoptosis*

Given that ICOS was dispensable for Foxp3$^+$ Treg proliferation during infection, we next asked if the defective Foxp3$^+$ Treg responses seen in helminth infected ICOS$^{-/-}$ mice were due to impaired survival. Therefore, we measured the *ex vivo* percentage of apoptotic Annexin V$^+$CD25$^+$CD4$^+$ T cells in the MLN of d 7 and d 10 *H. polygyrus* infected WT and ICOS$^{-/-}$ mice. CD25 was used as a surrogate marker for Foxp3$^+$ Tregs as co-detection of Annexin V and Foxp3 was not possible. The proportion of CD25$^+$ Treg undergoing apoptosis in naive mice was consistently greater in ICOS$^{-/-}$ mice when compared to WT, suggesting that ICOS contributes to CD25$^+$ Treg survival under normal homeostatic conditions (Fig. 4.6 A & B). Upon *H. polygyrus* infection, both WT and ICOS$^{-/-}$ mice showed an increase in the percentage of apoptotic Annexin V$^+$CD25$^+$ Tregs in the MLN, but the proportion of apoptotic Treg remained significantly greater in ICOS deficient animals (Fig. 4.6 A & B), which suggests ICOS also promotes Treg survival during *H. polygyrus* infection. The pattern in the rate of Treg apoptosis was similar in the CD25$^-$ Teff; the percentage of Annexin V$^+$CD25$^-$ cells was greater in both naïve and *H. polygyrus* infected ICOS deficient mice (Fig. 4.6 C). Thus, during homeostatic conditions and during *H. polygyrus* infection, ICOS co-stimulation promotes the survival of both CD25$^+$ Treg and CD25$^-$ Teff, potentially explaining the failed expansion of Treg in helminth infected ICOS$^{-/-}$ mice.

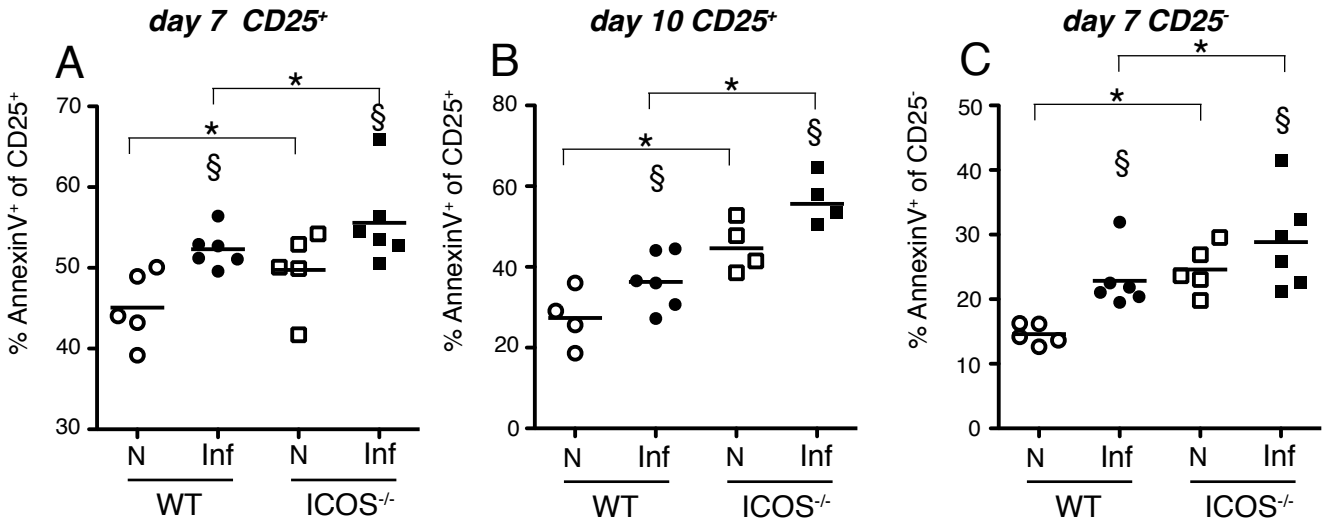



***Figure 4.6. ICOS deficiency leads to an increase in the rate of Treg apoptosis.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the percentage of Annexin V$^+$ CD25$^+$ cells of total CD4$^+$ in the MLN were quantified at d 7 (A) and d 10 (B) of infection. (C) Percentage of Annexin V$^+$ CD25$^-$ cells of total CD4$^+$ in the MLN at day 7. Symbols denote individual mice and lines denote means. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. § denotes significant increase due to infection * p = < 0.05, two way ANOVA followed by Tukeys post hoc test.

### *4.7 The attrition rate of BrdU labelled Foxp3$^+$ Treg is unchanged by* H. polygyrus *infection or ICOS signalling*

Annexin V staining gives a good measure of the rate of cellular apoptosis at a given time point. As a complement to Annexin V staining, we performed BrdU pulse chase experiments to measure the loss of Foxp3$^+$ Treg during *H. polygyrus* infection. WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus,* then at d 5 pi pulsed with BrdU by a single i.p. injection followed by 2 days of oral administration to label all Foxp3$^+$ Tregs that divided in this time period. The proportion of BrdU$^+$Foxp3$^+$ cells in the MLN measured on d 10 pi was compared to the baseline measurement on d 7 pi and the fold reduction was calculated to determine the loss of BrdU$^+$Foxp3$^+$ Treg over this period. Although the

individual experiments did not give consistent results, when the data from three separate experiments was combined there was no significant increment in the loss of $BrdU^{+}Foxp3^{+}$ cells between d 7 and d 10 in *H. polygyrus* infected mice when compared to naïve controls, and there were no differences between naïve or infected WT and $ICOS^{-/-}$ mice (Fig. 4.7 A). This would suggest that neither *H. polygyrus* infection nor ICOS signalling affects the loss of $BrdU^{+}Foxp3^{+}$ Treg.

As a control to ensure that any loss of $BrdU^{+}Foxp3^{+}$ Treg in the MLN did not simply reflect migration to the LP, we measured the percentage of $BrdU^{+}Foxp3^{+}$ Treg in the LP at d 7 and d 10 pi. Between d 7 and d 10 the percentage of $BrdU^{+}Foxp3^{+}$ Treg in the LP declined (Fig. 4.7 B), suggesting that the reduction in the percentage of $BrdU^{+}Foxp3^{+}$ Treg in the MLN is not due to trafficking to the LP. Further, the percentage of $BrdU^{+}Foxp3^{+}$ Treg in the LP of WT and $ICOS^{-/-}$ *H. polygyrus* infected mice was equal (Fig. 4.7 B), suggesting that similar to the MLN (Fig 4.4 A), ICOS co-stimulation is dispensable for $Foxp3^{+}$ Treg proliferation at the site of infection.

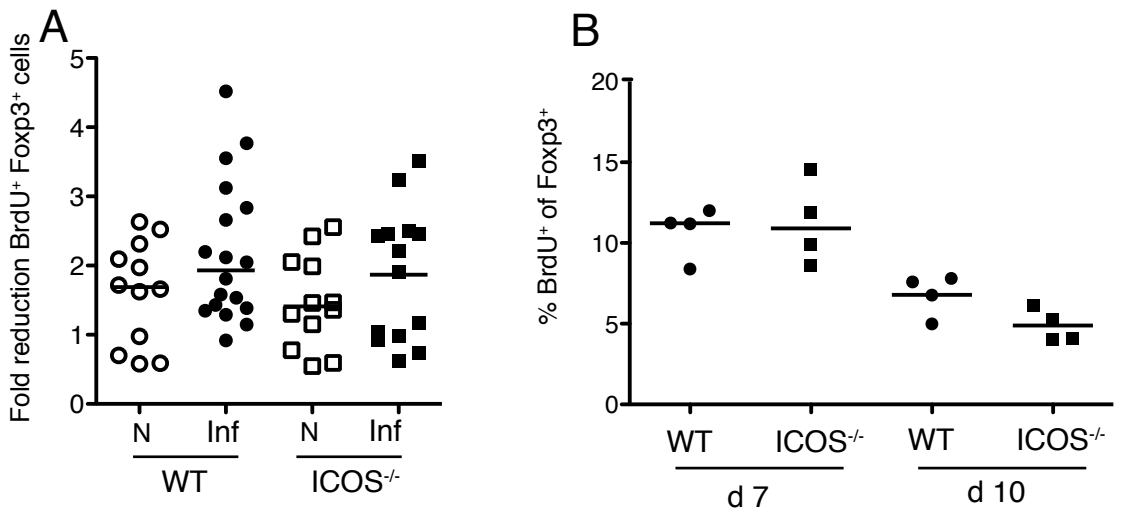



***Figure 4.7 The attrition rate of BrdU labelled Foxp3+ Treg is unchanged by* H. polygyrus *infection or ICSO signalling.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and 1 mg BrdU was administered to animals i.p. at d 5 pi and at 0.8 mg/ml in the drinking water from d 5 to d 7 pi. Mice were autopsied on d 7 and d 10 of infection. (A) Fold decrease in the number of BrdU$^{+}$Foxp3$^{+}$ Treg cells in the MLN between d 7 and d 10 of infection. (B) Percentage of CD4$^{+}$Foxp3$^{+}$ cells expressing BrdU in the LP at d 7 and d 10 of *H. polygyrus* infection. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Symbols denote individual mice and lines denote means.

### *4.8 IL-2 production from Foxp3- Teff cells during* H. polygyrus *infection is reduced in the absence of ICOS*

The T cell cytokine IL-2 is critically important for homeostasis and survival of Treg in the periphery. Conflicting early *in vitro* evidence suggests that ICOS co-stimulation is either dispensable for T cell IL-2 production (187), or that it contributes to IL-2 production (197). To determine if ICOS contributes to *in vivo* IL-2 production during *H. polygyrus* infection, we infected WT and ICOS deficient mice with *H. polygyrus* and FACS stained MLN CD4$^{+}$ T cells for intracellular IL-2 at d 7 and 14 pi. At d 7 and d 14 pi, in WT mice, *H. polygyrus* infection induced a significant increase in the proportion of CD4$^{+}$ T cells positive for IL-2 in the MLN, but in ICOS$^{-/-}$ mice the proportion of IL-2$^{+}$CD4$^{+}$ T cells did not increase (Fig 4.8 A

& B). This preliminary data suggests that ICOS is required for T cell IL-2 during *H. polygyrus* infection and the reduction in IL-2 availability could potentially explain why Treg survival is impaired in the absence of ICOS.

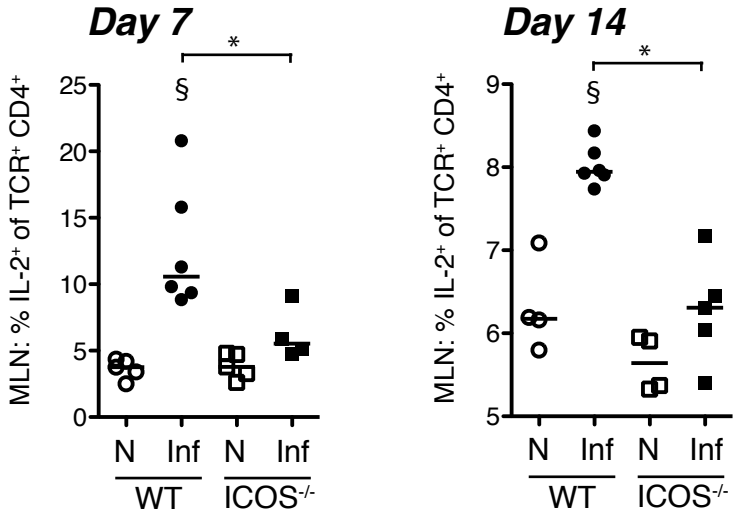



***Figure 4.8. IL-2 production from Foxp3⁻ Teff cells during* H. polygyrus *infection is reduced in the absence of ICOS.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the percentage of CD4$^{+}$ T cells positive for IL-2 in the MLN at d 7 and d 14 pi was determined by intracellular flow cytometry. (A) Percentage of CD4$^{+}$IL-2$^{+}$ T cells the MLN at d 7 pi. (B) Percentage of CD4$^{+}$IL-2$^{+}$ T cells the MLN at d 14 pi. Symbols denote individual mice and lines denote means. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. § denotes significant increase due to infection * p = < 0.05, Mann Whitney t-test.

### *4.9 Foxp3$^{+}$ Treg, but not Foxp3$^{-}$ Teff, require ICOS co-stimulation for IL-10 production during* H. polygyrus *infection.*

Secretion of IL-10 is one of the main mechanisms used by Foxp3$^{+}$ Treg for suppressor function. To assess the contribution of ICOS to Foxp3$^{+}$ Treg function during helminth infection, we infected WT and ICOS deficient mice with *H. polygyrus* and FACS stained Foxp3$^{+}$ and Foxp3$^{-}$ CD4$^{+}$ T cells in the MLN and LP for intracellular IL-10. During *H. polygyrus* infection, in the LP there was an increase in the percentage of IL-10$^{+}$ cells within the Foxp3$^{+}$ population in WT mice, but this was completely absent in ICOS deficient mice

(Fig. 4.8 A). Interestingly, the percentage of IL-10 producing cells in the Foxp3$^-$ Teff compartment was unchanged by *H. polygyrus* infection suggesting that Foxp3$^+$ Treg are the major source of IL-10 at the site of *H. polygyrus* infection (Fig. 4.8 B). Similar to the LP, in the MLN WT Foxp3$^+$ Treg increased IL-10 production on infection (Fig. 4.8 C) and although ICOS$^{-/-}$ Foxp3$^+$ Treg also increased IL-10 protein, this was to a significantly lower level than WT. In contrast to the LP, MLN Foxp3$^-$ Teff IL-10 increased on infection (Fig. 4.8 D). There was, however, no difference in the level of Teff IL-10 between WT and ICOS$^{-/-}$ mice. Taken together, these data suggest that ICOS differentially regulates Foxp3$^+$ Treg and Foxp3$^-$ Teff IL-10 production, and that the level of Foxp3$^+$ Treg requirement for ICOS driven IL-10 production depends on tissue location. In the intestinal LP Foxp3$^+$ Treg are dependent on ICOS co-stimulation for IL-10 protein during *H. polygyrus* infection. In the local lymph node, ICOS signalling contributed to Foxp3$^+$ Treg IL-10 production, but was dispensable for Foxp3$^-$ Teff IL-10 production.

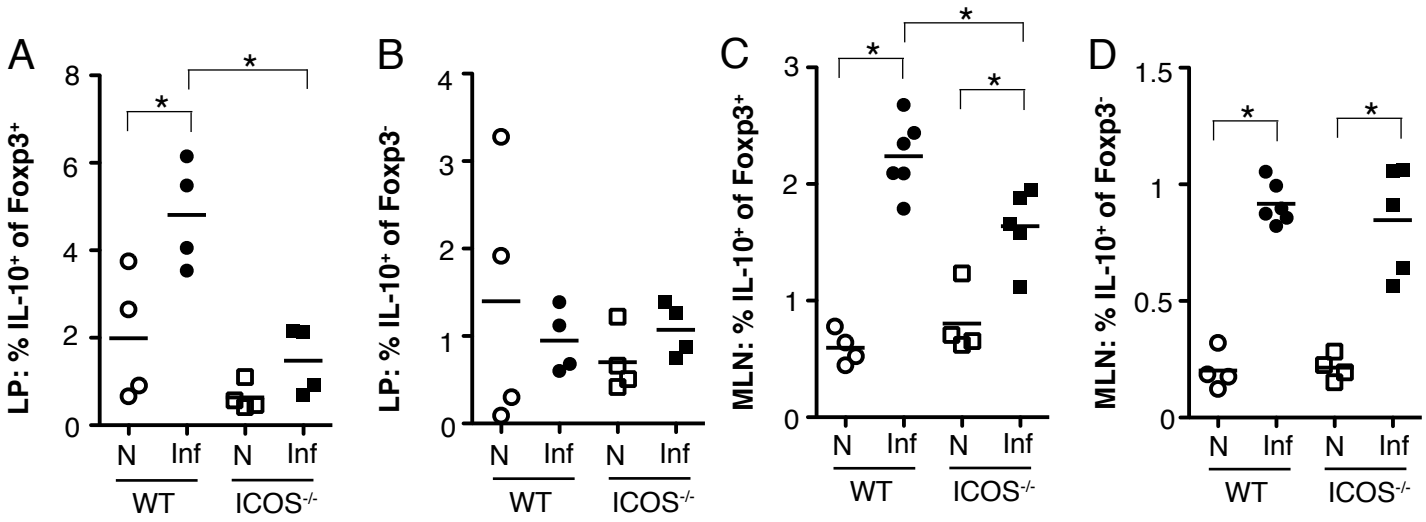



***Figure 4.9 ICOS is required for Foxp3$^+$ Treg, but not Foxp3$^-$ Teff, IL-10 protein production during* H. polygyrus *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the proportion of IL-10$^+$ cells within the CD4$^+$ Foxp3$^+$ and Foxp3$^-$ populations was determined in the LP and MLN by flow cytometry. (A) Percentage of IL-10$^+$ within the LP CD4$^+$Foxp3$^+$ and (B) Foxp3$^-$ population at d 7 pi. (C) Percentage of IL-10$^+$ within the MLN CD4$^+$Foxp3$^+$ and (D) Foxp3$^-$ population at d 7 pi. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Symbols denote individual mice and lines denote median values. Data are representative of two separate experiments. * denotes non-parametric significance tests p = < 0.05, Mann Whitney U.

### *4.10 In contrast to* H. polygyrus *infection, both Foxp3$^+$ Treg and Foxp3$^-$ Teff utilize ICOS signalling for optimal IL-10 production during S. mansoni infection*

IL-10 is critically important for protection from fatal immune pathology during the egg-phase of *S. mansoni* infection (30), and it has been suggested that ICOS contributes to the control of hepatic pathology through IL-10 (241). To investigate the contribution of ICOS to IL-10 production from Foxp3$^+$ and Foxp3$^-$ sources during *S. mansoni* infection, we infected

WT and ICOS$^{-/-}$ mice with *S. mansoni* and FACS stained CD4$^{+}$ T cells from the spleen and liver for intracellular IL-10 and intranuclear Foxp3. Similar to *H. polygyrus* infection, in the liver of *S. mansoni* infected WT mice, Foxp3$^{+}$ Tregs significantly increased production of IL-10 (Fig. 4.9 A). This increase was missing in ICOS deficient mice suggesting that ICOS promotes hepatic Foxp3$^{+}$ Treg IL-10 production during *S. mansoni* infection. In WT mice, in contrast to the *H. polygyrus* infection site where only Foxp3$^{+}$ Treg increased IL-10, Foxp3$^{-}$ Teff at the site of *S. mansoni* infection also upregulated IL-10 production (Fig. 4.9 B), suggesting that IL-10 from both Foxp3$^{+}$ Treg and Foxp3$^{-}$ Teff sources is required for the suppression of exuberant immune responses in the liver. In ICOS deficient mice, the percentage of hepatic Foxp3$^{-}$ IL-10$^{+}$ cells showed only a trend for an increase on *S. mansoni* infection and was significantly lower than that of WT mice (Fig. 4.9 B). Therefore, these data suggest that both Foxp3$^{+}$ Treg and Foxp3$^{-}$ Teff depend upon ICOS co-stimulation for IL-10 production at the site of *S. mansoni* infection.

Similar to the liver, the percentage of splenic IL-10$^{+}$Foxp3$^{+}$ Treg increased on infection in WT mice, but not in ICOS$^{-/-}$ mice (Fig. 4.9 C) suggesting that ICOS is required for Foxp3$^{+}$ Treg IL-10 production in the lymphoid tissue draining the site of *S. mansoni* infection. Again, the proportion of splenic Foxp3$^{-}$ Teff IL-10$^{+}$ cells increased on infection in WT mice, and also in ICOS$^{-/-}$ mice, although to a lesser extent than that of WT (Fig. 4.9 D). Thus, in contrast to *H. polygyrus* infection where only Foxp3$^{+}$ Treg require ICOS for IL-10, during *S. mansoni* infection Foxp3$^{+}$ Treg and Foxp3$^{-}$ Teff utilise ICOS signalling for IL-10 production.

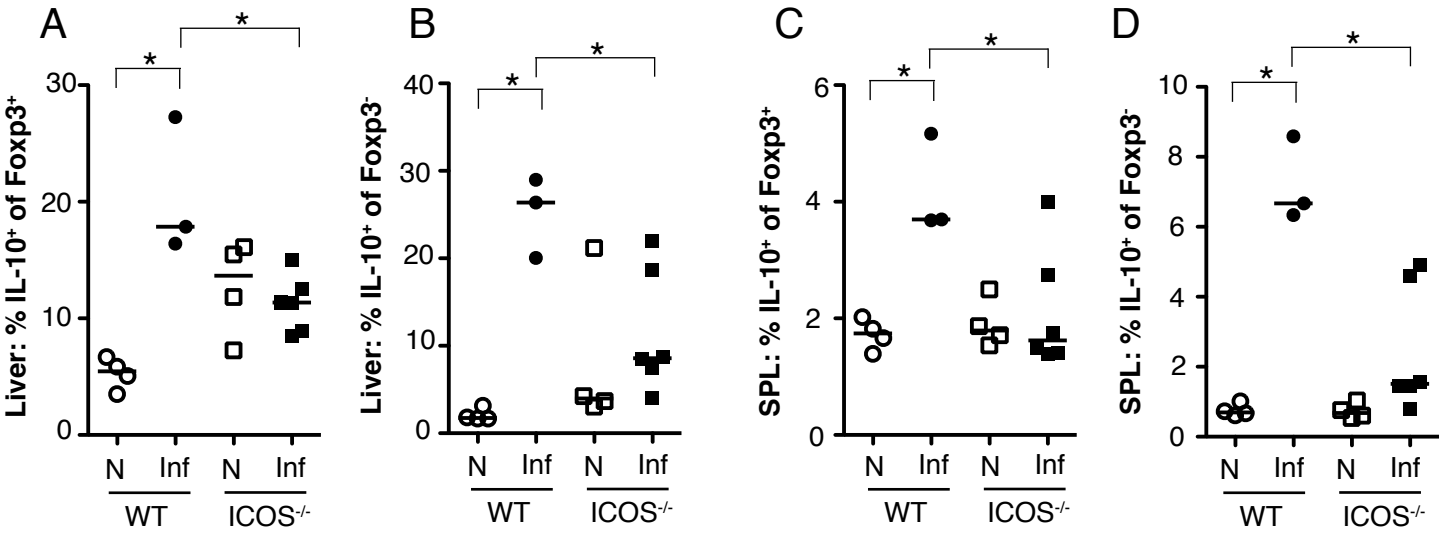



***Figure 4.10 In contrast to* H. polygyrus *infection, both Foxp3$^+$ Treg and Foxp3$^-$ Teff utilize ICOS signalling for optimal IL-10 production during* S. mansoni *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with *S. mansoni* and the proportion of IL-10$^+$ cells within the CD4$^+$Foxp3$^+$ and Foxp3$^-$ populations was determined in the liver and spleen by flow cytometry at wk 8 pi. (A) Percentage of IL-10$^+$ within the hepatic CD4$^+$Foxp3$^+$ and (B) Foxp3$^-$ population at wk 8 pi. (C) Percentage of IL-10$^+$ within the splenic CD4$^+$Foxp3$^+$ and (D) Foxp3$^-$ population at wk 8 pi. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Symbols denote individual mice and lines denote median values. Data show one experiment. * denotes non-parametric significance tests $p = < 0.05$, Mann Whitney U.

### *4.11 GFP expression in Foxp3$^+$ Treg from IL-10 GFP reporter mice does not increase on* H. polygyrus *infection and is unaffected by the absence of ICOS.*

To confirm the IL-10 flow cytometry data, we crossed ICOS$^{-/-}$ mice with IL-10 GFP reporter mice (TIGER mice). To date, one of the drawbacks of GFP reporter mice was that co-detection of GFP with Foxp3 was not possible by flow cytometry. The buffers required for intranuclear staining of Foxp3 degrade the GFP epitope, whereas the buffers used for intracellular staining of GFP do not perforate the nuclear membrane and impede staining of intranuclear Foxp3. However, I developed a novel protocol to fully detect both GFP and

Foxp3 by flow cytometry. This was achieved by adding a low concentration of TritonX-100 detergent to the manufacturer's intracellular staining buffers to simultaneously perforate both the cell membrane and nuclear membrane and fix them in this manner. Subsequently, a combination of antibodies was used to recover the GFP signal, and because the nuclear membrane had been perforated and fixed in an open configuration, Ab detection of Foxp3 was also possible. $ICOS^{-/-}$ TIGER mice were infected with *H. polygyrus* and GFP expression within the Foxp3 population was measured at d 7 pi in the MLN and LP. Although the basal proportion of LP $GFP^{+}$ $Foxp3^{+}$ Treg was significantly greater in $ICOS^{-/-}$ TIGER mice than WT, there was no increase in the proportion of LP $GFP^{+}$ $Foxp3^{+}$ Treg following *H. polygyrus* infection in either strain (Fig. 4.11 A & B). Similarly, there was no increase in the proportion of $GFP^{+}Foxp3^{-}$ Teff in WT or $ICOS^{-/-}$ mice following infection, but in contrast to $Foxp3^{+}$ Treg, within the $Foxp3^{-}$ Teff population there was no difference in the basal levels of GFP expression between WT and $ICOS^{-/-}$ mice (Fig. 4.11 C). In the MLN, there was no difference in the proportion of $GFP^{+}$ $Foxp3^{+}$ Treg or $GFP^{+}$ $Foxp3^{-}$ Teff between WT and $ICOS^{-/-}$ mice and no change in the proportion of these cells due to infection (Fig 4.11 D & E). These data suggest that *H. polygyrus* infection does not increase $Foxp3^{+}$ Treg or $Foxp3^{-}$ Teff GFP protein expression, and contrasts with the flow cytometry data that showed an ICOS dependent infection induced increase in $Foxp3^{+}$ Treg and $Foxp3^{-}$ Teff IL-10 protein production.

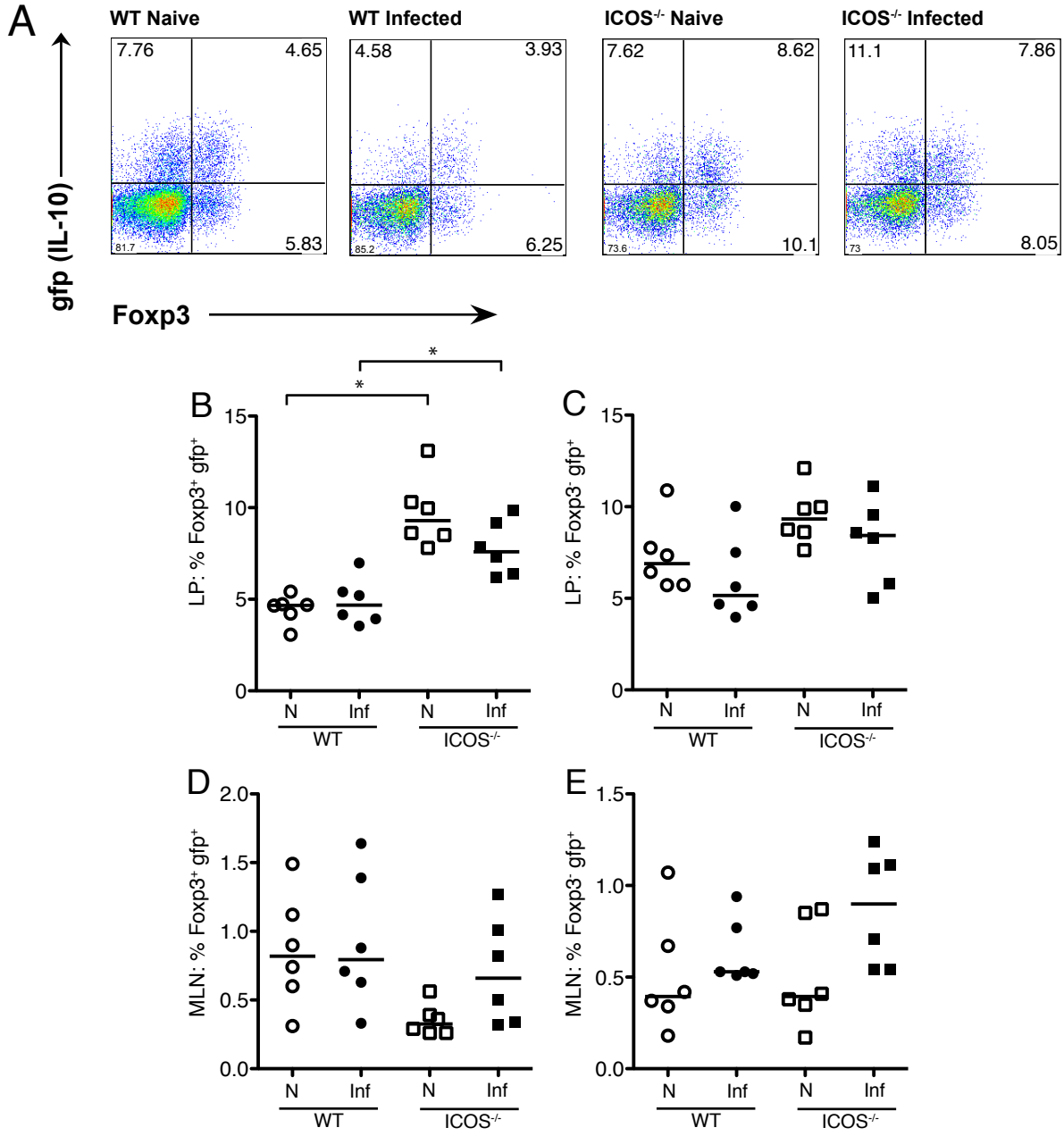



***Figure 4.11 GFP expression in Foxp3$^+$ Treg from IL-10 GFP reporter mice does not increase on* H. polygyrus *infection and is unaffected by the absence of ICOS.*** TIGER and ICOS$^{-/-}$ TIGER mice were infected with *H. polygyrus* and the proportion of GFP$^+$ cells within the CD4$^+$Foxp3$^+$ and Foxp3$^-$ populations was determined at d 7 pi by flow cytometry. (A) Representative FACS plots showing GFP versus Foxp3 in the LP at d 7 pi. (B) Percentage of GFP$^+$ within the CD4$^+$Foxp3$^+$ and (C) Foxp3$^-$ population in the LP at d 7 pi. (D) Percentage of GFP$^+$ within the CD4$^+$Foxp3$^+$ and (E) Foxp3$^-$ population in the MLN at d 7 pi. Open circles represent naïve WT mice and closed circles represent infected WT mice. Open squares represent naïve ICOS$^{-/-}$ mice and closed squares represent infected ICOS$^{-/-}$ mice. Symbols denote individual mice and lines denote mean values. Panels show combined data from two separate experiments. * p = < 0.05, two way ANOVA followed by Tukeys post hoc test.

### *4.12 Discussion*

The defective expansion and diminished maintenance of Foxp3$^+$ Treg in helminth infected ICOS deficient mice suggested a failure in one or more of the processes required for a complete Foxp3$^+$ Treg response. The first step in the generation of a T cell response is activation by cognate antigen in the MHC complex. If accompanied by appropriate co-stimulatory signals activated T cells undergo clonal expansion (268). The loss of ICOS signals during Foxp3$^+$ Treg priming could explain the impaired expansion of Foxp3$^+$ Treg that was evident in helminth infected ICOS$^{-/-}$ mice. One of the obstacles to measuring immune priming during helminth infection is the identification of recently antigen activated Foxp3$^+$ Treg. In the absence of a TCR transgenic to *H. polygyrus* antigens, we relied on the measurement of cell surface activation markers to give an indication of those Foxp3$^+$ Treg that have been primed by antigen and co-stimulatory signals. The activation phenotype of ICOS$^{-/-}$ Treg was similar to that of WT Treg suggesting that priming of Foxp3$^+$ Treg in the absence of ICOS was normal. This is in keeping with the observation that T cells only increase ICOS expression following TCR engagement (189) and suggests that the ICOS pathway contributes to the expansion of Foxp3$^+$ Treg downstream of immune priming.

After T cells have been activated during priming they begin to proliferate and expand in number (268). *In vivo* analysis of T cell BrdU uptake showed that ICOS deficient Foxp3$^+$ Treg were not impaired in their ability to proliferate in response to helminth infection. Therefore, diminished Foxp3$^+$ Treg proliferation does not explain the defective expansion of Foxp3$^+$ Treg seen in ICOS$^{-/-}$ mice during helminth infection. The division of Foxp3$^+$ Treg is probably driven by other co-stimulatory signals or cytokines during helminth infection. In support of co-stimulatory signals driving Treg division, antibody blockade of CD28 almost completely blocks the *in vivo* proliferation of Tregs (173), and CD28 co-stimulation alone (and not that of CD154, CD27, 4-1BB, OX-40 or ICOS) induces the proliferation of Foxp3$^+$

Treg *in vitro* (273). This would suggest that CD28 is unique amongst co-stimulators in its ability to promote proliferation of Foxp3$^+$ Treg. Whether cytokines have a role in driving helminth induced Foxp3$^+$ Treg proliferation has yet to be determined, but it is unlikely that IL-2 enhances Treg proliferation because evidence suggests that the principal function of IL-2 on Treg is to rescue them from apoptosis and maintain their survival (274-276)

In contrast to Foxp3$^+$ Treg, Foxp3$^-$ Teff cells showed an early dependency on ICOS for proliferative capacity during *H. polygyrus* infection. Similarly, ICOS has been shown to drive CD4$^+$ T cell proliferation in both Th1 and Th2 polarised helminth infections (203). This suggests that ICOS co-stimulation has different effects on Foxp3$^+$ Treg compared to Foxp3$^-$ Teff, and fits with a cell extrinsic effect of ICOS on T cell proliferation. ICOS deficiency results in reduced IL-2 *in vitro* (197) and my own data suggests that ICOS is required for IL-2 production from Foxp3$^-$ Teff during *H. polygyrus* infection. Whereas Foxp3$^-$ Teff require IL-2 for proliferation (277) and survival (278, 279), the role of IL-2 for Foxp3$^+$ Treg is exclusively one of survival (274-276). Therefore, reduced levels of IL-2 in helminth infected ICOS$^{-/-}$ mice would impair the rate of Foxp3$^-$ Teff proliferation, but not that of Foxp3$^+$ Treg. This model would further predict that ICOS deficiency would impede the survival of both Foxp3$^+$ Treg and Foxp3$^-$ Teff. Indeed, both ICOS$^{-/-}$ Foxp3$^+$ Treg and Foxp3$^-$ Teff showed an increased rate of apoptosis under homeostatic conditions and during *H. polygyrus* infection. Thus, ICOS aids the survival of both Treg and Teff and potentially explains the reduced proportions of Foxp3$^+$ Treg and failed expansion and maintenance of Foxp3$^+$ Treg seen in helminth infected ICOS$^{-/-}$ mice. In keeping with this, ICOS is known to aid the survival of T cells and NKT cells (207, 270, 271), and stimulates the downstream signalling molecule Akt (280) which is a known T cell survival factor.

Whether ICOS mediates Treg survival through IL-2 remains to be formally identified, but in support of this theory it has recently been shown that IL-2 rescues ICOS$^+$ Foxp3$^+$ Tregs from apoptosis, but has no such effect on ICOS$^-$ Foxp3$^+$ Treg (208). This suggests that

ICOS expression defines those Foxp3$^+$ Treg that show a greater dependency on IL-2 to maintain their survival. IL-2 signalling results in the downstream activation of pSTAT5 and this is also crucial for Foxp3$^+$ Treg homeostasis (281). We aimed to evaluate pSTAT5 levels in Foxp3$^+$ Treg from *H. polygyrus* infected ICOS$^{-/-}$ mice but we were unable to detect this protein *ex vivo* by flow cytometry (data not shown). To detect sufficient levels of pSTAT5 by flow cytometry, cells must be stimulated *ex vivo* with IL-2. However, this treatment would have masked any ICOS dependent *in vivo* IL-2 defect and we would not have been able to determine the impact of ICOS deficiency on pSTAT5.

Notably, following *H. polygyrus* infection ICOS$^{-/-}$ Foxp3$^+$ Treg failed to downregulate expression of the IL-7Rα chain (CD127). That ICOS$^{-/-}$ Foxp3$^+$ Treg retain IL-7Rα expression suggests they remain dependent on IL-7Rα signalling for survival. It is known that IL-7 is critical for the survival of naïve T cells under homeostatic conditions (282-285). However, following T cell antigen activation, the IL-7Rα is downregulated (286), and IL-2 has been shown to negatively regulate IL-7Rα expression (287) suggesting that T cells switch from a dependency on IL-7 under homeostatic conditions, to IL-2 dependency following activation by antigen. Therefore, I suggest that following *H. polygyrus* infection, WT Foxp3$^+$ Treg switch from IL-7 to IL-2 dependency for survival and downregulate the IL-7Rα as parasite responding Foxp3$^-$ Teff increase IL-2 availability. Due to the reduced Foxp3$^-$ Teff IL-2 seen in *H. polygyrus* infected ICOS$^{-/-}$ mice, ICOS$^{-/-}$ Foxp3$^+$ Treg remain partially dependent on IL-7 signalling for survival and retain surface expression of IL-7Rα following nematode infection. Further, because the IL-7Rα promiscuously pairs with either the common gamma chain γc cytokine receptor or the TSLPR to form the receptor for IL-7 or TSLP respectively (288, 289), it is possible that ICOS$^{-/-}$ Foxp3$^+$ Treg require either IL-7 or TSLP for their homeostasis in the low IL-2 environment of *H. polygyrus* infected ICOS$^{-/-}$ mice. In support of TSLP, it has been shown that despite the lymphopenia of IL-7$^{-/-}$ mice,

the proportion of Tregs remains intact (290), suggesting that IL-7 is dispensable for Treg homeostasis. In addition, GALT associated $CD103^+$ tolerogenic DCs producing TSLP are required for the generation of $Foxp3^+$ Treg (257), and given the immune location of *H. polygyrus* reactive $Foxp3^+$ Treg it is most likely that the failure of $ICOS^{-/-}$ $Foxp3^+$ Treg to downregulate the IL-7Rα reflects a continued dependency on TSLP for their maintenance due to the decreased IL-2 availability.

Following both *H. polygyrus* and *S. mansoni* infection, FACS staining for Foxp3 and IL-10 showed that ICOS was required for IL-10 protein production from $Foxp3^+$ Treg at the site of infection, and to a lesser extent in the lymph node. In keeping with this strict dependency of $Foxp3^+$ Treg on ICOS for IL-10 at the effector site, transfer of $ICOS^{-/-}$ T cells into diabetes prone NOD.$TCR\alpha^{-/-}$ mice showed that IL-10 production from pancreatic $Foxp3^+$ Treg was severely impaired (208). Similarly, ICOS expression has been shown to license human $Foxp3^+$ Tregs with the ability to suppress immune responses through IL-10 (234). In contrast to $Foxp3^+$ Treg, $ICOS^{-/-}$ $Foxp3^-$ Teff were unimpaired in their ability to produce IL-10 during *H. polygyrus* infection, suggesting that ICOS acts in a cell specific manner to drive IL-10. Diverse receptor expression and a distinct transcription factor signature between $Foxp3^+$ Treg and $Foxp3^-$ Teff could result in alternate outcomes downstream of ICOS stimulation. For example, $Foxp3^-$ Teff responding to *H. polygyrus* infection are Th2 differentiated (291) and express the transcription factor GATA-3 which can induce expression of IL-10 (292) independently of ICOS. ICOS deficiency did not completely ablate Treg IL-10 in the lymph node and future experiments should determine if the residual IL-10 is restricted to putative Th2 Treg expressing GATA3 (293).

In contrast to *H. polygyrus* infection, during *S. mansoni* infection cells of the $Foxp3^-$ Teff compartment were also partially dependent on ICOS for IL-10. This could be due to

differences in the Th subsets comprising the Foxp3$^-$ Teff population in the two helminth infections. Whilst the majority of Foxp3$^-$ Teff in *H. polygyrus* infection are Th2 cells, both Th2 and Tr1 cells will be present in the Foxp3$^-$ T cell response to *S. mansoni* infection (89, 150). Given that ICOS is crucial for IL-10 from cells with a Tr1 phenotype (205, 206), the diminished IL-10 in the Foxp3$^-$ compartment seen during *S. mansoni* infection of ICOS mice probably represents a loss of Tr1 cell IL-10, with the remaining IL-10 stemming from activated Th2 cells. Despite the reduction in T cell IL-10 in ICOS$^{-/-}$ mice we did not see any of the mortality or immunopathology which is characteristic of *S. mansoni* infected IL-10$^{-/-}$ mice (30). The reduction in IL-10 in ICOS$^{-/-}$ mice was not complete and was co-incident with a balanced Th2 cytokine response in the liver (Fig 6.9 F - H), which could account for the lack of immune pathology seen in these mice. This contrasts with a previous report showing that antibody blockade of ICOS during the egg phase of *S. mansoni* infection resulted in reduced liver IL-10 and increased liver egg granulomas (241). The increase in hepatic granuloma size was attributed to increased IFN-γ levels although Th2 cytokines were unaffected by anti-ICOS treatment. In ICOS$^{-/-}$ mice, intracellular cytokine staining showed that both Th1 and Th2 cytokines were unaffected potentially explaining why granuloma size was unaffected (Fig 6.8 A).

To confirm our data showing that ICOS is required for Foxp3$^+$ Treg IL-10 protein production, we crossed ICOS$^{-/-}$ mice with IL-10-IRES-GFP Tiger mice to mark cells expressing IL-10 during *H. polygyrus* and infection. Tiger mice have an internal ribosome entry site (IRES)- GFP cassette inserted via homologous recombination directly at the end of the last exon and before the polyA site of the IL-10 gene such that post-transcriptional regulation of IL-10 mRNA through the 3' UTR is unaffected (243). The resulting construct allows transcription of IL-10 in conjunction with GFP under the control of the endogenous IL-10 promoter. In contrast to results obtained from FACS staining of IL-10, results

obtained from *H. polygyrus* infected IL-10GFP reporter mice showed that IL-10 production was not increased by Foxp3$^+$ Treg or Foxp3$^-$ Teff and suggests that GFP protein expression does not fully reflect IL-10 protein production. Similarly, in 4get mice (that harbour an analogous IRES construct) around 90% of Th2 primed CD4$^+$ T cells expressed GFP protein, yet as few as 0.65% expressed IL-4 protein. Following re-stimulation of the primed CD4$^+$ T cells the proportion of cells expressing both GFP and IL-4 protein increased but only to a level approximately one-third that of the total GFP$^+$ population (294). The discrepancy between GFP and cytokine protein expression in the reporter cells most likely results from the different mechanisms governing their translation; whilst translation of GFP is mediated by an IRES, IL-10 translation is cap dependent. IRES elements can bypass the need for the translation initiation complex eIF4F (295), which is critically required for cap dependent translation (296), and further, translation of IRES elements can take place during cell division when global translation is shut down (297). Therefore, a greater proportion of GFP protein to cytokine protein can be present in reporter cells.

That IL-10 protein production increased on infection whilst GFP expression did not, could suggest that the regulation of IL-10 protein production is principally governed post-transcriptionaly. This in keeping with the observation that re-stimulation of primed CD4$^+$ T cells results in enhanced cytokine protein production, whilst the total GFP$^+$ population is unaffected (294). Because ICOS$^{-/-}$ Treg were unable to produce IL-10 protein during *H. polygyrus* infection, ICOS is implicated as a post-transcriptional regulator of IL-10. ICOS co-stimulation strongly activates the signalling molecule PI3K (280) resulting in the downstream activation of p38 (298). Activation of p38 has been shown to inhibit the RNA binding molecule tristetraprolin (TTP) that targets adenosine rich elements (ARE's) in the 3' UTR of IL-10 mRNA to induce its degradation (299, 300). Therefore, it is possible that

ICOS enhances IL-10 protein production by stabilising IL-10 mRNA through activation of p38. This is unlikely, however, as such a model would predict that GFP expression would decline as a result of reduced stability of IL-10 mRNA in the absence of ICOS, and this was not observed in $ICOS^{-/-}$ tiger mice. Interestingly, another downstream target of the ICOS pathway is mTOR, which has been shown to phosphorylate a family of proteins termed eIF4E-binding proteins (4E-BPs) (296). 4E-BPs inhibit formation of the translation initiation complex eIF4F, so that cap-dependent, but not IRES dependent, translation is inhibited. Phosphorylation of 4E-BPs weakens their capacity to bind elements within the eIF4F initiation complex, and allows cap dependent translation initiation to take place (296). Thus, ICOS activation of mTOR and the subsequent phosphorylation of 4E-BPs could account for the observation that ICOS deficiency leads to reduced IL-10 protein but does not affect IRES dependent GFP production. Such a mechanism may also explain why blockade of ICOS reduces the proportion of huCD2 IL-$4^{+}CD4^{+}$ T cells but does not affect $GFP^{+}CD4^{+}$ T cells following *Leishmania major* infection of 4get-KN2 mice (301).

***Summary***

- ICOS co-stimulation is dispensable for Foxp3$^+$ Treg proliferation during diverse helminth infections.
- In contrast to Foxp3$^+$ Treg, ICOS was required for optimal Foxp3$^-$ Teff proliferation during nematode and trematode infections.
- Priming and activation of Foxp3$^+$ Treg took place independently of ICOS during helminth infection.
- ICOS was required for the infection induced Foxp3$^+$ Treg downregulation of the IL-7Rα
- ICOS co-stimulation contributed to the maintenance of Foxp3$^+$ Treg under conditions of homeostasis and intestinal nematode infection.
- ICOS signalling was critically required for Foxp3$^+$ Treg IL-10 protein production at the site of intestinal nematode and blood trematode infection. ICOS also contributed to Foxp3$^+$ Treg IL-10 protein production in the local lymph nodes during helminth infection.

## *Chapter 5. Consequences of ICOS deficiency on Th2 immunity to* H. polygyrus

### *5.0 Introduction*

In the preceding chapters we described a novel role for ICOS in driving Foxp3$^+$ Treg cell responses during helminth infection. In the absence of ICOS, helminth induced Foxp3$^+$ Treg responses were markedly impaired suggesting alleviation of immune suppression. Removal of Treg immune suppression can result in stronger type 2 immunity such that resistance to helminth infection is increased. However, whilst ICOS co-stimulation promotes Foxp3$^+$ Treg responses, it is also an important component for a complete anti-helminth type 2 response. Thus, the ICOS pathway plays contradictory roles during helminth infection, driving both positive and negative immune responses.

The role of the ICOS pathway in conventional CD4$^+$ T cell effector immune responses to helminth infection has been investigated. For example, infection with the GI nematode *N. brasiliensis* results in increased levels of IL-4, IL-5, IL-10 and IFN-γ. Blockade of ICOS reduced the levels of both type 1 and type 2 cytokines, highlighting the importance of this costimulator in Teff cell responses (201). Infection of ICOS$^{-/-}$ mice with *T. muris* resulted in decreased IL-4 and IL-13, yet increased IFN-γ (203). Similarly, the use of an anti-ICOS Ab in mice infected with the GI nematode *T. spiralis,* led to increased in IFN-γ production, whilst IL-4 and IL-5 levels were reduced (218). Therefore, during intestinal nematode infections type 2 effector responses show a requirement for ICOS.

Whilst these studies demonstrated that ICOS signalling contributes to the helminth induced type 2 response, they focused on the lymph nodes draining the infection site. Some co-stimulators can have different effects depending on the immune location under investigation (245), and evidence suggests that type 2 responses at the tissue site of helminth infection show a more limited requirement for ICOS co-stimulation. For example, at the site of *B. malayi* infection, Th2 effector T cell responses were independent of ICOS

co-stimulation, and during *S. mansoni* infection, ICOS signalling was dispensable for IL-4 production from liver T cells (241, 302). Whether ICOS is required for type 2 responses at the site of *H. polygyrus* infection has not been investigated.

Recent data has shown that Tfh cell are the main source of IL-4 in the lymph node draining the site of *H. polygyrus* infection (303). Tfh are thought to be distinct from conventional Th1 and Th2 cells but whether this is indeed the case, or if the Tfh phenotype represents a stage of Th effector cell differentiation remains controversial. To properly distinguish differentiated Tfh requires their localisation within the lymphoid follicles. However, they express high levels of the follicular homing chemokine receptor CXCR5 and this serves as a good marker for their identification (304). ICOS is also highly expressed by Tfh, and Tfh cells have been shown to be critically dependent on ICOS co-stimulation for their differentiation and maintenance (227). Furthermore, blockade of ICOS during *L. major* infection led to a reduction in the percentage of IL-4 producing follicular resident $CXCR5^+$ Tfh cells suggesting that ICOS is important for Tfh during parasite infection (301). This new data raises the interesting possibility that ICOS contributes to type 2 immune responses generated in the lymphoid tissue during helminth infection primarily through its effects on Tfh cell.

A number of studies have recently described a new innate player in type 2 responses to helminth infection (219). These cells are collectively known as innate helper 2 cells (IHCs) and as an early source of type 2 cytokines they represent the first line of defence in the immune response to helminths (219). Significantly, IHCs express ICOS at high levels (34) suggesting that ICOS may also contribute to early innate type 2 responses. However, to date there is no evidence to suggest what the functional significance of ICOS expression by IHCs might be.

Disruption of Treg during helminth infection has been shown to increase Th2 effector cytokines and in some cases enhance parasite clearance (15, 146). However, it is now known that both regulatory and effector immune responses show a requirement for ICOS during helminth infection, and the level of resistance to helminth infection in ICOS deficient mice may depend on the importance of ICOS to either arm of immunity. If $Foxp3^+$ Treg have a greater degree of dependency on ICOS co-stimulation than Teff do, this could alleviate suppression of the effector response resulting in a more efficacious type 2 response and increased resistance to helminth infection. Conversely, if ICOS is more important for type 2 immune responses than $Foxp3^+$ Treg responses, the anti-parasite type 2 response will be impaired and this could increase susceptibility to infection.

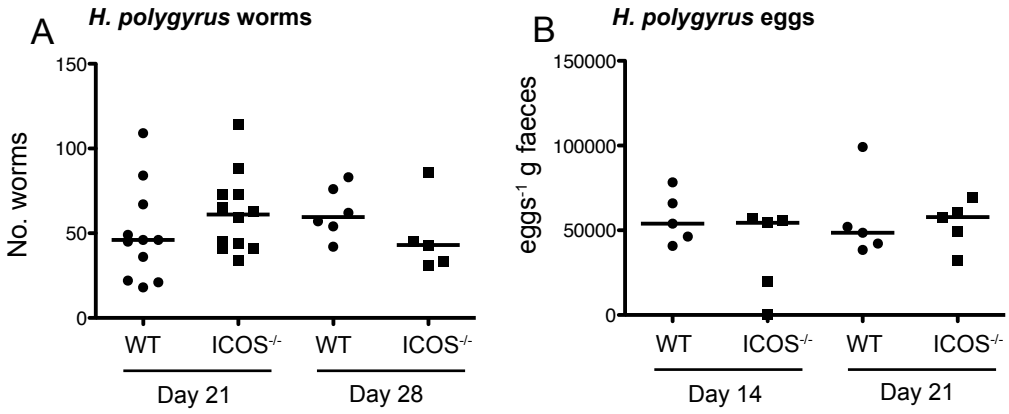
***Chapter Aims***

- Does ICOS deficiency affect resistance to *H. polygyrus* infection?
- Does ICOS contribute to Th2 responses in the MLN and infection site?
- Do IHCs utilise ICOS for expansion under Th2 polarising conditions *in vivo*?
- Is ICOS required for the expansion of Tfh during *H. polygyrus* infection?

## *Results*

### *5.1 ICOS deficiency does not impact susceptibility to* H. polygyrus *infection*

Depletion of Foxp3$^{+}$ Treg during helminth infection can lead to enhanced Th2 immunity and in some cases this results in increased parasite elimination (15, 146). Conversely, disruption of type 2 effector immune responses can increase susceptibility to helminth infection (19). ICOS deficiency led to a reduction in Foxp3$^{+}$ Treg responses during helminth infection, but on the other hand ICOS has been shown to promote type 2 immunity to helminth infection. To determine the consequences of ICOS deficiency on resistance to *H. polygyrus* infection, we measured *H. polygyrus* worm and egg numbers from infected WT and ICOS$^{-/-}$ mice. There were no differences in worm numbers at d 21, or at d 28 of infection between infected WT and ICOS$^{-/-}$ mice (Fig. 5.1 A), and no differences in egg burden (Fig. 5.1 B). Therefore, ICOS deficiency has no impact on resistance to *H. polygyrus* infection.

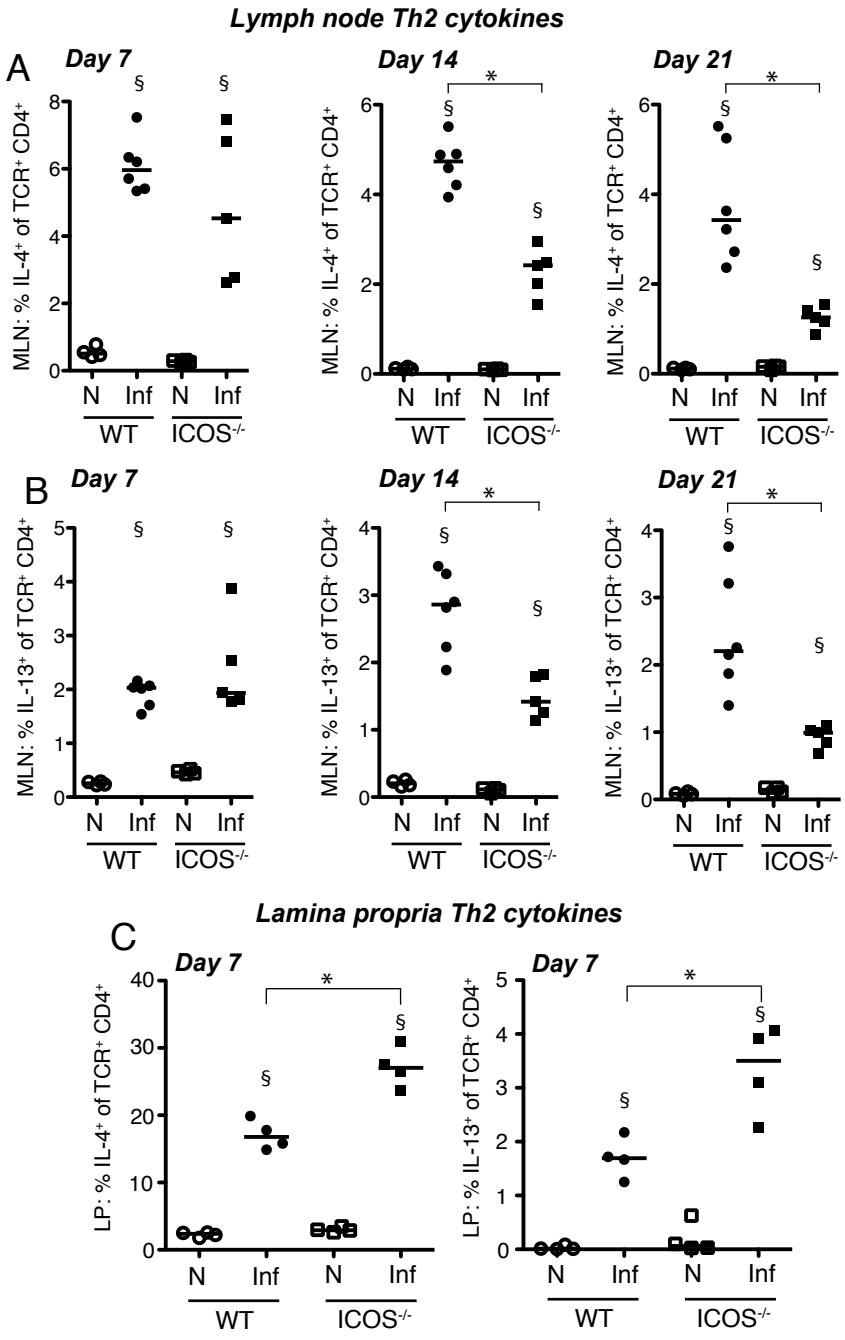



***5.1 ICOS deficiency does not impact susceptibility to* H. polygyrus *infection*.** WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the parasite burdens were assessed at d 21 and d 28 of infection (A), and the number of *H. polygyrus* eggs in the faeces were quantified at d 14 and d 21 (B). Data points represent individual mice and bars show median values. Closed circles denote *H. polygyrus* infected WT mice, closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice.

### *5.2 ICOS has differential effects on anti-helminth type 2 cytokines depending on immune location.*

To determine the effects of ICOS deficiency on the type 2 response to *H. polygyrus*, we infected WT and ICOS$^{-/-}$ mice with *H. polygyrus* and measured CD4$^{+}$ T cell type 2 cytokine responses in the MLN and LP of the small intestine by flow cytometry. In the MLN, at d 7 of infection, the percentage of IL-4$^{+}$ and IL-13$^{+}$ CD4$^{+}$ T cells increased in both WT and ICOS$^{-/-}$ mice indicating that ICOS is not required for early type 2 priming events (Fig. 5.2 A & B). However, by d 14 of infection, the percentage of MLN IL-4 and IL-13$^{+}$ CD4$^{+}$ T cells was significantly reduced in ICOS$^{-/-}$ mice compared to WT (Fig. 5.2 A & B). Levels of T cell IL-4 and IL-13 remained lower in ICOS$^{-/-}$ mice throughout infection (Fig. 5.2 A & B). This data suggests that ICOS co-stimulation is not required for early Th2 priming but that it is required for promoting the type 2 response within the MLN once it has been established.

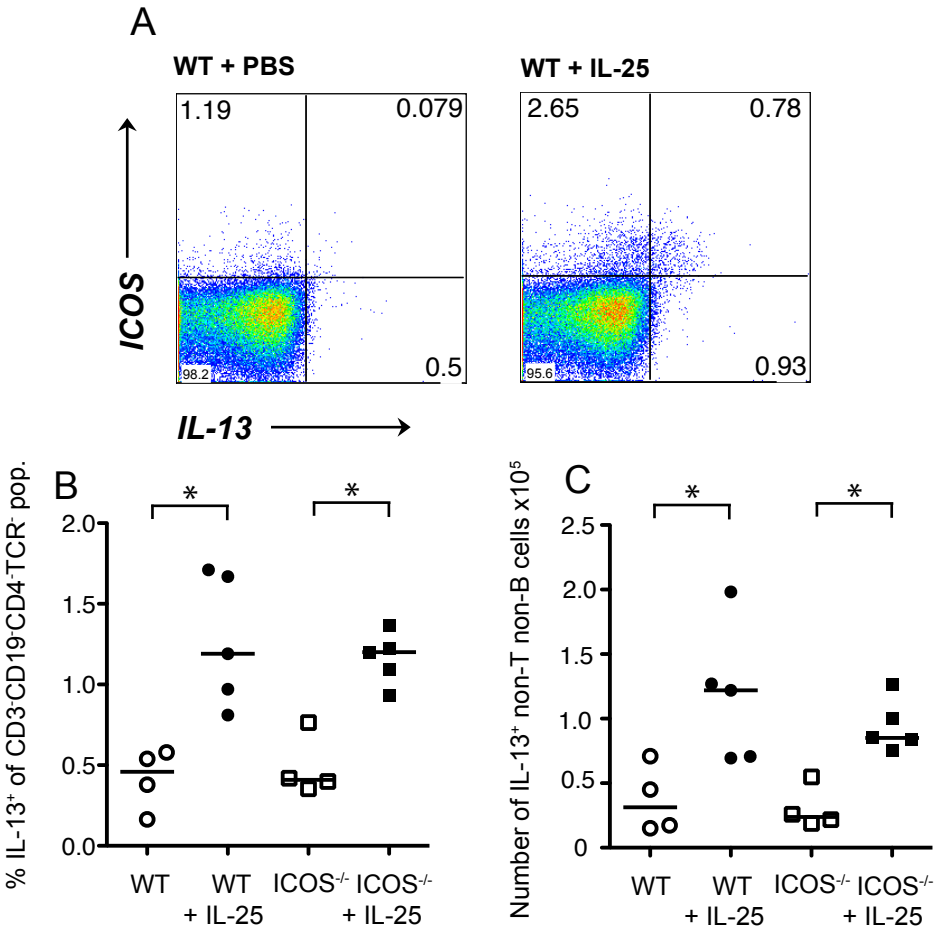
Given that Th2 responses in the local lymph node are impaired, why are ICOS$^{-/-}$ mice not more susceptible to *H. polygyrus* infection? In stark contrast to the MLN, in the LP the proportion of CD4$^{+}$ T cells positive for IL-4 and IL-13 was markedly increased in *H. polygyrus* infected ICOS$^{-/-}$ mice compared to WT (Fig. 5.2 C) suggesting that ICOS acts to downregulate Th2 cytokine production at the infection site. Thus, during *H. polygyrus* infection, ICOS has differential effects on T cell Th2 cytokine production in different tissue sites, promoting the ongoing Th2 response in the local lymph node, but acts to downregulate Th2 cytokines at the site of infection. The differences in the magnitude of the Th2 response between the lymph node and infection site could potentially explain why resistance to *H. polygyrus* infection is unchanged in ICOS$^{-/-}$ mice.




***5.2 ICOS has differential effects on anti-helminth type 2 cytokines depending on immune location.*** WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the percentage of CD4$^+$ TCR$^+$ IL-4$^+$ and IL-13$^+$ cells in the MLN and LP was measured by intra cellular flow cytometry. (A) Percentage of IL-4$^+$ cells within the CD4$^+$TCR$^+$ population in the MLN at d 7, 14 and 21 (B) Percentage of IL-13$^+$ cells within the CD4$^+$TCR$^+$ population in the MLN at d 7, 14 and 21. (C) Percentage of IL-4$^+$ and IL-13$^+$ cells within the CD4$^+$ TCR$^+$ population in the LP at d 7. Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *H. polygyrus* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice. § = significant increase due to infection. * p= <0.05 Mann Whitney test. Panels are representative of three separate experiments.

### *5.3 ICOS is not required for IL-25 mediated expansion of innate helper cells*

IHCs are now recognised as an essential early source of type 2 cytokines in response to helminth infection (219). IHCs can be distinguished by expression of ICOS (in conjunction with other markers) (219) yet the functional significance of ICOS expression by IHCs has not been investigated. We wanted to ask if ICOS is required for the expansion and effector function of IHCs, but IHCs are of low abundance during *H. polygyrus* infection (Dr Katherine Smith, Dr Rick Maizels, unpublished observations), and in the absence of IL-13 reporter mice they can be difficult to identify. IHCs are, however, critically dependent on IL-25 for their induction (219). Therefore, to ask if ICOS was required for IHCs development, we administered recombinant IL-25 to WT and $ICOS^{-/-}$ mice and measured the proportion of $IL\text{-}13^{+}$ non-T non-B cells in the MLN. As the first line of immune defence IHCs are most prominent at mucosal sites so I intended to measure IHCs in the LP. Unfortunately, this was not possible because IL-25 treatment resulted in the death of all LP mononuclear cells, possibly due to excessive mucus production. Indeed, IL-25 is a potent inducer of type 2 responses at mucosal surfaces (219) potentially driving increased goblet cell hyperplasia and increased mucus secretion. In the MLN, following IL-25 treatment the proportion and absolute numbers of $CD3^{-}CD19^{-}CD4^{-}TCR^{-}IL\text{-}13^{+}$ cells increased to similar levels in both WT and $ICOS^{-/-}$ mice (Fig. 5.3 A-C), suggesting that ICOS is not required for the IL-25 driven expansion of IHCs. Thus, the loss of type 2 cytokines in the MLN of helminth infected $ICOS^{-/-}$ mice is not due to impaired innate help for the adaptive response.




***5.3 ICOS is not required for IL-25 mediated expansion of nuocytes*** WT and ICOS$^{-/-}$ mice were administered recombinant IL-25 or PBS as a control and the percentage of CD3$^-$CD19$^-$CD4$^-$TCR$^-$IL-13$^+$ cells in the MLN were measured at d 7. (A) Representative FACS plots showing ICOS versus IL-13 gated on CD3$^-$CD19$^-$CD4$^-$TCR$^-$ cells. (B) Percentage of IL-13$^+$ cells of the CD3$^-$CD19$^-$CD4$^-$TCR$^-$ population. Data points represent individual mice and bars show median values. Open circles denote PBS treated WT mice and closed circles denote IL-25 treated WT mice. Open squares denote PBS treated ICOS$^{-/-}$ mice and closed squares denote IL-25 treated ICOS$^{-/-}$ mice. * p= < 0.05 Mann Whitney test. Panels are representative of two separate experiments.

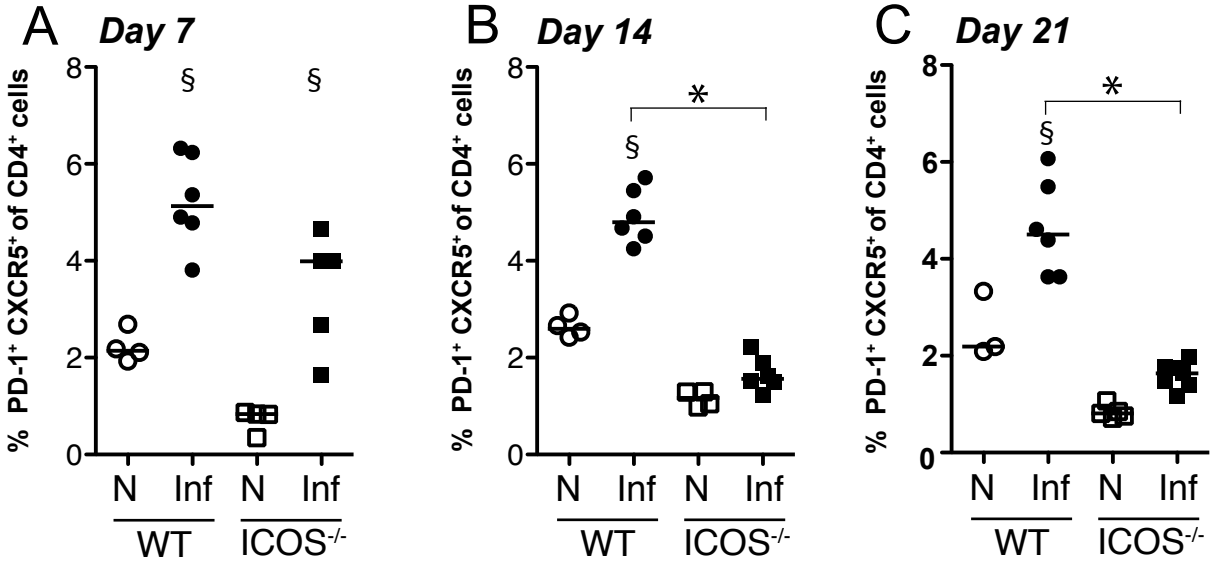
### *5.4 The reduced type 2 cytokines in the LN of* H. polygyrus *infected ICOS$^{-/-}$ mice is due to a loss of IL-4 secreting Tfh.*

ICOS is highly expressed by the Tfh cell subset and these cells have been shown to be the main source of IL-4 in the reactive lymph node during helminth infections (233, 303). In the absence of ICOS the Tfh population is severely impaired (227). We found that the

effects of ICOS deficiency on IL-4 were tissue specific, with reduced IL-4 in the MLN and increased IL-4 in the LP, and because Tfh are abundant in the lymph nodes but absent from the infection site, this raises the interesting question that the LN type 2 defect of helminth infected $ICOS^{-/-}$ mice is predominantly due to a loss of IL-4 secreting Tfh. To test this, we measured Tfh responses in the MLN of *H. polygyrus* infected WT and $ICOS^{-/-}$ mice to see if the reduction in MLN IL-4 seen in $ICOS^{-/-}$ mice was mirrored by a loss of Tfh.

Tfh express high levels of CXCR5, ICOS and PD-1. ICOS cannot be used as a marker for Tfh in $ICOS^{-/-}$ mice, so Tfh are marked here based on expression of CD4, CXCR5, and PD-1. $CD4^+$ T cells do not commit to the Tfh lineage and become localised within the follicles until d 10 of infection (301), so at d 7 of *H. polygyrus* infection $CD4^+CXCR5^+PD\text{-}1^+$ T cells most likely represent those cells in the process of differentiation to the Tfh subset. Although the basal proportion of $CXCR5^+PD\text{-}1^+$ cells was reduced in $ICOS^{-/-}$ mice, at d 7 of *H. polygyrus* infection the percentage of $CXCR5^+PD\text{-}1^+$ cells increased in both WT and $ICOS^{-/-}$ mice (Fig. 5.4 A), suggesting that ICOS is not required for the initial infection induced expansion of $CXCR5^+PD\text{-}1^+$ T cells, before they have committed to the Tfh lineage. In contrast, at d 14 of infection, when Tfh are fully committed, the expansion of $CXCR5^+PD\text{-}1^+$ Tfh seen in WT mice was absent in $ICOS^{-/-}$ mice (Fig. 5.4 B), and $CXCR5^+PD\text{-}1^+$ Tfh remained reduced in $ICOS^{-/-}$ mice at day 21 of infection (Fig. 5.4 C). This failure in the expansion of $CXCR5^+PD\text{-}1^+$ Tfh directly mirrored the reduction in the proportions of MLN $CD4^+$ T cells producing IL-4 at d 14 and d 21 pi. Thus, ICOS is not required for the early d7 expansion of pre-Tfh cells during *H. polygyrus* infection, and at this stage the levels of MLN IL-4 were unaffected by ICOS deficiency. However, from d14 pi, once Tfh have fully commited to their lineage they become heavily dependent on ICOS for their maintenance, and from this juncture onwards the reduction in the Tfh population was followed by the loss of lymph node IL-4, suggesting that ICOS primarily contributes to

the MLN type 2 response to *H. polygyrus* by maintaining the IL-4 producing Tfh cell population.

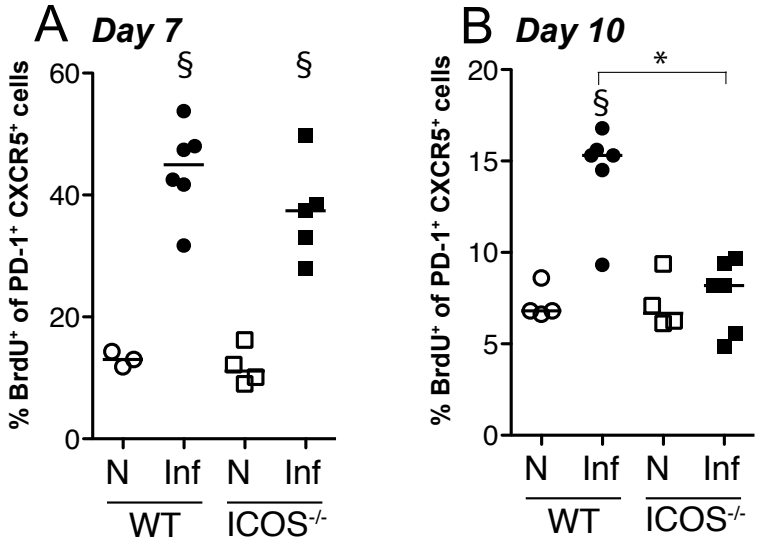



***5.4 ICOS promotes lymph node Th2 responses through maintenance of Tfh.*** WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the percentage of CD4$^{+}$CXCR5$^{+}$PD-1$^{+}$ cells in the MLN was measured at d 7, 14 and 21. Percentage of CXCR5$^{+}$PD-1$^{+}$ cells within the CD4$^{+}$ population in the MLN at d 7 (A), d 14 (B), and d 21 (C). Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *H. polygyrus* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice. § = significant increase due to infection * p = < 0.05 Man Whitney test. Panels are representative of three separate experiments.

### *5.5 ICOS co-stimulation promotes the proliferation of committed Tfh cells during* H. polygyrus *infection*

ICOS was required for the early proliferation of Foxp3$^{-}$CD4$^{+}$ Teff during *H. polygyrus* infection (Fig 4.5 A). To test if the reduced expansion of CXCR5$^{+}$ Tfh seen in ICOS$^{-/-}$ was due to a failure to proliferate, we administered BrdU to *H. polygyrus* infected WT and ICOS$^{-/-}$ mice 1 d prior to autopsy and measured the proportion of BrdU$^{+}$CXCR5$^{+}$PD-1$^{+}$CD4$^{+}$ T cells in the MLN. At d 7 of *H. polygyrus* infection, the proportion of proliferating BrdU$^{+}$CXCR5$^{+}$PD-1$^{+}$ T cells significantly increased in both WT and ICOS$^{-/-}$ mice (Fig. 5.5

A) suggesting that ICOS is dispensable for the initial infection induced $CXCR5^{+}PD\text{-}1^{+}$ pre-Tfh cell proliferative burst. However, by d 10 of infection the percentage of $BdU^{+}$ proliferating $CXCR5^{+}PD\text{-}1^{+}$ Tfh was severely reduced in $ICOS^{-/-}$ mice compared to WT (Fig. 5.5 B). This would suggest that ICOS is required for the proliferation of differentiated Tfh cells during *H. polygyrus* infection. The delayed reduction in the rate of Tfh proliferation was similar to the pattern of delayed expansion in the proportion of Tfh seen in *H. polygyrus* infected $ICOS^{-/-}$ mice. Taken together, these data suggest that the initial expansion of pre-Tfh takes place independently of ICOS but once these early Tfh cells differentiate and commit to the Tfh lineage they switch to a dependency on ICOS for proliferative capacity.

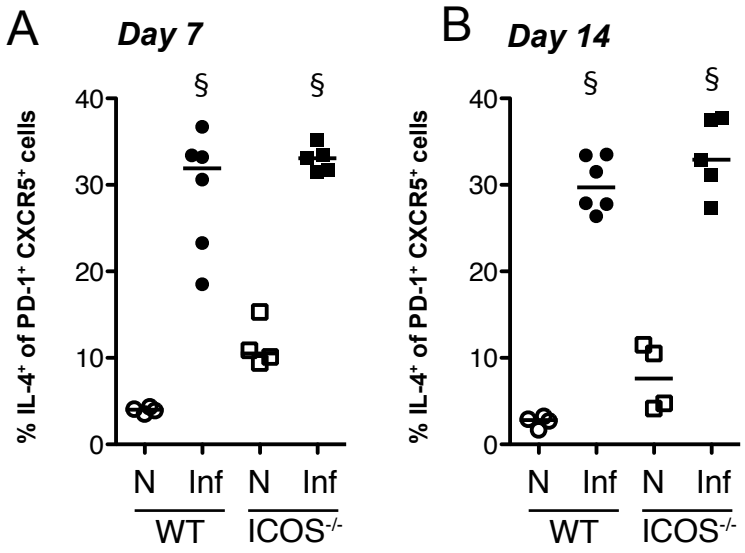



***5.5 During* H. polygyrus *infection early Tfh proliferation is independent of ICOS, but ICOS is essential for continued Tfh proliferation.*** WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the percentage of CD4$^{+}$ CXCR5$^{+}$ PD-1$^{+}$ cells positive for BrdU in the MLN was measured at d 7, 10 and 14. Percentage of BrdU$^{+}$ cells within the CD4$^{+}$ CXCR5$^{+}$ PD-1$^{+}$ population in the MLN at d 7 (A), d 10 (B). Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *H .polygyrus* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice. § = significant increase due to infection. * p= < 0.05 Mann Whitney test. Panels are representative of three separate experiments.

### *5.6 Tfh IL-4 production is independent of ICOS*

In the absence of ICOS the *H. polygyrus* induced expansion of Tfh was severely impaired and this was associated with reduced IL-4 in the MLN. Therefore, a reduced proportion of IL-4 secreting Tfh could account for the reduction in MLN IL-4 levels, but ICOS signals could also act in a cell intrinsic manner to promote Tfh cell IL-4 production. Therefore, to determine if Tfh require ICOS for IL-4 production, we measured the proportion of IL-4$^{+}$ cells within the CXCR5$^{+}$PD-1$^{+}$CD4$^{+}$ T cell population from *H. polygyrus* infected WT and ICOS$^{-/-}$ mice by flow cytometry. Taken as a percentage of the CXCR5$^{+}$PD-1$^{+}$ population, IL-4 production from CXCR5$^{+}$PD-1$^{+}$ was increased at d 7 of *H. polygyrus* infection in both WT and ICOS$^{-/-}$ mice (Fig. 5.6 A). Similarly, at d 14 of *H. polygyrus* infection the proportion

of $CXCR5^{+}PD\text{-}1^{+}$ Tfh positive for IL-4 was increased in both WT and $ICOS^{-/-}$ mice (Fig. 5.6 B). This preliminary data suggests that ICOS is not required for IL-4 production by Tfh cells, and that the reduced type 2 response in $ICOS^{-/-}$ mice is due to the reduced proportion of Tfh.

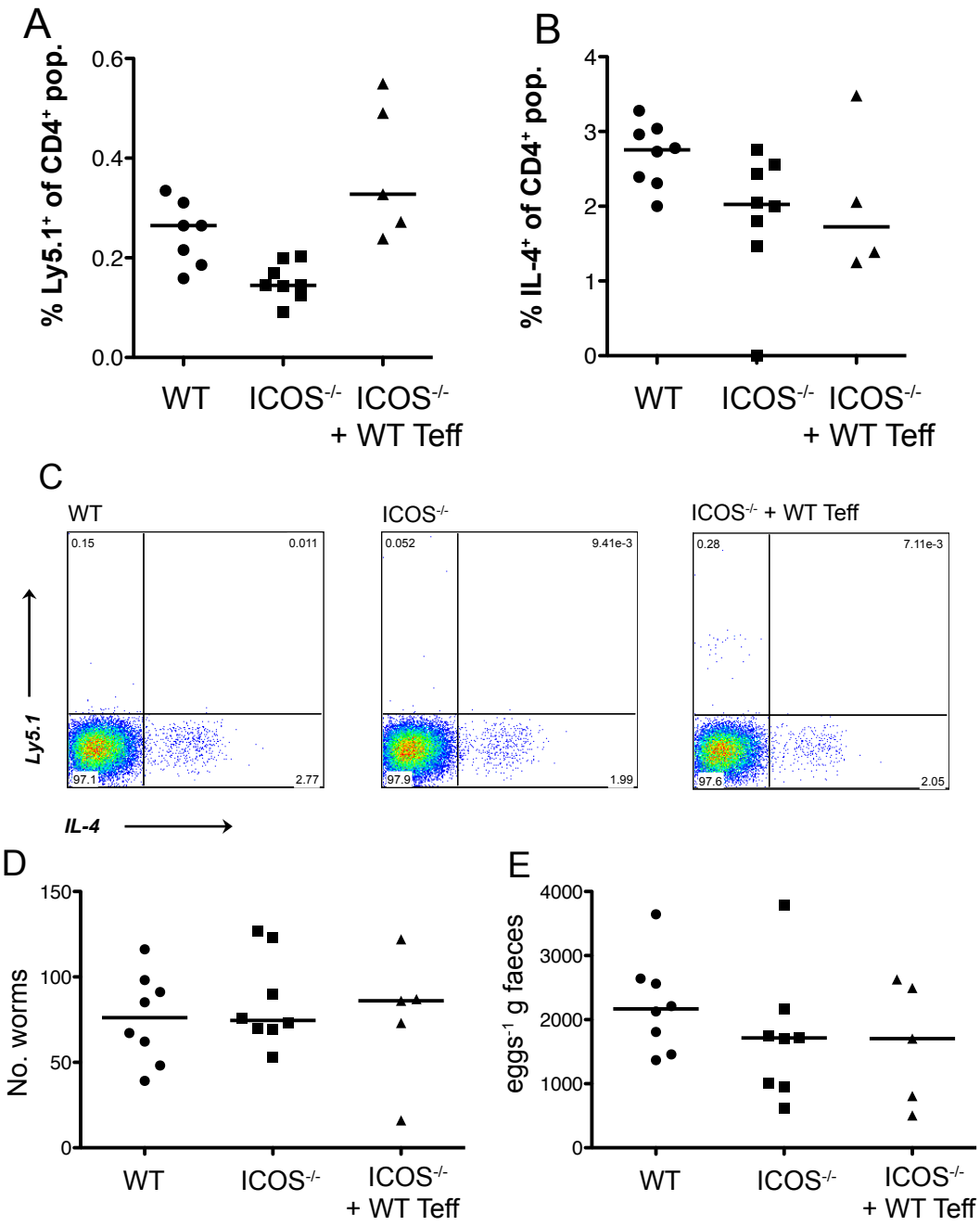



***5.6 Tfh IL-4 production is independent of ICOS.*** WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus* and the percentage of CD4$^{+}$CXCR5$^{+}$PD-1$^{+}$ cells positive for IL-4 in the MLN was measured at d 7 and d 14. Percentage of IL-4$^{+}$ cells within the CD4$^{+}$CXCR5$^{+}$ PD-1$^{+}$ population in the MLN at d 7 (A) and d 14 (B). Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *H. polygyrus* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice. § = significant increase due to infection. * p= < 0.05 Mann Whitney test. Panels show one experiment.

### *5.7 Addition of WT effector T cells into ICOS$^{-/-}$ recipient mice*

Through its effects on Teff and Treg, ICOS drives both positive and negative immune responses, and in ICOS deficient mice both regulatory and effector responses are impaired. To properly investigate the consequences of Foxp3$^{+}$ Treg ICOS deficiency on ICOS sufficient Th2 effector cells requires the generation of mixed bone marrow chimaeras or of a floxed ICOS mouse to be used with current Foxp3$^{CRE}$ mice. To preliminarily address this question, we sought to provide a source of WT ICOS sufficient Foxp3$^{-}$ CD4$^{+}$ T cells for recipient ICOS$^{-/-}$ mice. Thus, Ly5.1$^{+}$ GFP$^{-}$ ICOS$^{+/+}$ Teff cells from

Foxp3 GFP reporter mice were FACS sorted and transferred into ICOS$^{-/-}$ recipients. One day after transfer, recipient mice were infected with *H. polygyrus* and autopsied at day 28 of infection. Unfortunately, the donor population did not expand so that only a small proportion of the donor cells were detectable on autopsy (Fig. 5.7 A) and these cells did not produce IL-4 (Fig. 5.7 B & C) suggesting that they were not parasite specific. Given that only a small proportion of donor WT Teff survived in ICOS$^{-/-}$ recipient mice and they were not responding to the parasite, it was not unexpected that there was no impact on resistance to *H. polygyrus* infection (Fig. 5.7 D & E).




***5.7 Addition of WT effector T cells into ICOS$^{-/-}$ recipient mice.*** Ly5.1$^{+}$Foxp3$^{-}$ cells FACS sorted from Foxp3$^{GFP}$ mice were transferred into ICOS$^{-/-}$ mice (ICOS$^{-/-}$ + WT Teff), which were subsequently infected with *H. polygyrus.* As a control WT and ICOS$^{-/-}$ mice were infected with *H. polygyrus.* At d 28 of infection, mice were autopsied and the percentage of Ly5.1$^{+}$ (A), and CD4$^{+}$IL-4$^{+}$ cells (B) in the MLN were measured by flow cytometry. (C) Representative FACS plots showing Ly5.1 versus IL-4. (D) Parasite burdens were assessed at d 28 and and the number of *H. polygyrus* eggs in the faeces were quantified at d 21. Data points represent individual mice and bars show median values. Closed circles denote *H. polygyrus* infected WT mice, closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice, closed triangles denote *H. polygyrus* infected ICOS$^{-/-}$ + WT Teff mice.

### *5.8 Discussion*

Alongside its role in driving Foxp3$^+$ Treg responses, ICOS co-stimulation was also required for optimal lymph node type 2 responses during *H. polygyrus* infection. Indeed, ICOS deficiency has previously been associated with impaired Th2 cytokine production within secondary lymphoid tissue (201, 203, 218). Interestingly, recent work indicates this may be predominantly due to the loss of ICOS dependent IL-4 secreting Tfh cells (233, 301, 303), and there is evidence that ICOS-ICOS-L interactions are not necessary for T cell IL-4 production (218, 302). IL-4 competent CD4$^+$ T cells commit to the Tfh lineage and enter the follicles between d 6 – 10 of *L. major* infection (301), and within this time frame (d 7 of *H. polygyrus* infection) we found IL-4 production by MLN CD4$^+$ T cells was indeed unaffected by ICOS deficiency. Cells of the Tfh lineage can be further subdivided into interfollicular zone pre-Tfh (305-307), and the later arising germinal centre resident Tfh (230, 308). Of these two Tfh subsets, only the GC resident Tfh are capable of producing IL-4 (308), which suggests that the early source of IL-4 during *H. polygyrus* infection is Th2 cells and not pre-committed Tfh cells. That early MLN type 2 cytokines were unaffected by the absence of ICOS suggests that ICOS is not required for the initial Th2 priming events, and is in keeping with the hypothesis that conventional Th2 cells can produce IL-4 independently of ICOS.

Most significantly, in *H. polygyrus* infected ICOS$^{-/-}$ mice the loss of MLN IL-4 protein occurred at later time points following the failure of MLN CXCR5$^+$ Tfh cells to expand, suggesting that ICOS signalling contributes to MLN Th2 cytokines primarily through IL-4 secreting Tfh cells. The failed expansion of CXCR5$^+$ Tfh cells was accompanied by a reduction in the proportion of BrdU$^+$ Tfh, and although BrdU uptake is principally used to measure proliferation, the loss of BrdU$^+$ Tfh$^+$ cells in the absence of ICOS could also reflect death of Tfh, or result from fewer cells committing to the Tfh lineage. In support of commitment, it has been shown that ICOS signalling at the time of T cell priming by DCs

promoted up-regulation of the transcription factor Bcl-6 which is crucial for Tfh differentiation (230). ICOS is also important for the Tfh signature cytokine IL-21 (309), and IL-21 is required to maintain the number of Tfh as well as enhancing Tfh cell formation (228, 310, 311). Thus, ICOS signalling could act indirectly through IL-21 to promote both differentiation and survival of Tfh during helminth infection. Interestingly, although the reduced proportion of Tfh could explain the low IL-4 levels in the MLN of $ICOS^{-/-}$ mice, on a per cell basis ICOS was not required for Tfh IL-4 production. This contrasts with in vitro data in which ICOS augmented IL-4 production under type 2 polarising conditions (212). However, recent data suggests that regulation of IL-4 production in Tfh is distinct from conventional Th2 cells (312). In Tfh, IL-4 production was strictly dependent on a 3' enhancer in the IL-4 locus known as hypersensitivity site V (HSV), whereas Th2 cells were far less dependent on HSV for IL-4 (312). This would suggest that ICOS signalling could have differential effects on different Th subsets depending on the particular downstream transcriptional program of the Th cell.

Whether IL-4 secreting Tfh and conventional $CXCR5^{-}$ Th2 cells represent distinct T cell lineages, or if IL-4 secreting Tfh simply represent differentiating Th2 cells interacting with B cells prior to migration to the effector site is somewhat controversial but mounting evidence suggests that they are indeed distinct lineages. For example, in mice infected with *N. brasiliensis*, $IL\text{-}4^{+}$ T cells from the lungs produce much less of the Tfh signature cytokine IL-21 when activated than $IL\text{-}4^{+}$ T cells from the draining lymph node (301). This does not exclude the possibility that $IL\text{-}4^{+}IL\text{-}21^{low}$ lung T cells do not represent Tfh that have migrated from the LN and switched off IL-21 production following differentiation into effector Th2 cells. However, Tfh cell appear to retain their phenotype in vivo because IL-4 secreting cells isolated from the lungs, but not IL-4 secreting cells from the lymph node, are able to recruit eosinophils to the lung following adoptive transfer into IL-4/IL-13 dual deficient mice infected with *N. brasiliensis,* a process which is uniquely dependent on

conventional Th2 cells. Therefore, IL-4 producing T cells in the lymph node are phenotypically and functionally distinct from peripheral Th2 cells and have the cardinal characteristics of Tfh (301). Indeed, if Tfh were not a distinct lineage and simply represented a stage in the differentiation of Th2 cells, then the ICOS dependent Tfh deficiency would be predicted to impact Th2 responses both in the MLN and site of infection.

Importantly, at the infection site, in the absence of Tfh cells, we found that ICOS deficiency actually led to an increased percentage of $CD4^+$ T cells producing IL-4 and IL-13 protein, suggesting that Tfh cell are indeed distinct from Th2 cells. Thus, in contrast to IL-4-secreting $CXCR5^+$ Tfh cells, not only are Th2 effector cell responses efficiently generated in $ICOS^{-/-}$ mice, it appears that ICOS is in fact involved in suppressing Th2 cell effector responses at the infection site. $Foxp3^+$ Tregs accounted for the majority of $CD4^+$ T cell derived IL-10 within the LP (Fig 4.8 A), even though $Foxp3^+$ Treg functions in *H. polygyrus* infection are reported to be IL-10 independent (88, 143). IL-10 and adaptive $Foxp3^+$ Tregs are known to suppress Th2 cytokine production at mucosal surfaces (205, 313, 314), and so the increased Th2 responses seen within the LP of *H. polygyrus* infected $ICOS^{-/-}$ mice may be a consequence of functionally impaired $Foxp3^+$ Tregs.

Within the intestinal tissue, IHCs are amongst the first cells of the immune system to respond to helminth infection and produce Th2 cytokines (219). In the absence of ICOS the IL-25 induced expansion of IL-13 producing IHCs was normal. This suggests that early innate type 2 immune responses, which help to augment the adaptive type 2 response, do not require ICOS co-stimulation, further indicating that early type 2 immune priming is independent of ICOS. The functional significance of ICOS expression by IHCs remains to be identified but it is possible that ICOS aids the survival of IHC because it has a similar role in promoting the survival of innate NKT cells (271). Furthermore, IHCs depend on IL-7 for survival (219) and ICOS regulates IL-7R expression on $Foxp3^+$ Tregs, suggesting that

ICOS could increase IHC sensitivity to IL-7 signalling. Future experiments should aim to quantify the rate of apoptosis in ICOS$^{-/-}$ IHCs.

My data suggests that the effects of ICOS co-stimulation on type 2 immunity are tissue specific, such that reduced type 2 cytokines in the MLN are countered by increased type 2 responses in the LP. The difference in the magnitude of the Th2 response between the two sites could potentially explain why ICOS deficiency did not alter protection to *H. polygyrus* infection. Accordingly, ICOS has also been shown to be dispensable for protective immunity to a number of other helminths, even though reduced LN Th2 responses were reported. For example, immunity to *N. brasiliensis*, which is strictly dependent on IL-4R signalling (315), was independent of ICOS despite contracted type 2 responses (201). In addition, blockade of ICOS had no effect on *T. spiralis* parasite numbers regardless of reduced IL-4 (218), and ICOS$^{-/-}$ mice are able to expel *T. muris*, albeit with delayed kinetics, in spite of attenuated type 2 immunity (203). Alleviated suppression at mucosal surfaces and subsequently enhanced type 2 immunity at the site of parasite infection (countered by the reduced LN type 2 response) could also account for these findings.

As ICOS is implicated in both negative and positive immune responses it was difficult to dissect its effects on Foxp3$^{+}$ Treg suppression of the anti-parasite type 2 response. In the absence of a floxed ICOS mouse, or bone marrow chimeras, we sought to populate ICOS$^{-/-}$ mice with congenically marked ICOS-sufficient Foxp3$^{-}$ WT Teff to investigate the consequences of Foxp3$^{+}$ Treg ICOS deficiency on WT Th2 effector cells. Unfortunately, only a small number of transferred cells were detectable at day 28, and there was no increase in the Th2 response in mice receiving WT Teff. Expansion of transferred cells was probably limited by competition from host effector cells. It would have been possible to reconstitute RAG mice with WT Teff and ICOS$^{-/-}$ Treg distinguished by expression of CD25. This would suffer from contaminating Foxp3$^{+}$ cells in both CD25$^{+}$ and CD25$^{-}$

populations and a better system would be to generate $ICOS^{-/-}$ $Foxp3^{GFP}$ mice to isolate $ICOS^{-/-}$ $Foxp3^{+}$ Treg. However, using transferred $ICOS^{-/-}$ $Foxp3^{+}$ Treg and WT $Foxp3^{-}$ Teff would only limit ICOS deficiency to natural $Foxp3^{+}$ Treg because $Helios^{-}$ $Foxp3^{+}$ adaptive Treg could arise from the transferred WT Teff population, and adaptive $Foxp3^{+}$ Treg dominated the intestinal $Foxp3^{+}$ Treg response to *H. polygyrus.* Indeed, the proper characterisation of the effects of ICOS on Treg Th2 suppression awaits the development of a mouse with a $Foxp3^{+}$ Treg specific ICOS deletion.

Thus, in addition to its novel role in driving Treg responses during *H. polygyrus* infection, ICOS is also highly important for the generation of lymph node IL-4 secreting Tfh*,* but acts to downregulate conventional $CXCR5^{-}$ Th2 responses at the site of infection. This finding potentially explains why previous studies have demonstrated that ICOS is important for LN type 2 responses during helminth infection, whereas Th2 immunity at the site of helminth infection is not dependent on ICOS co-stimulation. Despite reduced lymph node IL-4, ICOS deficiency did not alter susceptibility to *H. polygyrus* infection, possibly as a result of decreased regulation and increased Th2 cytokines at the infection site itself. As ICOS controls both positive and negative immune responses and can have opposing roles depending on location, an understanding of the consequences of these contradictory effects will be important when considering targeting ICOS therapeutically.

***Summary***

- ICOS deficiency does not impact resistance to *H. polgyyrus* infection.
- At day 7 the MLN type 2 response to *H. polygyrus* infection is independent of ICOS.
- Post-day 7, ICOS signalling contributes to the generation and maintenance of IL-4 producing Tfh in the local lymph node following *H. polygyrus* infection.
- ICOS acts to down-regulate Th2 responses at the site of *H. polygyrus* infection.

## *Chapter 6. Does early* S. mansoni *infection elicit a Foxp3$^+$ Treg response?*

### *6.0 Introduction*

Foxp3$^+$ Treg are activated and suppress Th2 responses controlling immunpathology during the egg-phase phase of *S. mansoni* infection (16, 85, 148), but little is known of their role and induction in the early larval lung transit phase. The outcome of long-term helminth infection may be determined in the first weeks of infection by the character of the initial immune response. Indeed, in both filarial and intestinal nematode infection, early expansion and activation of Foxp3$^+$ Treg assuages late stage effector immunity to the detriment of host protection (15). Thus, nematode infections bias early immune responses toward regulation to benefit their own survival, but it is not known if the trematode parasite *S. mansoni* also induces early Foxp3$^+$ Treg responses to increase its fitness. IL-6 deficiency leads to enhanced Th2 responses and increased protective immunity to lung stage *S. mansoni* larvae (316), and new data suggests the absence of IL-6 can impair Foxp3$^+$ Treg function during *H. polygyrus* infection (Dr Katherine Smith, Dr Rick Maizels, submitted manuscript), potentially implicating Foxp3$^+$ Treg in the suppression of protective Th2 responses to *S. mansoni* larvae in the lungs.

The immune mechanisms underlying host resistance to *S. mansoni* infection are poorly understood, and those mechanisms that have been speculated to be important for resistance were characterised during secondary infection of animals vaccinated with irradiated *S. mansoni* cercariae. In this regard, protective immunity to challenge infection is proposed to manifest in the lung (317). Radiation attenuation prolongs cercarial transit through the skin allowing priming of Th1 responses in the local lymph nodes (318), so that following challenge infection, CD4$^+$ T cells with a Th1 phenotype are rapidly recruited to the lung parenchyma (319, 320) where they initiate focal aggregates of mononuclear cells through IFN-$\gamma$ production (321). It is thought that these inflammatory foci represent a

physical barrier to the migration of lung stage schistosomula, rather than exhorting an acute lethal hit (26). In the absence of IL-10, inflammatory aggregates in the lung are increased resulting in a lower number of recovered challenge parasites, suggesting that protective immunity to *S. mansoni* larvae in the lung is diminished by the suppressive cytokine IL-10 (322). At the tissue site of *H. polygyrus* infection, $Foxp3^+$ Treg were the main source of IL-10, and $Foxp3^+$ Treg IL-10 production was strictly ICOS dependent. Although $Foxp3^+$ Tregs are not thought to be the dominant source of IL-10 during egg-stage *S. mansoni* infection (85, 87), it is not known if $Foxp3^+$ Treg are an important source of IL-10 in the lungs during early *S. mansoni* infection. Further, the T cell requisite on ICOS for IL-10 production (205, 234, 236) suggests that ICOS contributes to susceptibility to *S. mansoni* infection through suppression of protective immunity by IL-10. Whether ICOS deficiency renders mice more resistant to *S. mansoni* infection has not been investigated.

During the egg phase of *S. mansoni* infection, the Th2 immune response functions to protect the host liver from the damaging egg toxins, rather than bring about parasite expulsion (30). This is achieved through formation of granulamatous collagenous lesions containing macrophages, eosinophils and $CD4^+$ T cells, which surround the individual eggs. (8) Th2 cytokines also serve to suppress host detrimental Th1 polarised inflammatory responses to egg antigens (30). However, exuberant Th2 effector immunity can also be deleterious to the host, resulting in uncontrolled immune-pathology if not kept in check (16, 85, 148). The suppressive effect of Treg in this context is IL-10 independent (85), although IL-10 from other sources, such as $Foxp3^-$ Th2 cells, and possibly Tr1 like cells, can contribute to the regulation of egg-induced immune granulomas (87, 323). We demonstrated in chapter 3 that ICOS promotes expansion of $Foxp3^+$ Treg numbers during egg phase *S. mansoni* infection, and that both $Foxp3^+$ and $Foxp3^-$ T cells require ICOS for optimal IL-10 production (Fig 4.9 A-D). Furthermore, previous work has shown that blockade of ICOS at the onset of egg-laying results in larger hepatic granulomas (241).

Thus, the ICOS pathway is implicated in the regulation of hepatic immunopathology during *S. mansoni* infection. However, in the preceding chapter we saw that alongside its role in Foxp3$^+$ Treg responses, ICOS drives Th2 cytokine production through its effects on IL-4 secreting Tfh, and Tfh cells are also an important source of IL-4 during *S. mansoni* infection (233). Thus, ICOS may play contradictory roles in egg induced granuloma formation, promoting both regulatory and effector immune responses.

***Chapter Aims***

- Do *S. mansoni* parasites induce an early Foxp3$^+$ Treg immune response to aid their survival?
- Does ICOS co-stimulation contribute to the suppression of protective immune responses in the lungs through IL-10 and/or Foxp3$^+$ Treg?
- Does ICOS deficiency lead to more severe hepatic immune pathology due to a lack of Foxp3$^+$ Tregs during the acute egg-phase of *S. mansoni* infection?

## *Results*

### *6.1 Schistosomula lung transit does not induce expansion of Foxp3$^+$ Treg*

Early larval stage *L. sigmodontis* infection induces a dominant Foxp3$^+$ Treg response (15), and immune regulation is a common feature of early stage of GI nematode infection (31, 32). Although Foxp3$^+$ Tregs have been shown to control pathology during the egg phase of *S. mansoni* infection (85), it is not known if Foxp3$^+$ Tregs play a role in immunity to early stage *S. mansoni* larvae transiting the lung. In addition, because ICOS was required for optimal early Foxp3$^+$ Treg responses to *H. polygyrus* infection, we hypothesised that ICOS would also be important for Foxp3$^+$ Treg during early *S. mansoni* infection. To address this issue, we infected C57BL/6 WT and ICOS$^{-/-}$ mice with 70 *S. mansoni* cercariae and measured Foxp3 responses in the lungs and thoracic lymph nodes at d 7, 14 and 21 of infection to span the time frame of *S. mansoni* larvae transit through the lungs (24). Interestingly, throughout this period, there was no difference in the percentage of CD4$^+$ T cells expressing Foxp3 in the lungs between naïve and *S. mansoni* infected WT mice (Fig. 6.1 A & B). Although there was a trend for an increase in the absolute numbers of Foxp3$^+$ cells in the lungs of infected mice this did not reach statistical significance (Fig. 6.1 C). Similar to the lungs, in the tLN the percentage of Foxp3$^+$ CD4$^+$ T cells was unchanged by *S. mansoni* infection (Fig. 6.1 D), and whilst the numbers of Foxp3$^+$ cells trended towards an increase this was not of statistical significance (Fig. 6.1 E). Like in WT mice, in ICOS deficient mice, despite lower proportions of Foxp3$^+$ Treg, as previously noted (207), there was no change in the percentage or numbers of Foxp3$^+$ CD4$^+$ T cells in the lungs or tLN in response to *S. mansoni* infection (Fig. 6.1 B-E). Therefore, in contrast to other helminth infections, early stage *S. mansoni* infection does not induce expansion of Foxp3$^+$ Treg in the infected tissue site or local lymph node.

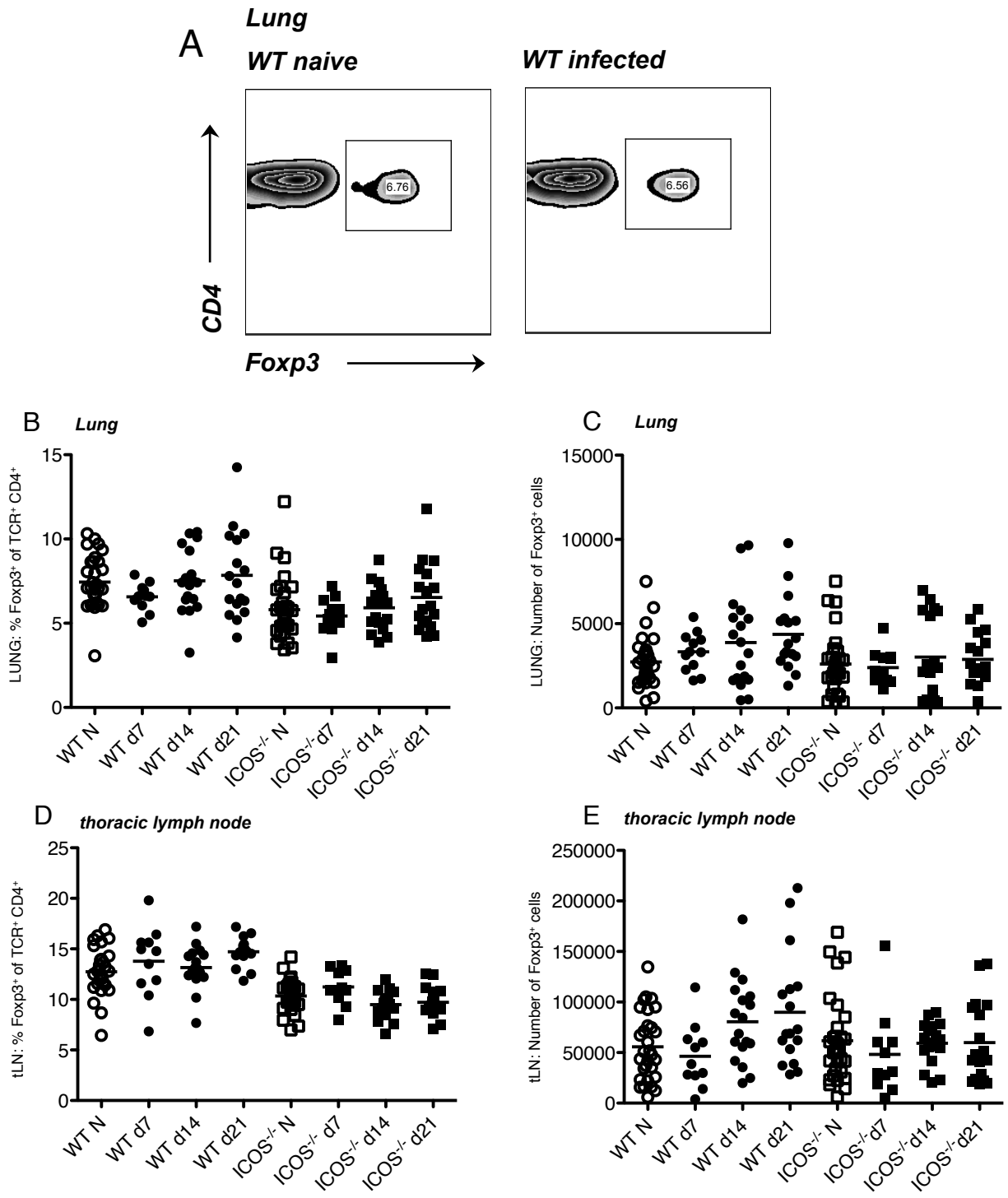



***Figure 6.1. Schistosomula lung transit does not induce expansion of Foxp3$^+$ Treg.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and Foxp3 responses were measured in the lung and tLN at d 7, 14 and 21 of infection. (A) Representative FACS plots showing percentage of Foxp3$^+$ cells within the CD4$^+$ population in the lung. (B) Percentage and (C) number of Foxp3$^+$ cells within the CD4$^+$ population in the lung. (D) Percentage and (E) number of Foxp3$^+$ cells within the CD4$^+$ population in the tLN. Data points represent individual mice from three separate experiments, and bars show mean values. Open circles denote naïve WT mice and closed circles denote *S. mansoni* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *S. mansoni* infected ICOS$^{-/-}$ mice.

### *6.2 Foxp3$^+$ Treg do not proliferate following challenge with* S. mansoni *larvae*

Early Foxp3$^+$ Treg expansion is associated with increased proliferation in *H. polygyrus* (Fig 4.4 A) and *L. sigmodontis* infection (15). To test if Foxp3$^+$ Treg proliferation is induced in response to schistosomula lung passage, we administered BrdU to *S. mansoni* infected WT and ICOS$^{-/-}$ 1 d prior to autopsy on d 7, 14 and 21 pi and measured the percentage of labeled Foxp3$^+$ cells in the lungs and tLN by flow cytometry. In both the lungs and tLN of *S. mansoni* infected mice, there was no increase in the proportion of BrdU$^+$ Foxp3$^+$ T cells when compared to naïve controls (Fig. 6.2 A & B). As previously noted, ICOS had no influence on the rate of Foxp3$^+$ Treg proliferation. Thus, consistent with no change in the numbers of Foxp3$^+$ Treg, Foxp3$^+$ Treg do not proliferate in response to early *S. mansoni* infection.

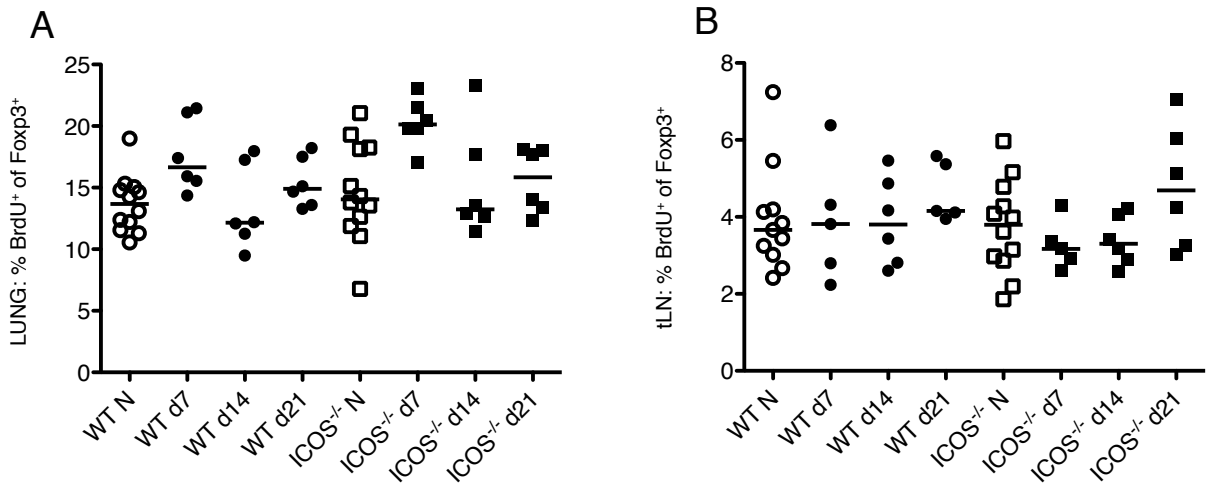



***Figure 6.2. The rate of Foxp3$^+$ Treg proliferation is unaffected by S. mansoni larvae lung passage.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and given 1mg of BrdU 24 hours prior to autopsy. At d 14 of infection the percentage of BrdU$^+$ cells within the Foxp3$^+$ population was measured in the lungs (A) and tLN (B). Data points represent individual mice and bars show mean values. Open circles denote naïve WT mice and closed circles denote *S. mansoni* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *S. mansoni* infected ICOS$^{-/-}$ mice. Panels are representative of two separate experiments.

### *6.3 Ratio of Foxp3$^+$Helios$^+$ to Foxp3$^+$Helios$^-$ cells remains constant throughout* S. mansoni *larval lung transit*

The rapid increase in Foxp3$^+$ Treg during early stage *L. sigmodontis* infection argues for expansion of pre-existing natural Foxp3$^+$ Tregs (15), whereas the expansion of Foxp3$^+$ Tregs at the site of *H. polygyrus* infection was dominated by adaptive Foxp3$^+$Helios$^-$ Treg, although both natural and adaptive Foxp3$^+$ Treg expanded in the MLN. To test if the ratio of natural to adaptive Foxp3$^+$ Treg was altered at the tissue site and reactive LN of early *S. mansoni* infection, we measured Foxp3 and Helios expression in lung and tLN CD4$^+$ T cells on d 14 pi by flow cytometry. Following *S. mansoni* infection, there was no change in the proportion of CD4$^+$TCR$^+$Foxp3$^+$Helios$^+$ or Foxp3$^+$Helios$^-$ cells in the lungs (Fig. 6.3 A &

B) or tLN (Fig. 6.3 C) suggesting that lung transiting *S. mansoni* larvae do not preferentially induce a natural or an adaptive Foxp3$^{+}$ Treg response.

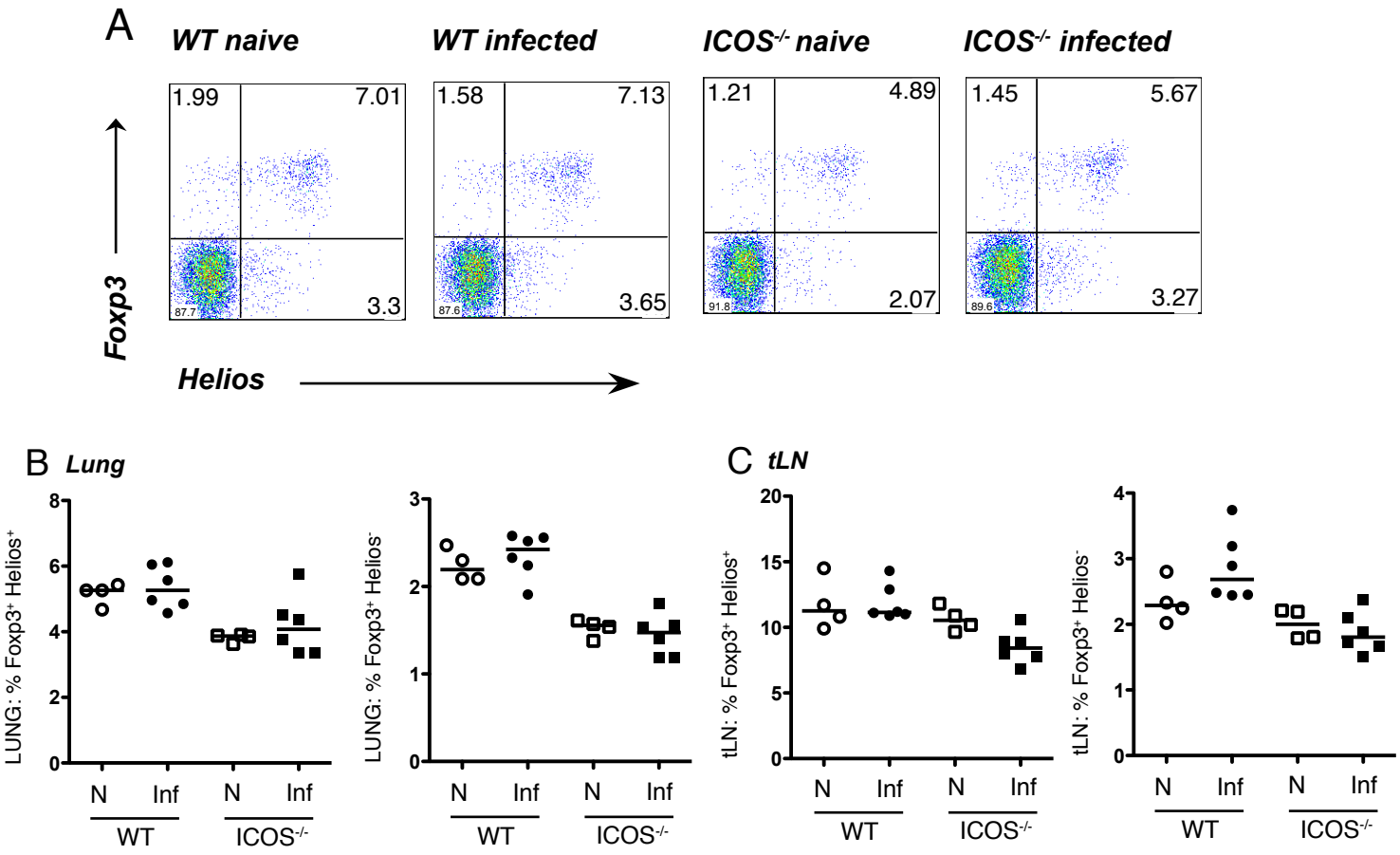



***Figure 6.3. Ratio of Foxp3$^+$Helios$^+$ to Foxp3$^+$Helios$^-$ cells remains constant throughout* S. mansoni *larval lung transit.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and the proportion of Foxp3$^+$ and Helios$^+$ cells within the CD4$^+$ T cell population were measured in the lung and tLN at d 14 of infection. (A) Representative FACS plots showing Foxp3 versus Helios in the lung. (B) Percentage of Foxp3$^+$Helios$^+$ and Foxp3$^+$Helios$^-$ cells within the CD4$^+$ population in the lung. (C) Percentage of Foxp3$^+$Helios$^+$ and Foxp3$^+$ Helios$^-$ cells within the CD4$^+$ population in the tLN. Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *S. mansoni* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *S. mansoni* infected ICOS$^{-/-}$ mice. Panels are representative of two separate experiments.

### *6.4 Lung Foxp3$^+$ Treg increase CD103 expression, but not CD25, during early* S. mansoni *infection.*

During the egg-phase of *S. mansoni* infection, the proportion of Foxp3$^+$ cells in the liver and spleen remains relatively constant. However, these Foxp3$^+$ Treg are in a heightened state of activation denoted by increased expression of CD103 and CD25, and the

activated phenotype is associated with enhanced suppression of immunity (31, 32). Similar to the egg-phase, our own observations showed that the proportion of Foxp3$^+$ Treg was unaltered during the larval stage of *S. mansoni* infection, but it is possible that the Tregs present were in a heightened state of activation. Therefore, in order to determine if Foxp3$^+$ Treg are activated during the early stage of *S. mansoni* infection, we measured CD103 and CD25 expression on Foxp3$^+$ cells in the lungs and local lymph node at wk 2 of *S. mansoni* infection. Low cell numbers in the lungs and tLN prevented us from measuring a more extensive panel of Treg activation markers. Notably, similar to late stage *S. mansoni* infection, Foxp3$^+$ Treg in the lung increased expression of CD103 suggesting increased activation (Fig. 6.4 A & B). However, there was no increase in the percentage of Foxp3$^+$ Treg expressing CD25 (Fig. 6.4 C). These data suggest that early *S. mansoni* infection induces an increase in Foxp3$^+$ Treg with an activated phenotype. Thus, despite the lack of Foxp3$^+$ Treg expansion, there is some indication that Foxp3$^+$ Treg are activated during early *S. mansoni* infection, which could suggest a Foxp3$^+$ Treg response but one much more subtle in nature than in other helminth infections.

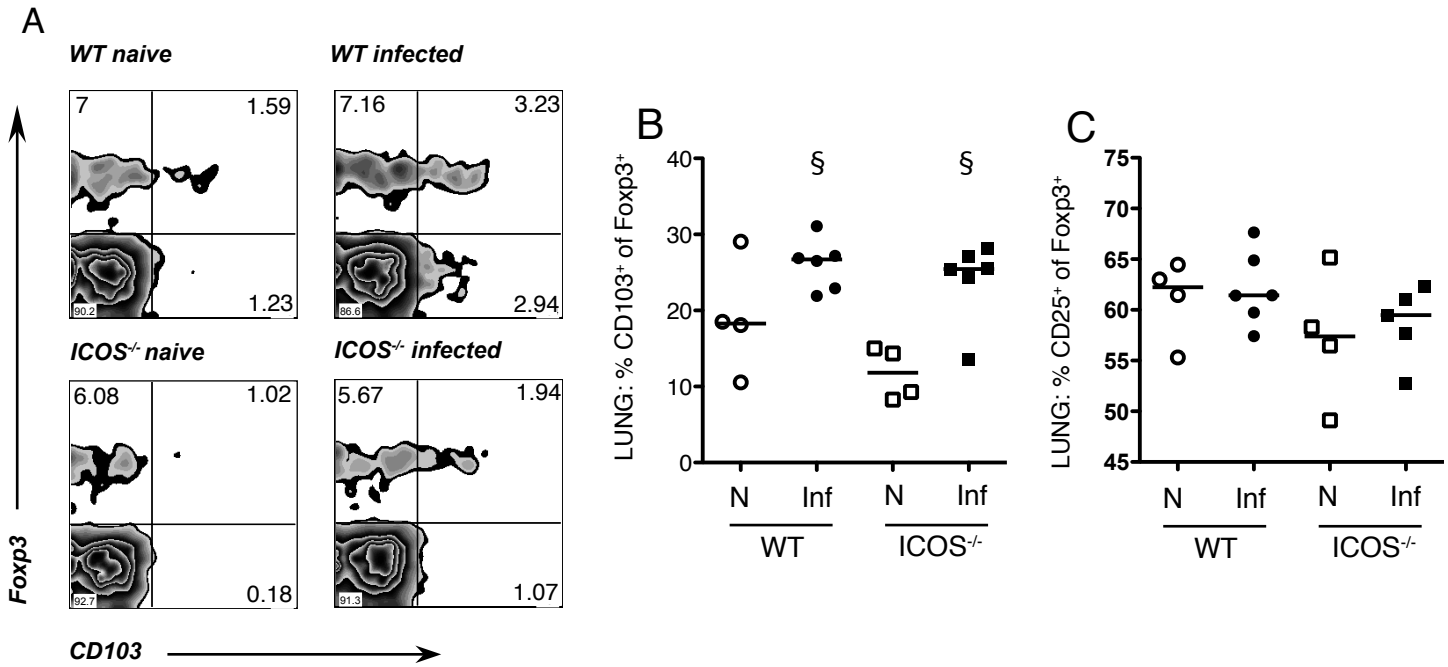



***Figure 6.4. Lung Treg increase CD103 expression, but not CD25, during early* S. mansoni *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and the expression of CD103 and CD25 by Foxp3$^{+}$CD4$^{+}$ T cells in the lung was measured at d 14 of infection. (A) Representative FACS plots showing Foxp3 versus CD103 in the lungs at d 14 of *S. mansoni* infection of WT and ICOS$^{-/-}$ mice. (B) Graph showing percentage of CD4$^{+}$Foxp3$^{+}$ cells expressing CD103 in the lung. (C) Graph showing percentage of CD4$^{+}$Foxp3$^{+}$ cells expressing CD25 in the lung. Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *S. mansoni* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *S. mansoni* infected ICOS$^{-/-}$ mice. § denotes significant increase on infection. Panels are representative of two separate experiments.

### *6.5 Foxp3$^{+}$ Tregs are not a major source of IL-10 in the early stage of* S. mansoni *infection*

The suppressive cytokine IL-10 has been shown to inhibit protective immunity following vaccination with attenuated *S. mansoni* cercariae (87, 322). The main source of IL-10 was

shown to be a population of T cells expressing CD25 (87). At the site of *H. polygyrus* infection, Foxp3$^{+}$ Tregs were a major source of IL-10 and this was dependent on ICOS signalling. However, during the egg-stage of *S. mansoni* infection Foxp3$^{+}$ Treg are not the most prominent source of IL-10 (85, 87), but whether Foxp3$^{+}$ Tregs produce IL-10 during early *S. mansoni* infection has not been investigated. To ask if Foxp3$^{+}$ Tregs were also important producers of IL-10 during early *S. mansoni* infection, and to investigate the contribution of ICOS co-stimulation to IL-10 production from these cells, we infected WT and ICOS$^{-/-}$ mice with 70 *S. mansoni* cercariae and co-stained CD4$^{+}$ T cells for Foxp3 and IL-10 by flow cytometry at d 14 of infection. Unfortunately, due to low cell numbers from the lung homogenates, our analysis was restricted to the tLN and the spleen. In both the tLN and spleen, there was no increase in the percentage of Foxp3$^{+}$ cells expressing IL-10 upon infection (Fig. 6.5 A & B). This would suggest that Foxp3$^{+}$ Treg are not a major source of IL-10 during early *S. mansoni* infection. In contrast to the Foxp3$^{+}$ population, there was a small trend for an increase in the percentage of Foxp3$^{-}$CD4$^{+}$ T cells producing IL-10 in both the tLN and spleen (Fig. 6.5 C & D), which could suggest that effector T cells, or possibly Tr1 cells, produce IL-10 in the larval stage of *S. mansoni* infection.

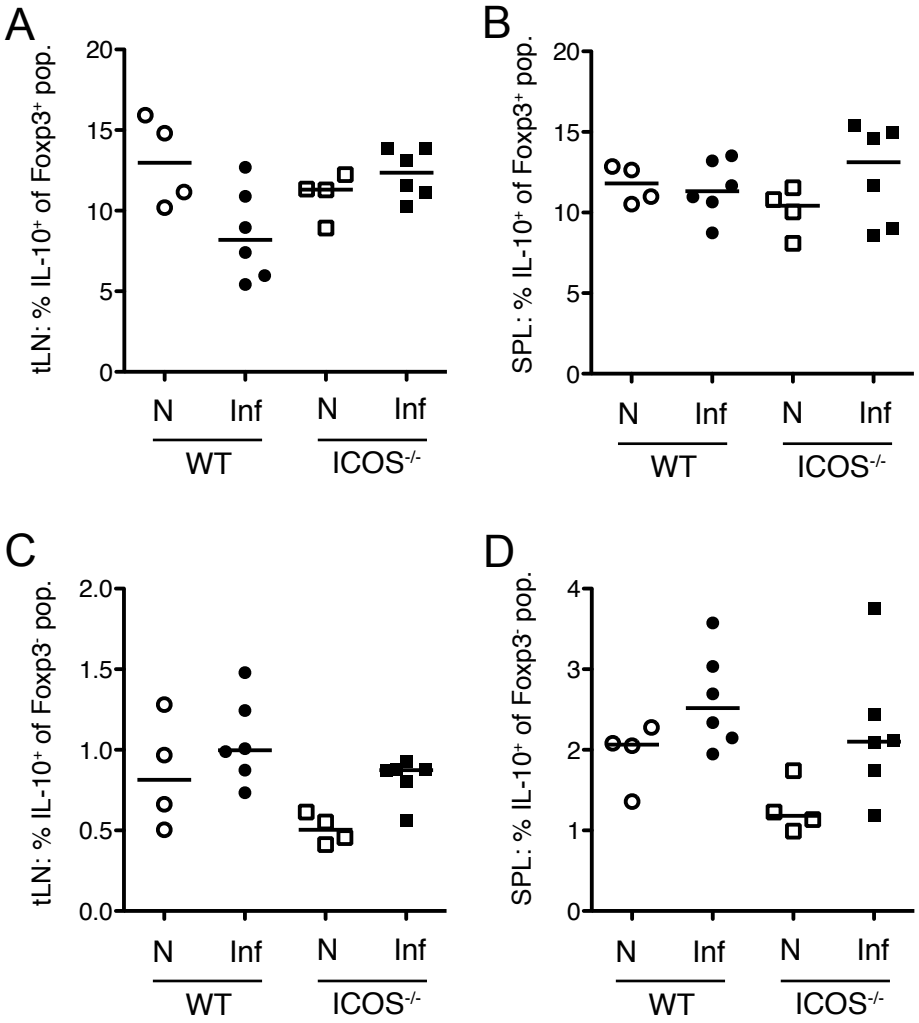



***Figure 6.5.Foxp3$^+$ Tregs are not a major source of IL-10 in the early stage of* S. mansoni *infection.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and the proportion of IL-10$^+$Foxp3$^+$CD4$^+$ T cells was measured in the spleen and tLN at d 14 of infection. Percentage of IL-10$^+$ cells within the CD4$^+$Foxp3$^+$ population in the tLN (A) and spleen (B). Percentage of IL-10$^+$ cells within the CD4$^+$Foxp3$^-$ population in the tLN (C) and spleen (D). Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *S. mansoni* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *S. mansoni* infected ICOS$^{-/-}$ mice. Panels are representative of two separate experiments.

### *6.6 Blockade of ICOS during early* S. mansoni *infection reduces IL-10 from CD25$^+$ T cells in the lung, which is associated with increased lung CD4$^+$ T cell IFN-γ*

Because low lung cell numbers prevented us from measuring Foxp3 and IL-10 by intracellular cytokine staining at this site, to try and measure lung T cell IL-10 we infected IL-10 GFP reporter mice (Tiger mice) with 70 *S. mansoni* cercariae and treated them with

an anti-ICOS Ab or IgG control and measured GFP expression in the lungs at d 14 of infection. In this particular experiment we recovered sufficient cells for intracellular cytokine analysis so this was performed alongside GFP detection. In the lungs, at d 14 pi, there was a significant increase in the percentage of $CD25^{+}CD4^{+}$ T cells expressing GFP (Fig. 6.6 A), suggesting that infection induced an increase in lung $CD25^{+}$ T cell IL-10. Blockade of ICOS had no effect on the percentage of $CD25^{+}CD4^{+}$ T cells expressing GFP (Fig. 6.6 A). In keeping with previous reports (319, 320), the proportion of IFN-$\gamma^{+}CD4^{+}$ T cells in the lungs increased due to infection, and this was further augmented by ICOS blockade, suggesting that ICOS acts to downregulate lung $CD4^{+}$ T cell IFN-$\gamma$ (Fig. 6.6 B). Infection also induced an increase in the percentage of IL-$4^{+}$ $CD4^{+}$ T cells in the lung but this was unaffected by treatment with anti- ICOS Ab (Fig. 6.6 C). Therefore, these preliminary data suggest during early *S. mansoni* infection, $CD25^{+}$ T cells are a source of IL-10 in the lungs, and that ICOS signalling downregulates lung T cell Th1 cytokine production.

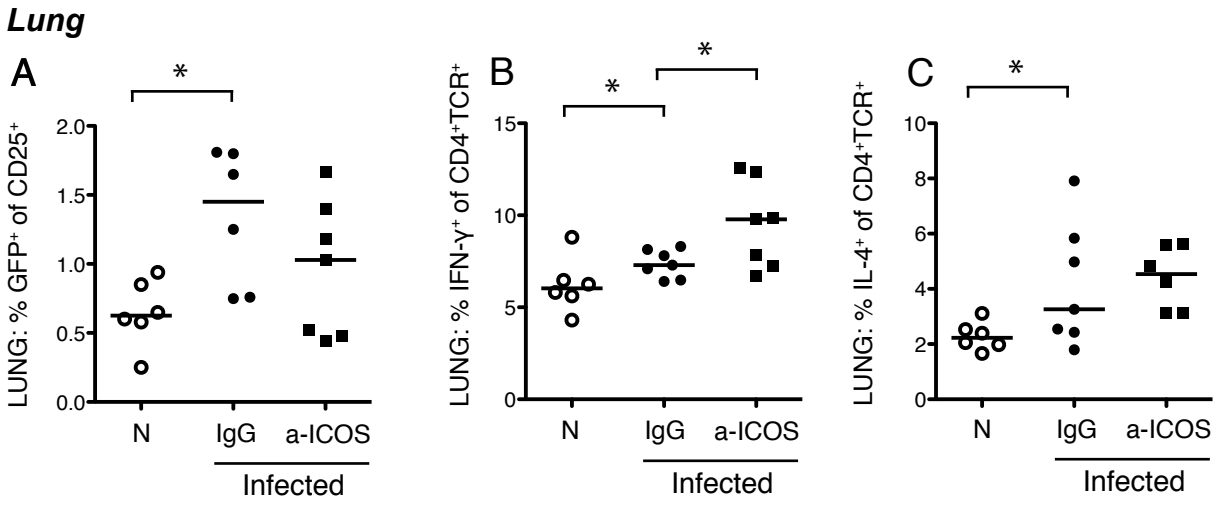



***Figure 6.6. Blockade of ICOS during early* S. mansoni *infection reduces Treg IL-10 in the lung, which is associated with increased lung CD4⁺ T cell IFN-γ.*** IL-10GFP mice were infected with 70 *S. mansoni* cercariae and treated with an anti-ICOS Ab or IgG control and GFP expression and cytokine responses in the lung were measured by flow cytometry. (A) The percentage of $CD4^+$ $CD25^+$ cells expressing GFP in the lungs at d 14 of infection. (B) Percentage of IFN-$\gamma^+$ $CD4^+$ cells and (C) IL-$4^+$ $CD4^+$ cells in the lungs at d 14 of infection. Open circles denote naïve IL-10GFP mice. Closed circles denote *S. mansoni* infected IL-10GFP mice treated with IgG Ab and closed squares denote *S. mansoni* infected IL-10GFP mice treated with anti-ICOS Ab. * p= < 0.05 Mann Whitney test.

### *6.7 ICOS deficiency leads to delayed migration of* S. mansoni *larvae but does not affect susceptibility to infection.*

T cell IFN-γ production in the lung is associated with protection during challenge infection of vaccinated mice (320, 321, 324), and ICOS was shown to downregulate T cell IFN-γ during primary infection. Moreover, ICOS is associated with T cell IL-10 production, and IL-10 impedes blockade of *S. mansoni* larval lung passage (322). Therefore, we hypothesised that lung transit of *S. mansoni* parasites would be impaired in $ICOS^{-/-}$ mice. To test this we recovered *S. mansoni* larvae from infected WT and $ICOS^{-/-}$ mice via hepatic perfusion at wk 2 and wk 3 of infection. In order to recover enough *S. mansoni* larvae to account for variation and allow statistical analysis, mice were hyper-infected with 200 *S. mansoni* infective cercariae. In these experiments, at wk 2 of infection we consistently

detected significantly fewer *S. mansoni* larvae in ICOS$^{-/-}$ mice when compared to WT (Fig. 6.7 A). However, by wk 3 of infection the numbers of *S. mansoni* larvae in WT and ICOS$^{-/-}$ mice were similar (Fig. 6.7 B) suggesting that ICOS deficiency delayed migration of *S. mansoni* larvae. By wk 8 of infection there was no difference in the number of adult *S. mansoni* parasites between WT and ICOS$^{-/-}$ mice (Fig. 6.7 C), and no difference in the number of *S. mansoni* eggs in the liver (Fig. 6.7 D). Thus, ICOS deficiency affects the early immune response in such a way that *S. mansoni* migration is delayed, possibly as a result of increased lung IFN-γ, but this was not sufficient to kill the migrating larvae.

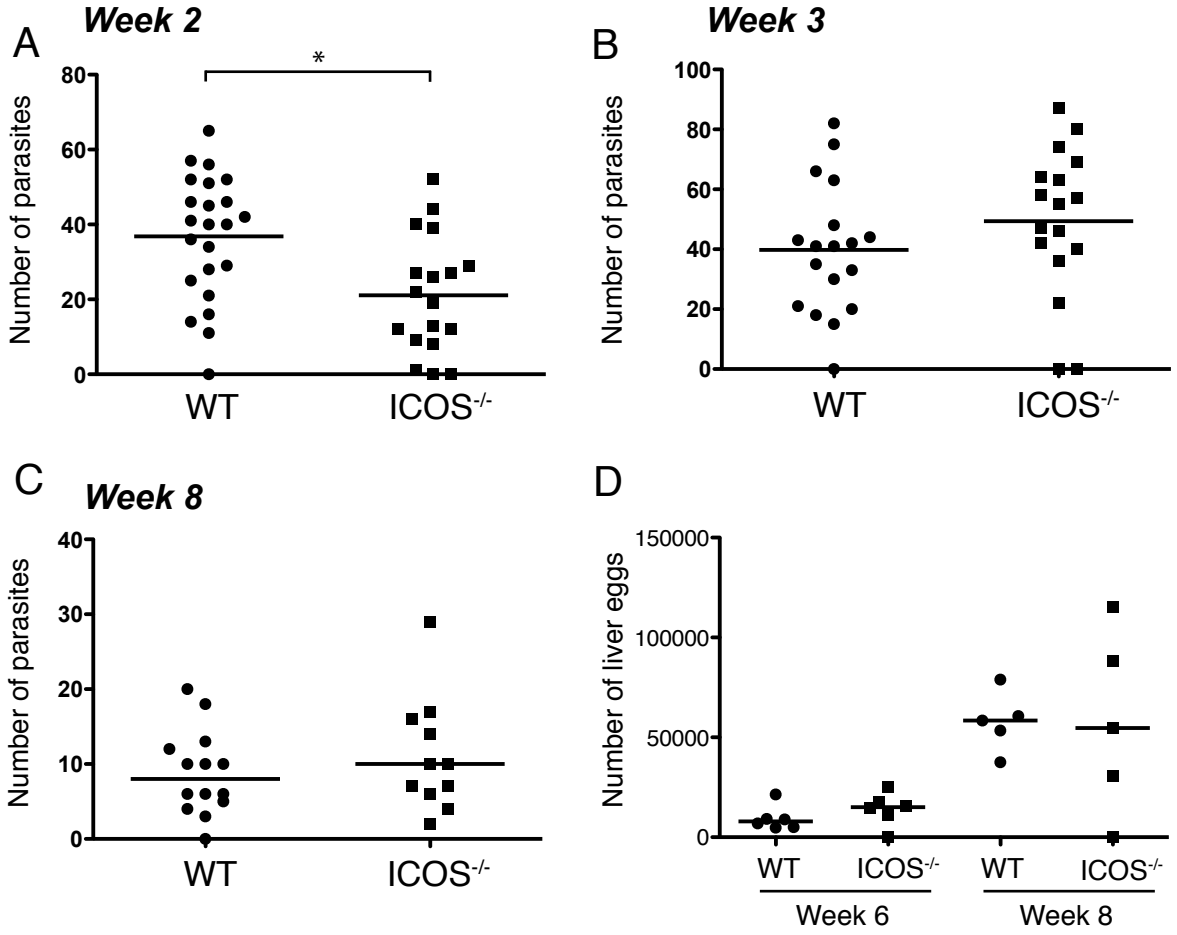



***Figure 6.7. ICOS deficiency leads to delayed migration of* S. mansoni *larvae but does not affect susceptibility to infection.*** (A & B) C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 200 *S. mansoni* cercariae and the numbers of schistosmula were enumerated at wk 2 and wk 3 of infection respectively. (C) C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and the numbers of adult parasites were enumerated at wk 8 of infection. (D) C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and the numbers of eggs in the liver were counted at wk 6 and wk 8 of infection. Closed circles denote *S. mansoni* infected WT mice and closed squares denote *S. mansoni* infected ICOS$^{-/-}$ mice. Panels show combined data from two separate experiments. * = $p < 0.05$ t-test.

### *6.8* S. mansoni *egg-induced hepatic immunopathology is unaltered by ICOS deficiency*

Previous evidence suggests that antibody blockade of ICOS at the onset of *S. mansoni* egg laying results in larger egg-induced granulomas, and this was associated with reduced

hepatic IL-10 (241). To confirm these observations in ICOS deficient mice, and to investigate the mechanisms underlying regulation of egg-induced immune pathology, we measured the area of egg-induced granulomas in the livers of wk 8 *S. mansoni* infected WT and $ICOS^{-/-}$ mice. Surprisingly, in contrast to the previous report (241), there was no difference in the size of egg-induced granulomas between WT and ICOS deficient mice (Fig. 6.8 A). This suggests that, despite the reduced $Foxp3^{+}$ Treg responses of *S. mansoni* infected $ICOS^{-/-}$ mice (Fig. 3.4 A & B) ICOS is not required for the control of hepatic egg-induced immunopathology.

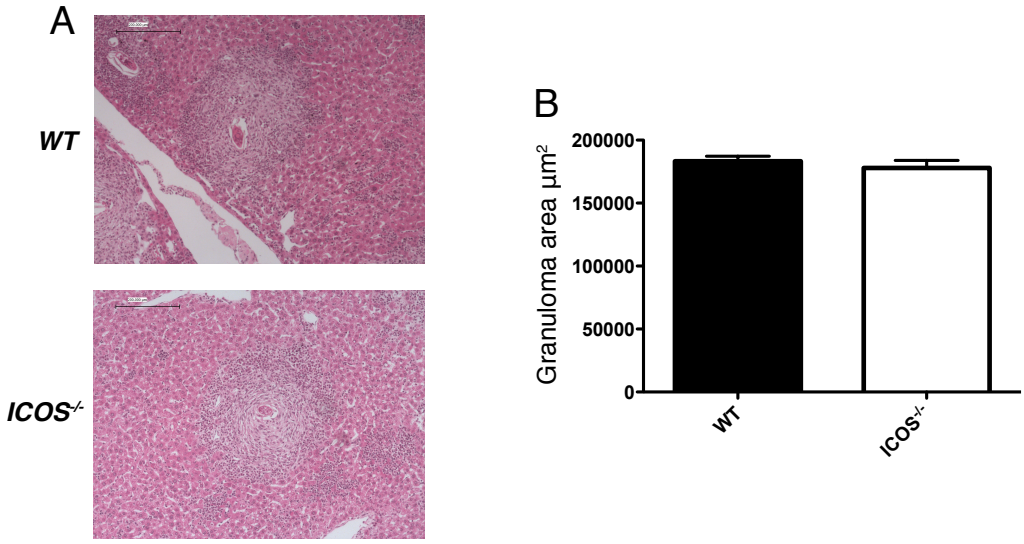



***Figure 6.8.*** **S. mansoni** ***egg-induced hepatic immunopathology is unaltered by ICOS deficiency.*** C57BL/6 WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and autopsied at wk 8 of infection. Liver sections were stained with H & E and the area of immune infiltrate surrounding individual eggs was measured using a compound microscope and Leica analysis software. (A) Representative images of H & E stained granulomas. Scale bar = 200 μm. (B) Area in μm$^2$ of hepatic granulomas. Closed bar denotes *S. mansoni* infected WT mice and open bar denotes *S. mansoni* infected ICOS$^{-/-}$ mice.

### *6.9 Th2 cytokines in late stage* S. mansoni *infection are intact in the absence of ICOS*

Foxp3$^+$ Treg control hepatic immunopathology through suppression of exuberant Th2 responses (85). In the absence of ICOS, Foxp3$^+$ Treg responses during late stage *S. mansoni* infection were impaired but immunopathology was unaffected. In the previous chapter we saw that during *H. polygyrus* infection ICOS promoted type 2 responses in the MLN through IL-4 secreting Tfh, but acted to downregulate type 2 cytokines at the infection site, highlighting the role of ICOS in both positive and negative immune responses. To ask if ICOS co-stimulation also drives type 2 immune responses following *S. mansoni* infection, we infected WT and ICOS$^{-/-}$ mice with 70 *S. mansoni* cercariae and measured CD4$^+$ T cell cytokines in the spleen and liver via flow cytometry. At wk 6 of

infection, the proportion of splenic $CD4^+$ $IL\text{-}4^+$ and $IL\text{-}13^+$ T cells was increased in both WT and $ICOS^{-/-}$ mice to a similar level, which suggests that ICOS is not required for the early egg-stage type 2 response (Fig. 6.9 A & B). At wk 8 of infection, there was no difference in the proportion of splenic $IL\text{-}13^+$ T cells between WT and $ICOS^{-/-}$ mice (Fig. 6.9 D). However, at this stage of infection the proportion of $IL\text{-}4^+$ T cells was significantly lower in $ICOS^{-/-}$ mice than WT (Fig. 6.9 C), suggesting that ICOS is required for T cell IL-4 during the acute egg-phase. Similar to *H. polygyrus* infection, the reduction in splenic IL-4 in $ICOS^{-/-}$ mice was co-incident with a failure of expansion of $CXCR5^+$ $CD4^+$ T cells (Fig. 6.9 E), indicating that the reduced IL-4 was due to fewer IL-4 secreting Tfh cells. In the liver, there was no difference in the proportion of $IL\text{-}4^+$ or $IL\text{-}13^+$ $CD4^+$ T cells between WT and $ICOS^{-/-}$ mice (Fig. 6.9 F-H) suggesting ICOS is not required for Th2 cytokines in the liver. Therefore, the lack in change in the level of Th2 cytokines could explain why ICOS deficiency had no effect on hepatic immunopathology, and further suggests that other mechanisms must compensate for the decreased $Foxp3^+$ Tregs and IL-10.

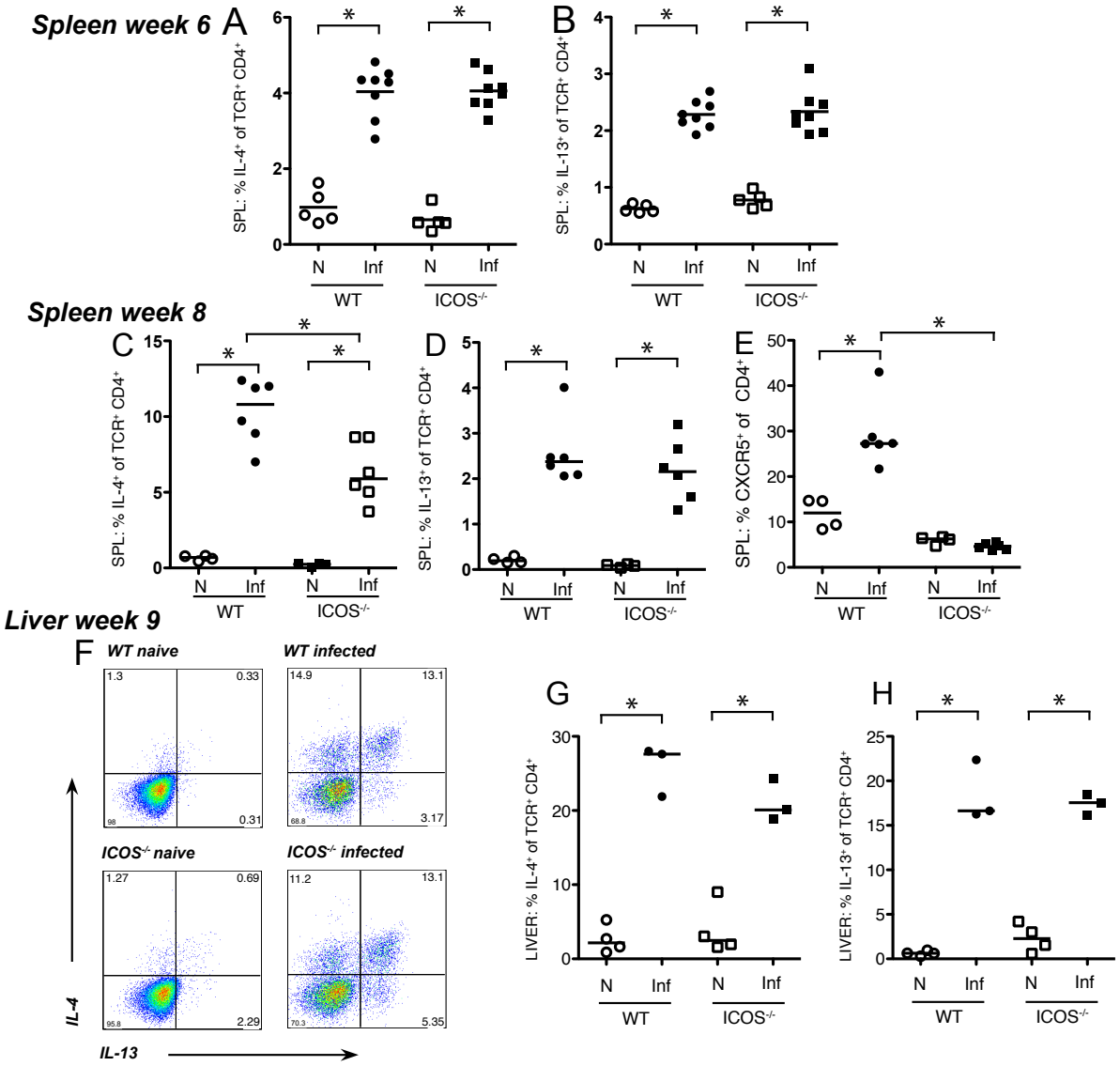



***Figure 6.9. Type 2 cytokines in late stage* S. mansoni *infection are intact following ICOS deficiency*** WT and ICOS$^{-/-}$ mice were infected with 70 *S. mansoni* cercariae and the percentage of CD4$^{+}$TCR$^{+}$IL-4$^{+}$ and IL-13$^{+}$ cells in the spleen and liver was measured by intracellular flow cytometry. (A & B) Percentage of IL-4$^{+}$ and IL-13$^{+}$ cells within the CD4$^{+}$TCR$^{+}$ population in the spleen at wk 6. (C & D) Percentage of IL-4$^{+}$ and IL-13$^{+}$ cells within the CD4$^{+}$TCR$^{+}$ population in the spleen at wk 8. (E) Percentage of CD4$^{+}$CXCR5$^{+}$ T cells in the spleen at wk 8 (F) Representative FACS plots showing percentage of IL-4 against IL-13$^{+}$ CD4$^{+}$ T cells in the liver at wk 8 of *S. mansoni* infection (G & H) Percentage of IL-4$^{+}$ and IL-13$^{+}$ cells within the CD4$^{+}$TCR$^{+}$ population in the liver at wk 8 of infection. Data points represent individual mice and bars show median values. Open circles denote naïve WT mice and closed circles denote *H .polygyrus* infected WT mice. Open squares denote naïve ICOS$^{-/-}$ mice and closed squares denote *H. polygyrus* infected ICOS$^{-/-}$ mice. * p= < 0.05 Mann Whitney test. Panels are representative of two separate experiments.

### *6.10 Discussion*

Rapid expansion of Foxp3$^+$ Treg occurs in the early phase of nematode infections. For example, during larval migration of *L. sigmodontis* parasites, Foxp3$^+$ Treg increase their rate of proliferation and expand in proportion (15). Furthermore, these Foxp3$^+$ Treg are in a heightened state of activation and effectively suppress effector immunity (15). Similar to filarial infection, during early *H. polygyrus* infection, rapid expansion and activation of Foxp3$^+$ Treg results in the suppression of type 2 cytokines (17, 31, 32), and Foxp3$^+$ Tregs must be depleted in the first few days of infection of *S. ratti* infection to effectively restore protective type 2 immunity (146). In contrast to nematode infection, during larval lung transit phase of *S. mansoni* infection, Foxp3$^+$ Treg showed no increase in proliferation and consequently did not expand in proportion or number, although there was some increase in activation status. This could suggest, that in contrast to other helminths, co-option of Treg function is not a survival strategy used by *S. mansoni* parasites to avoid host immune attack.

What other modes of immune subversion are used by *S. mansoni* to its advantage? Whereas nematodes secrete molecules that mimic the activity of host TGF-β to promote Foxp3$^+$ Treg responses to aid their survival (144, 325), *S. mansoni* parasites have acquired the ability to hide from the host immune defence. For example, *S. mansoni* parasites secrete a membranocalyx that surrounds them and shields vunerable surface proteins from immune recognition (26). Further, host erythrocyte blood group antigens are embedded in the membranocalyx, disguising the parasites from immune attack (326). Intriguingly, both lung stage and skin stage schistosomula coat their surface with host MHC molecules to camouflage themselves against the host immune response (327). Thus, *S. mansoni* parasites are poorly immunogenic, exposing only limited antigen for recognition by the host immune system, and those antigens that are visible to the immune system are often host derived and therefore do not elicit an adaptive immune response. In

terms of immunogenicity and their ability to induce protection to challenge infection, lung stage *S. mansoni* larvae are less immunogenic than skin stage *S. mansoni* larvae, which are less immunogenic than the cercariae (328). Therefore, the weak antigenicity of *S. mansoni* parasites gives them a low immune profile, and the lack of an early lung stage $Foxp3^+$ Treg response could reflect the fact that *S. mansoni* does not need to elicit a $Foxp3^+$ Treg response to evade immune attack.

Whilst low cell numbers prevented us from detecting IL-10 protein with Foxp3 in the lungs of *S. mansoni* infected $ICOS^{-/-}$ mice, the lack of IL-10 from $Foxp3^+$ Treg in the tLN could suggest that suppression through IL-10 is not a major $Foxp3^+$ Treg mechanism at this stage. Indeed, $Foxp3^+$ Treg suppression of exuberant immune responses during the egg-phase of *S. mansoni* infection seems to be largely IL-10 independent (85). Evidence suggests that the majority of IL-10 produced during *S. mansoni* infection comes from $Foxp3^-$ sources, representing either IL-10 secreting activated Th2 cells or cells with a Tr1 phenotype. For example, following drug cure of *S. mansoni* infection, in response to the egg-phase of a challenge infection the dominant source of IL-10 is a population of $CD4^+GITR^+CD25^+$ T cells that were suggested to be distinct from $Foxp3^+$ Treg (87). In keeping with this, infection of $IL\text{-}10^{GFP}$ reporter mice showed a significant increase in IL-10 from $CD4^+CD25^+$ T cells in the lungs during the early stage of *S. mansoni* infection, suggesting that $IL\text{-}10^+$ cells with this phenotype transcend the different stages of infection. Whether the $CD25^{high}IL\text{-}10^+$ population includes a proportion of $Foxp3^+$ Treg remains to be determined, but the observation that 50% of $GITR^+$ T cells express Foxp3 during *S. mansoni* infection (87) excludes full dismissal of $Foxp3^+$ Treg as contributors to the pool of IL-10.

In the absence of ICOS, schistosomula migration through the lungs was delayed. Following challenge infection, $IL\text{-}10^{-/-}$ mice vaccinated with radiation attenuated (RA) cercariae show increased protective immunity associated with increased T cell IFN-γ in the

lung (322). Increased lung T cell IFN-γ promotes the development of lung inflammatory foci that have been postulated to impede the passage of schistosomula through the lungs (329), resulting in their death in the alveolar spaces. Although blockade of ICOS in S. mansoni infected IL-10$^{GFP}$ reporter mice did not decrease IL-10 from lung CD25$^{+}$ T cells, it did result in a significant increase in CD4$^{+}$ T cell IFN-γ, a phenotype similar to that of resistant RA cercariae vaccinated IL-10$^{-/-}$ mice (322). This suggests that ICOS signalling controls Th1 effector immune responses in the lungs, and although not sufficient to induce protective immunity, increased IFN-γ in the lungs of ICOS$^{-/-}$ mice could increase the formation of inflammatory foci and account for the slow passage of schistosomula through the lungs.

During *H. polygyrus* infection, ICOS mediated effects on MLN type 2 cytokines through IL-4 secreting Tfh whilst conventional Th2 cells and initial Th2 priming events were less dependent on ICOS. Similar to *H. polygyrus* infection, Tfh are a major source of IL-4 in the lymphoid tissue during *S. mansoni* infection (233), whereas in the hepatic tissue conventional Th2 cells chiefly produce IL-4 (233). ICOS deficiency during *S. mansoni* infection led to a reduction in lymphoid IL-4 during the acute phase of the egg-stage, and this was co-incident with a failure in CXCR5$^{+}$ cells, suggesting that like in *H. polygyrus* infection, ICOS contributes to type 2 immunity through Tfh cells. At the onset of egg-production, at a time when egg-antigens prime cognate T cells, ICOS was dispensable for IL-4 production in the lymphoid tissue, which is in keeping with the observation that ICOS co-stimulation was not required for Th2 priming events during *H. polygyrus* infection. In further support of the hypothesis that ICOS contributes to type 2 immunity predominantly through Tfh, in the liver, which is devoid of Tfh, the proportion of conventional IL-4$^{+}$ Th2 cells was unaffected by the absence of ICOS.

In contrast to *H. polygyrus* infection where ICOS deficiency led to a reduction in MLN IL-13, $CD4^+$ T cell IL-13 was unaffected by the absence of ICOS throughout the egg-phase of *S. mansoni* infection. It is possible that the reduction in IL-13 during *H. polygyrus* infection is a downstream consequence of the ICOS dependent Tfh IL-4 defect, because IL-4 signalling promotes Th2 induction throughout the reactive LN (291). Thus, reduced IL-4 from Tfh could influence cytokine production from conventional Th2 cells outside the follicles. However, the reason why IL-13 did not follow IL-4 during *S. mansoni* infection remains unclear. Perhaps early IL-4 from innate cells that are independent of ICOS (330, 331) is sufficient to prime $CD4^+$ T cells to produce IL-13, rendering IL-4 from Tfh less important in this process. In addition, chronic immune responses are less dependent on ICOS signalling suggesting compensatory mechanisms take over. For example, in ICOS deficient mice infected with *T. muris*, early type 2 defects show a later recovery (203) suggesting other mechanisms can compensate. Similarly, chronic filarial infection of $ICOS^{-/-}$ mice does not result in significant Th2 immune defects, which suggests some level of redundancy with other co-stimulators (302). Indeed, overlap exists between ICOS and CD28 because both can activate the intracellular signalling molecule PI3K leading to downstream activation of the MAP kinases JNK, p38 and ERK (280, 298, 332). Intriguingly, over expression of ICOS in CD28 deficient mice substitutes CD28 dependent defects in primary antibody responses, germinal centre formation and $Foxp3^+$ Treg homeostasis (333). Therefore, CD28 may compensate for ICOS in chronic infection settings.

$Foxp3^+$ Treg control egg-induced hepatic immune granulomas through suppression of Th2 responses during acute *S. mansoni* infection (16, 30, 85, 148). ICOS deficiency did not affect egg-induced hepatic immunopathology despite reduced $Foxp3^+$ Treg responses. This could suggest that $Foxp3^+$ Treg are not the main governors of exuberant immunity in the liver, and is in keeping with the hypothesis that IL-10 is the principal regulator of

hepatic immune responses (30, 58). However, ICOS deficiency also led to a reduction in IL-10 from both $Foxp3^-$ T cells and $Foxp3^+$ T cells, and previous work has shown that blockade of ICOS from the onset of egg laying led to a significant increase in the size of egg-induced granulomas associated with decreased IL-10 and increased IFN-γ (241), raising the question as to why ICOS deficiency did not lead to enhanced hepatic immunopathology? Enhanced immunopathology is associated with prolific Th1 or Th2 responses (30, 85), and in *S. mansoni* infected $ICOS^{-/-}$ mice although $Foxp3^+$ Treg and IL-10 were reduced, there was no concurrent increase in IFN-γ (data not shown), and no increase in type 2 cytokines in the spleen or at the site of infection. The discrepancy between $ICOS^{-/-}$ mice and Ab blockade of ICOS could arise from idiosyncrasies in the different methods of ICOS ablation. Indeed, differences in immunity between $ICOS^{-/-}$ mice and Ab blockade of ICOS in WT mice in the same disease setting have occurred. For example, $ICOS^{-/-}$ mice are more susceptible to MOG induced EAE disease symptoms (197), yet Ab blockade of ICOS can reduce the symptoms of EAE (224).

Thus, in contrast to filarial and intestinal nematode infection, early trematode infection does not induce local expansion of $Foxp3^+$ Treg possibly as a result of the low immune profile of invading larvae. However, lung $Foxp3^+$ Treg showed some increase in activation status during the early stages of *S. mansoni* infection, and similar to nematode infection this was independent of ICOS signalling. The ICOS pathway contributes to the downregulation of T cell IFN-γ production in the lung following passage of schistosomula, and in doing so potentially facilitates their passage at this vulnerable stage. Despite delayed migration, ICOS deficiency did not alter late stage protective immunity to infection, and although ICOS is required for optimal $Foxp3^+$ Treg responses and IL-10 production during the egg phase of *S. mansoni* infection, its absence did not affect Th2 immunity or exacerbate hepatic immunopathology.

***Summary***

- Early *S. mansoni* infection does not induce rapid expansion of Foxp3$^+$ Treg, contrasting with infections with filarial and intestinal nematodes
- *S. mansoni* larval passage through the lungs induces IL-10 production by CD25$^+$ lung T cells
- ICOS deficiency delayed migration of *S. mansoni* larvae through the lung potentially through increased IFN-γ.
- During the egg-stage of *S. mansoni* infection ICOS promoted lymphoid IL-4 through Tfh and was not required for conventional Th2 cells at the tissue site of infection.
- Hepatic egg-induced immunopathology was not increased by ICOS deficiency despite reduced expansion of Foxp3$^+$ Treg.

## *7.0 Final Discussion*

Helminth parasites infect almost 2 billion people globally imparting a major health and economic burden on the worlds developing regions, yet so far therapies to combat these infections have proved inadequate and new strategies are required. The immunoregulatory mechanisms induced by helminths impede protection to infection and perturb potential vaccination. Inhibiting the mechanisms that helminths use for immune suppression could potentially restore protective immunity and boost the efficacy of helminth prophylaxis. At the same time, the immune downregulation elicited by helminth parasites protects the infected host from damaging immunopathology, and helminth infections are linked to the amelioration of allergy and autoimmune diseases. In this regard, understanding the mechanisms underlying helminth-induced immune suppression is of principal importance for the development of therapeutics for these conditions (334).

Foxp3$^+$ Tregs play a major role in immune suppression during helminth infection but the co-stimulatory signals governing their induction and function in this context are largely unclear. Targeting Treg co-stimulators offers a potential strategy to manipulate Treg function for therapeutic use in helminth infections, anti-tumour immunity, autoimmune disease and allergies. Growing evidence suggested that the ICOS pathway is involved for the development and function of Treg in a number of autoimmune settings, but knowledge of its role in helminth infections has been restricted to Th2 immunity. Therefore, we hypothesised that ICOS was also important for the generation and maintenance of a Foxp3$^+$ Treg response to helminth infection.

### *7.1 ICOS acts on Foxp3$^+$ Tregs in a tissue specific manner*

Interestingly, under homeostatic conditions ICOS exerted its effects on Foxp3$^+$ Treg in a tissue specific manner. In the LN of naive ICOS$^{-/-}$ mice, in keeping with previous studies, the Foxp3$^+$ Treg population was decreased, but in contrast, I found that in the small intestine LP not only was the proportion of natural Helios$^+$Foxp3$^+$ Tregs expanded in the absence of ICOS, these Treg were in a heightened state of activation. A similar tissue specific effect has been described for OX40 co-stimulation, in that the absence of OX-40 results in a tissue specific loss of Treg in the intestine, whereas LN Treg populations are independent of OX40 signals (245). The factors accounting for the increased Foxp3$^+$ Treg in the intestines of naive ICOS$^{-/-}$ mice are unknown but given that intestinal ICOS$^{-/-}$ Foxp3$^+$ Treg were severely impaired in their ability to produce IL-10, the increased proportion of Foxp3$^+$ Treg could result from a compensatory expansion by this population in an effort to recover loss of IL-10 function. In the steady state, Foxp3$^+$ Treg IL-10 controls immune responses at environmental interfaces (81), and mice with a Foxp3$^+$ Treg specific deletion of IL-10 spontaneously develop colitis (81). Notably, reminiscent of the ICOS$^{-/-}$ mice, in these mice the proportion of intestinal Foxp3$^+$ Tregs was expanded, perhaps to control the intestinal immune inflammation resulting from the IL-10 deficiency (81). Furthermore, colitis also occurs in mice whose Foxp3$^+$ Treg lack the IL-10 receptor, and these Foxp3$^+$ Treg have an activated phenotype (335), similar to the intestinal Foxp3$^+$ Treg of ICOS$^{-/-}$ mice, suggesting that impaired IL-10 signalling leads to an increase in Treg activation in the gut. Therefore, I propose that the increased proportion and activation status of intestinal Treg in naïve ICOS$^{-/-}$ mice is due to their specific loss of IL-10. Tregs potentially expand in this manner in an effort to control the increased IL-17 responses that result from IL-10 deficiency, as even in the steady state IL-10 is critical for the suppression of IL-17 produced in the intestines (336-338) via stimulation by commensal bacteria (339).

Even though natural Helios$^{+}$Foxp3$^{+}$ Treg activity was increased in the intestines of naïve ICOS$^{-/-}$ mice, following *H. polygyrus* infection ICOS$^{-/-}$ mice failed to mount an adaptive Helios$^{-}$Foxp3$^{+}$ Treg response. This finding contrasts with an *in vivo* transfer model which demonstrated that ICOS$^{-/-}$ CD25$^{-}$ T cells are as able as WT CD25$^{-}$ T cells to switch on Foxp3 to become adaptive Treg following transfer into irradiated recipient mice, suggesting that ICOS is not required for adaptive Foxp3$^{+}$ Treg (340). However, the following factors could account for the discord. The transfer model measured Foxp3$^{+}$ Treg conversion in the LN during conditions of lymphopenic expansion, whereas I measured intestinal Foxp3$^{+}$ Treg conversion in response to *H. polygyrus*. It is clear from my own data that ICOS signalling has much different effects on Foxp3$^{+}$ Treg depending on tissue location, and in the *H. polygyrus* infected intestines the immune conditions are much different from that of the homeostatic lymph node. Factors such as IL-4, TSLP, IL-33 and TGF-β, and even DC derived IL-6 induced by commensals, (341) will be present in significant amounts in the *H. polygyrus* infected intestine, all of which can affect Foxp3$^{+}$ Treg responses (257, 261, 342, 343), and could potentially account for the incongruent results. Furthermore, in the transfer model, only the donor cells were ICOS deficient so that cytokines from recipient T cells that could affect Treg conversion, for example IL-2 (344, 345), will be present that are lacking in the global ICOS$^{-/-}$ mice. This could suggest that the effects of ICOS on Treg are primarily extrinsic and this possibility will be discussed later. Finally, because CD25$^{-}$ T cells were used as the donor population, it is likely that some of the transferred cells were Foxp3$^{+}$ CD25$^{-}$ T cells, and their outgrowth during lymphopenic expansion cannot be discounted. Further investigation of the effects of ICOS on adaptive versus natural Treg, and also into the general importance of these subsets during helminth infection, requires refinement of the in vivo transfer system that I set up using congenically marked GFP$^{+}$ and GFP$^{-}$ T cells from Foxp3$^{GFP}$ mice.

### *7.2 ICOS co-stimulation has differential effects on different T cell subsets.*

ICOS signalling also had differential effects on the effector Th2 response depending on tissue location. Previous studies have shown that ICOS contributes to the anti-helminth LN Th2 response during *N. brasiliensis* (201), *T. muris* (203) and *T. spiralis* (218) infections, yet in these studies the Th2 immune response at the site of infection was not investigated. ICOS is known to be critical for the development of Tfh cell (204, 227, 230, 346) and recent work has demonstrated that Tfh are the main source of IL-4 in the LN during helminth infection (40, 233, 301). At the tissue site of helminth infection, conventional Th2 cells predominantly produce IL-4 (233, 301). In *H. polygyrus* infected $ICOS^{-/-}$ mice the reduction in LN Th2 cytokines was mirrored by the loss of Tfh suggesting that the ICOS contributes to LN IL-4 primarily through its effects on Tfh. Indeed, in the first wk of *H. polygyrus* infection, before Tfh have fully commited to their lineage and produce IL-4 (301, 308), Th2 cytokines were un-affected by the absence of ICOS. This suggests that ICOS is not required for early Th2 priming and that conventional Th2 cells are less dependent on ICOS co-stimulation, raising the possibility that the loss of Th2 cytokines in the helminth infected $ICOS^{-/-}$ mice of previous studies (201, 203, 218) could also be ascribed to a failure in IL-4 secreting Tfh. Proper distinction of Tfh requires their identification within the lymphoid follicles, and future studies should use fluorescence microscopy to visualise the IL-4 producing cells in the lymph node of *H. polygyrus* infected $ICOS^{-/-}$ mice, in order to confirm that the loss of LN type 2 cytokines was due to fewer IL-4 secreting Tfh. To this end, the generation of $ICOS^{-/-}$ IL-4 reporter mice would prove valuable for the distinction of $IL\text{-}4^{+}$ follicular resident Tfh by fluorescence microscopy.

In keeping with the hypothesis that conventional Th2 cells are less dependent on ICOS, some evidence suggests that type 2 responses at the tissue site of helminth infection do not require ICOS signalling (302). In support of this, I found that at the site of *H. polygyrus,* not only were Th2 responses efficiently mounted in the absence of ICOS, they were much greater than that of WT mice suggesting that conventional Th2 cells act independently of ICOS, and that ICOS has a further role in downregulating type 2 immunity at the site of infection (Fig 7.2). As $Foxp3^+$ Treg IL-10 is important for suppression of mucosal immunity (81), and ICOS was essential for intestinal $Foxp3^+$ Treg IL-10 during *H. polygyrus* infection, the reduced IL-10 from $ICOS^{-/-}$ $Foxp3^+$ Treg in the intestines could explain the observed increase in type 2 cytokines at this site. To further investigate if $Foxp3^+$ Treg IL-10 is important for the control of type 2 immunity in the intestines during *H. polygyrus* infection $Foxp3^{CRE}$ x $IL\text{-}10^{flox/flox}$ mice could be used to measure the intestinal anti-helminth type 2 response in the absence of Treg IL-10. If these mice had a similar phenotype to the ICOS deficient mice, it would support the idea that ICOS acts to downregulate type 2 immunity through Treg IL-10.

In contrast to the intestine where only Treg produced IL-10 following *H. polygyrus* infection, in the local lymph node both $Foxp3^+$ Treg and $Foxp3^-$ Teff produced IL-10, but only $Foxp3^+$ Treg showed a partial requirement on ICOS for IL-10, whereas $Foxp3^-$ Teff were able to produce IL-10 in the absence of ICOS, suggesting that ICOS has differential effects on different cell types. $Foxp3^+$ Treg express different cytokine receptors and co-stimulatory molecules to $Foxp3^-$ Teff so that the interaction of downstream signalling pathways activated by ICOS on Treg will be different to that of Teff. Moreover, differences in the transcriptional machinery of Treg and Teff could account for the discord between the two cell subsets. For example, $Foxp3^+$ Treg suppression of mucosal immunity through IL-10 is dependent on STAT3 (335) and future studies should aim to investigate if ICOS co-stimulation results in STAT3 activation in both $Foxp3^+$ Treg and $Foxp3^-$ Teff. In addition,

the Th2 master transcription factor GATA-3 can induce IL-10 production (292) so that Foxp3$^{-}$ Th2 effector cells generated by *H. polygyrus* infection can potentially bypass the requirement on ICOS signalling for IL-10 production. Future studies should aim to determine if the residual Foxp3$^{+}$ IL-10$^{+}$ Treg seen in the MLN of *H. polygyrus* infected ICOS$^{-/-}$ mice co-express GATA-3.

A picture is emerging in which ICOS can co-stimulate a number of T cell subsets but that some of these subsets are more dependent on ICOS co-stimulation than others. Indeed, both Tfh and Treg generated during helminth infection showed a much greater dependency on ICOS co-stimulation than conventional Th2 cells. These findings could suggest that it may be possible to block ICOS on Treg for the *in vivo* treatment of helminth infection, or perhaps concomitantly with an anti-helminth vaccine to inhibit Treg suppression of T cell memory responses, because conventional anti-helminth Th2 cells would be largely unaffected by such a regime. However, ICOS blockade during helminth infection would also eliminate the IL-4 secreting Tfh population, the importance of which in resistance to helminth infection is not yet clear and must be delineated before proceeding with such an intervention.

Numerous studies have now highlighted the importance of Treg IL-10 in the control of colitis (80, 81, 347, 348), and in humans mutations in the IL-10R gene have been associated with IBD (349). Because ICOS was critically important for Treg IL-10 production in the intestines it might be possible to target ICOS therapeutically to boost intestinal Treg IL-10 in IBD patients to ameliorate colitis symptoms. However, this approach must be exercised with caution because ICOS signalling is also essential for the maintenance of pre-differentiated Th17 (204) cells so it carries the risk of simultaneously activating the pathogenic Th17 cells which are responsible for colitic inflammation.

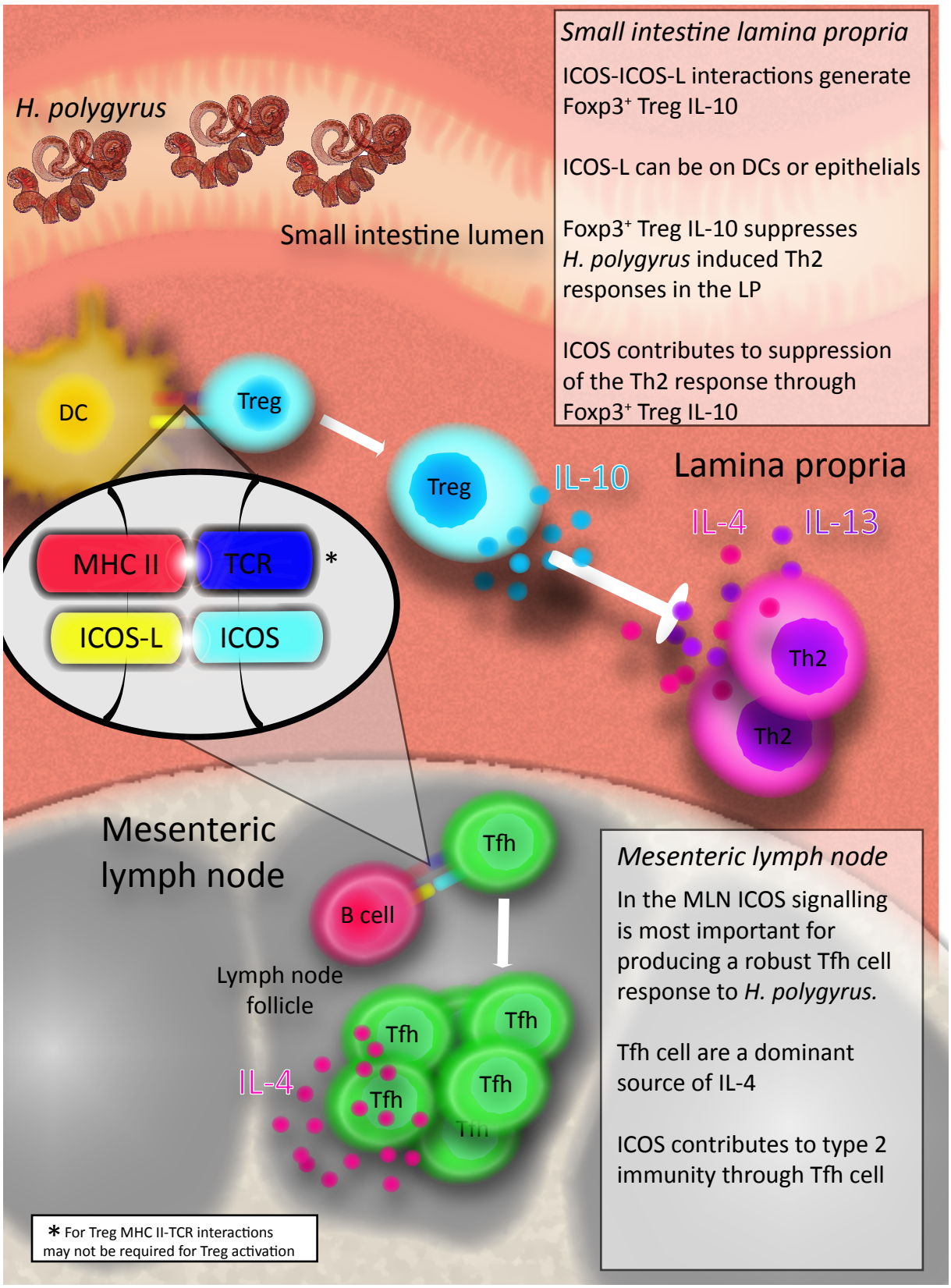



***Figure 7.2.1 Hypothesis of the effects of ICOS costimulation on type 2 cytokines in different tissues***

### *7.3 Mechanisms underlying the reduced expansion of Foxp3$^+$ Treg in helminth infected ICOS$^{-/-}$ mice.*

In the absence of ICOS, expansion of Foxp3$^+$ Treg in the secondary lymphoid tissue in response to *H. polygyrus* infection, and to the egg-phase of *S. mansoni* infection, was impaired. To generate an adaptive immune response requires TCR activation in the presence of co-stimulatory signals before T cells can begin to proliferate and differentiate. We hypothesised that the reduced expansion of Foxp3$^+$ Treg in helminth infected ICOS$^{-/-}$ mice was due to defective immune priming, proliferation and/or survival.

#### *7.3.1 Priming of Foxp3$^+$ Treg does not require ICOS*

During helminth infection the activation status of lymphoid tissue Foxp3$^+$ Treg was unaffected by ICOS deficiency, indicating that ICOS co-stimulation was not required for priming of the helminth induced Foxp3$^+$ Treg response. This is in keeping with the demonstration that ICOS is only expressed at low levels on naive T cells and is only increased following TCR and CD28 stimulation (189), and could suggest that in the early stages of helminth infection Foxp3$^+$ Treg surface expression of ICOS is presumably not sufficient for Treg activation. However, immune priming during helminth infection is a difficult aspect to measure because it requires the identification of T cells with a TCR specific to parasite antigens. In the initial stages of the immune response such T cells will only be present at very low frequencies. If the parasite antigen were known then it may be possible to use MHC tetramers to identify them. Alternatively, TCR transgenics specific for parasite antigens are required to measure antigen responsive T cells and this technique was beyond the scope of this study.

### *7.3.2 ICOS maintains Foxp3$^+$ Treg survival*

Interestingly, analysis of Treg BrdU expression showed that ICOS was also dispensable for the helminth induced *in vivo* proliferation of Foxp3$^+$ Treg, demonstrating that the failed expansion of Foxp3$^+$ Treg in helminth infected ICOS$^{-/-}$ mice was not the result of defective proliferation. Instead, ICOS signalling contributed to the survival of Treg under both conditions of homeostasis and helminth infection, so that reduced Treg survival potentially explains the reduction in Foxp3$^+$ Treg. However, this must be interpreted with the caveat that CD25 was used as a surrogate marker to identify Treg undergoing apoptosis and a proportion of these cells may be Foxp3$^-$CD25$^+$ activated Teff cells. It would be possible to measure activated caspases in conjunction with Foxp3 but these proteins are difficult to detect by flow cytometry *ex vivo* giving only a very weak signal. Therefore, more sensitive assays that can be used to measure Foxp3$^+$ Treg undergoing apoptosis are required to verify our findings, but in support of the observation that ICOS maintains Treg survival, the ICOS pathway has also been shown to aid the survival of antigen specific Teff cells (207) and for NKT cells (271), and ICOS potently activates the signalling molecule Akt (280), a known survival factor for T cells.

### *7.3.3 Does ICOS act in a cell extrinsic or intrinsic manner to aid Foxp3$^+$ Treg survival?*

Whether ICOS signals act in a cell intrinsic and/or extrinsic fashion to promote Treg survival remains to be determined. The action could be primarily extrinsic, arising as a consequence of the ICOS dependent reduction in Teff IL-2. Whilst both Treg and Teff require IL-2 to maintain their survival (274-276, 278, 279), only Teff show a further requirement on IL-2 to proliferate (277). Therefore, an ICOS dependent reduction in IL-2 availability could explain why both Foxp3$^+$ Treg and Foxp3$^-$ Teff showed reduced survival, but only Foxp3$^-$ Teff showed a reduction in the rate of proliferation during helminth

infection. IL-2 signalling results in the downstream activation and phosphorylation of the signal transducer and activator of transcription-5 (STAT5) (281) and a reduction in pSTAT5 in $ICOS^{-/-}$ $Foxp3^{+}$ Tregs could indicate impaired IL-2 signalling. Phosphorylated STAT proteins rapidly lose their phosphate moiety following the removal of the activating stimulus making them difficult to detect *ex vivo* by flow cytometry. In order to detect pSTAT5 ex-vivo by flow cytometry requires stimulation with recombinant IL-2, and such a treatment would disguise the effects of an *in vivo* ICOS dependent IL-2 deficiency on STAT5 activation in Treg. Whilst evidence suggests ICOS maintains Treg survival extrinsically through IL-2, it remains possible that ICOS signalling acts in a cell intrinsic manner to aid Treg survival. Indeed, CD28 signalling contributes to Treg homeostasis through IL-2, but it can also act independently of IL-2 to maintain the peripheral (173) and thymic Treg populations (350), suggesting that co-stimulatory molecules can function in a cell intrinsic manner for Treg homeostasis. The generation of floxed ICOS mice to restrict ICOS deficiency to $Foxp3^{+}$ Treg using $Foxp3^{CRE}$ mice would be a valuable tool for investigating if reduced Treg survival in the absence of ICOS was a cell intrinsic effect, or due to a deficiency of Teff IL-2.

### *7.3.4 ICOS co-stimulation could be used for in vitro expansion of Treg for cellular therapies for graft tolerance.*

Intriguingly, new Treg cellular therapies for promoting tolerance to allografts are now being pioneered. For example, $Foxp3^{+}$ Treg from HLA matched donor sources have been isolated and expanded *in vitro* prior to infusion into Leukeamic patients undergoing umbilical cord blood transplantation. Treatment with Treg successfully prevented allogeneic graft versus host disease (351). One of the problems associated with this technique is the difficulty of *in vitro* expansion of Treg as survival rate in vitro is generally poor and mitogenic stimulation favours expansion of contaminating Teff cells. Current protocols use monoclonal antibodies to stimulate CD3 and CD28, and the Treg are

cultured with recombinant human IL-2. As ICOS co-stimulation is also important for Treg survival, perhaps targeting ICOS in the Treg culture could enhance in vitro Treg recoveries.

### *7.4 Consequences of ICOS deficiency on protective immunity and pathology during helminth infection.*

One of the drawbacks of using $ICOS^{-/-}$ mice to investigate the effects of ICOS on helminth generated $Foxp3^{+}$ Treg was that other T cell subsets, such as Tfh, implicated in the generation of protective type 2 immune responses to helminth infection were also impaired by the absence of ICOS and could potentially explain why resistance to *H. polygyrus* infection was unchanged by the absence of ICOS. This could also explain why hepatic immune pathology was not more severe in *S. mansoni* infected $ICOS^{-/-}$ mice in the face of reduced Treg and IL-10 responses. Indeed, in most cases this would result in heightened immune pathology associated with an increased mixed Th1/Th2 response (30) response. This was not the case in $ICOS^{-/-}$ mice as both Th2 and Th1 cytokines were comparable to WT animals, possibly as a result of the overall balancing effect of ICOS deficiency on both regulatory and effector responses. Mice with a $Foxp3^{+}$ Treg specific deletion of ICOS could be used to determine if Treg require ICOS for suppression of type 2 immune responses during helminth infection, without the complication of global $ICOS^{-/-}$ in which both Treg and Teff populations are impaired. An alternative solution to this problem would be to create mixed bone marrow chimaeras with $ICOS^{+/+}Foxp3^{-/-}$ and $ICOS^{-/-}Foxp3^{+/+}$ donor marrow to restrict ICOS deficiency to Treg. Whether such mice would show increased resistance to *H. polygyrus* infection, or worse immune pathology during *S. mansoni* infection remains to be determined.

### *7.5 Immune regulation in lung stage S. mansoni infection*

Some helminths potently induce Foxp3$^+$ Treg during the initial stages of infection to bias early immune responses toward regulation resulting in suppression of late stage protective immunity (15). In this manner the outcome of an infection that can potentially last 10 years can be determined in the first few weeks or even days after infection. Whilst IL-10 has been implicated in the suppression of lung stage protective immunity to early secondary *S. mansoni* infection following vaccination with attenuated cercariae (322), less is known of the regulatory mechanisms in the early stage of primary *S. mansoni* infection. Notably, *S. mansoni* lung larval passage did not result in the proliferation or expansion of Foxp3$^+$ Treg in the lung, although Foxp3$^+$ Treg showed some signs of enhanced activation. The activated phenotype could potentially indicate a Foxp3$^+$ Treg response but one more subtle than that of other helminths. Data had hinted of a role for Foxp3$^+$ Treg in suppression of protective immune responses to *S. mansoni* larvae in the lung. For example, IL-6 deficiency leads to enhanced Th2 responses in the lung and increased protective immunity (316). In this case, the Treg populations were not examined but new evidence suggests that, contrary to expectations, Foxp3$^+$ Treg responses during *H. polygyrus* infection are impaired in the absence of IL-6, and future work should aim to characterise the lung Treg phentoype of *S. mansoni* infected IL-6$^{-/-}$ mice.

Suppression by IL-10 in the lung forms a barrier to protective immunity to secondary *S. mansoni* infection but less is known of its role in primary infection. Given the T cell requisite on ICOS for IL-10 we supposed that ICOS deficiency would lead to enhanced protection through reduced IL-10. Although ICOS deficiency did not enhance killing of parasites, there was a slower passage of larvae through the lung. Detection of lung T cell cytokines by flow cytometry proved difficult and warrants further refinement. However preliminary data suggests ICOS blockade had no effect on lung IL-10, but that it did result in increased T cell IFN-γ. As IFN-γ initiates the development of pulmonary foci, which have

been proposed to impede passage of larvae, it is possible that the increased IFN-γ seen in the lungs of ICOS mice could explain the delayed lung migration but this was not strong enough effect to completely block lung passage and further analysis of lung pathology is required to confirm this.

### *7.6 Conclusion*

Co-stimulatory molecules that were previously thought to drive positive immune responses are now also implicated in the suppression of immunity when expressed by Treg. ICOS had been classically associated with Th2 responses to helminth infections but this study described a novel role for ICOS co-stimulation in the generation and maintenance of $Foxp3^+$ Tregs during diverse helminth infections. ICOS signalling contributed to the maintenance of Treg survival and was highly important for $Foxp3^+$ Treg function in terms of IL-10 production. The effects of ICOS co-stimulation on Treg and Teff responses to helminth infection were both tissue specific and T cell subset dependent and future studies using T cell subset specific ICOS deficient transgenics will be required to delineate the importance of ICOS to the different T cell subsets. This foundational research has elucidated the key cellular affects of ICOS on helminth generated Treg which will be of importance when considering future Treg therapies for allergies and autoimmune disease, and also for restoring protective immunity to helminth infection.

### *References*


1. Hotez, P. J., P. J. Brindley, J. M. Bethony, C. H. King, E. J. Pearce, and J. Jacobson. 2008. Helminth infections: the great neglected tropical diseases. *J Clin Invest* 118:1311-1321.
2. Hotez, P. J., A. Fenwick, L. Savioli, and D. H. Molyneux. 2009. Rescuing the bottom billion through control of neglected tropical diseases. *Lancet* 373:1570-1575.
3. King, C. H. 2010. Parasites and poverty: the case of schistosomiasis. *Acta Trop* 113:95-104.
4. Hotez, P. J., J. M. Bethony, D. J. Diemert, M. Pearson, and A. Loukas. 2010. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nat Rev Microbiol* 8:814-826.
5. Hotez, P. J. 2009. Mass drug administration and integrated control for the world's high-prevalence neglected tropical diseases. *Clin Pharmacol Ther* 85:659-664.
6. Bethony, J., S. Brooker, M. Albonico, S. M. Geiger, A. Loukas, D. Diemert, and P. J. Hotez. 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* 367:1521-1532.
7. Hotez, P. J., J. Bethony, M. E. Bottazzi, S. Brooker, and P. Buss. 2005. Hookworm: "the great infection of mankind". *PLoS Med* 2:e67.
8. MacDonald, A. S., M. I. Araujo, and E. J. Pearce. 2002. Immunology of parasitic helminth infections. *Infect Immun* 70:427-433.
9. Taylor, M. J., A. Hoerauf, and M. Bockarie. 2010. Lymphatic filariasis and onchocerciasis. *Lancet* 376:1175-1185.
10. Molyneux, D. H. 2012. Tropical lymphedemas--control and prevention. *N Engl J Med* 366:1169-1171.
11. Maizels, R. M., and M. Yazdanbakhsh. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 3:733-744.
12. Maizels, R. M., A. Balic, N. Gomez-Escobar, M. Nair, M. D. Taylor, and J. E. Allen. 2004. Helminth parasites--masters of regulation. *Immunol Rev* 201:89-116.
13. Taylor, M. D., A. Harris, S. A. Babayan, O. Bain, A. Culshaw, J. E. Allen, and R. M. Maizels. 2007. CTLA-4 and CD4+ CD25+ regulatory T cells inhibit protective immunity to filarial parasites in vivo. *J Immunol* 179:4626-4634.
14. Taylor, M. D., L. LeGoff, A. Harris, E. Malone, J. E. Allen, and R. M. Maizels. 2005. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J Immunol* 174:4924-4933.
15. Taylor, M. D., N. van der Werf, A. Harris, A. L. Graham, O. Bain, J. E. Allen, and R. M. Maizels. 2009. Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection. *Eur. J. Immunol.* 39:192-206.
16. Layland, L. E., R. Rad, H. Wagner, and C. U. P. da Costa. 2007. Immunopathology in schistosomiasis is controlled by antigen-specific regulatory T cells primed in the presence of TLR2. *Eur. J. Immunol.* 37:2174-2184.
17. Rausch, S., J. Huehn, C. Loddenkemper, M. Hepworth, C. Klotz, T. Sparwasser, A. Hamann, R. Lucius, and S. Hartmann. 2009. Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3(+) cells. *Eur. J. Immunol.* 39:3066-3077.
18. Maizels, R. M., D. A. Bundy, M. E. Selkirk, D. F. Smith, and R. M. Anderson. 1993. Immunological modulation and evasion by helminth parasites in human populations. *Nature* 365:797-805.

19. Else, K. J., F. D. Finkelman, C. R. Maliszewski, and R. K. Grencis. 1994. Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med* 179:347-351.
20. Else, K. J., and R. K. Grencis. 1991. Cellular immune responses to the murine nematode parasite Trichuris muris. I. Differential cytokine production during acute or chronic infection. *Immunology* 72:508-513.
21. Else, K. J., L. Hültner, and R. K. Grencis. 1992. Cellular immune responses to the murine nematode parasite Trichuris muris. II. Differential induction of TH-cell subsets in resistant versus susceptible mice. *Immunology* 75:232-237.
22. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 2005. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. 1986. *J Immunol* 175:5-14.
23. Mosmann, T. R., and K. W. Moore. 1991. The role of IL-10 in crossregulation of TH1 and TH2 responses. *Immunol Today* 12:A49-53.
24. Wilson, R. 2009. The saga of schistosome migration and attrition. *Parasitology*:1-12.
25. Curwen, R. S., P. D. Ashton, S. Sundaralingam, and R. A. Wilson. 2006. Identification of novel proteases and immunomodulators in the secretions of schistosome cercariae that facilitate host entry. *Mol Cell Proteomics* 5:835-844.
26. Wilson, R. A., and P. S. Coulson. 2009. Immune effector mechanisms against schistosomiasis: looking for a chink in the parasite's armour. *Trends Parasitol* 25:423-431.
27. Anthony, R. M., L. I. Rutitzky, J. F. Urban, M. J. Stadecker, and W. C. Gause. 2007. Protective immune mechanisms in helminth infection. *Nat Rev Immunol* 7:975-987.
28. Urban, J. F., I. M. Katona, W. E. Paul, and F. D. Finkelman. 1991. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc Natl Acad Sci USA* 88:5513-5517.
29. Fallon, P. G., and D. W. Dunne. 1999. Tolerization of mice to Schistosoma mansoni egg antigens causes elevated type 1 and diminished type 2 cytokine responses and increased mortality in acute infection. *J Immunol* 162:4122-4132.
30. Hoffmann, K. F., A. W. Cheever, and T. A. Wynn. 2000. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol* 164:6406-6416.
31. Finney, C. A., M. D. Taylor, M. S. Wilson, and R. M. Maizels. 2007. Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. *Eur. J. Immunol.* 37:1874-1886.
32. Rausch, S., J. Huehn, D. Kirchhoff, J. Rzepecka, C. Schnoeller, S. Pillai, C. Loddenkemper, A. Scheffold, A. Hamann, R. Lucius, and S. Hartmann. 2008. Functional analysis of effector and regulatory T cells in a parasitic nematode infection. *Infect Immun* 76:1908-1919.
33. Svetić, A., K. B. Madden, X. D. Zhou, P. Lu, I. M. Katona, F. D. Finkelman, J. F. Urban, and W. C. Gause. 1993. A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *J Immunol* 150:3434-3441.
34. Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. A. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, H. E. Jolin, and A. N. J. Mckenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*:1-5.
35. Price, A. E., H. E. Liang, B. M. Sullivan, R. L. Reinhardt, C. J. Eisley, D. J. Erle, and R. M. Locksley. 2010. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA* 107:11489-11494.
36. Saenz, S. A., M. C. Siracusa, J. G. Perrigoue, S. P. Spencer, J. F. Urban, J. E. Tocker, A. L. Budelsky, M. A. Kleinschek, R. A. Kastelein, T. Kambayashi, A. Bhandoola, and D. Artis. 2010. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464:1362-1366.

37. Voehringer, D., T. A. Reese, X. Huang, K. Shinkai, and R. M. Locksley. 2006. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J Exp Med* 203:1435-1446.
38. Katona, I. M., J. F. Urban, and F. D. Finkelman. 1988. The role of L3T4+ and Lyt-2+ T cells in the IgE response and immunity to Nippostrongylus brasiliensis. *J Immunol* 140:3206-3211.
39. Vignali, D. A., P. Crocker, Q. D. Bickle, S. Cobbold, H. Waldmann, and M. G. Taylor. 1989. A role for CD4+ but not CD8+ T cells in immunity to Schistosoma mansoni induced by 20 krad-irradiated and Ro 11-3128-terminated infections. *Immunology* 67:466-472.
40. King, I., and M. Mohrs. 2009. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med*.
41. Anthony, R. M., J. F. Urban, F. Alem, H. A. Hamed, C. T. Rozo, J.-L. Boucher, N. van Rooijen, and W. C. Gause. 2006. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med* 12:955-960.
42. Patel, N., T. Kreider, J. F. Urban, and W. C. Gause. 2009. Characterisation of effector mechanisms at the host:parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. *Int J Parasitol* 39:13-21.
43. Morimoto, M., M. Morimoto, J. Whitmire, S. Xiao, R. M. Anthony, H. Mirakami, R. A. Star, J. F. Urban, and W. C. Gause. 2004. Peripheral CD4 T cells rapidly accumulate at the host: parasite interface during an inflammatory Th2 memory response. *J Immunol* 172:2424-2430.
44. Cliffe, L. J., N. E. Humphreys, T. E. Lane, C. S. Potten, C. Booth, and R. K. Grencis. 2005. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* 308:1463-1465.
45. Hashimoto, K., R. Uchikawa, T. Tegoshi, K. Takeda, M. Yamada, and N. Arizono. 2009. Depleted intestinal goblet cells and severe pathological changes in SCID mice infected with Heligmosomoides polygyrus. *Parasite Immunol* 31:457-465.
46. Hasnain, S. Z., C. M. Evans, M. Roy, A. L. Gallagher, K. N. Kindrachuk, L. Barron, B. F. Dickey, M. S. Wilson, T. A. Wynn, R. K. Grencis, and D. J. Thornton. 2011. Muc5ac: a critical component mediating the rejection of enteric nematodes. *J Exp Med* 208:893-900.
47. Hasnain, S. Z., H. Wang, J.-E. Ghia, N. Haq, Y. Deng, A. Velcich, R. K. Grencis, D. J. Thornton, and W. I. Khan. 2010. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* 138:1763-1771.
48. Herbert, D. R., J. Q. Yang, S. P. Hogan, K. Groschwitz, M. Khodoun, A. Munitz, T. Orekov, C. Perkins, Q. Wang, F. Brombacher, J. F. Urban, M. E. Rothenberg, and F. D. Finkelman. 2009. Intestinal epithelial cell secretion of RELM-{beta} protects against gastrointestinal worm infection. *J Exp Med*.
49. Grencis, R. K., K. J. Else, J. F. Huntley, and S. I. Nishikawa. 1993. The in vivo role of stem cell factor (c-kit ligand) on mastocytosis and host protective immunity to the intestinal nematode Trichinella spiralis in mice. *Parasite Immunol* 15:55-59.
50. McDermott, J. R., R. E. Bartram, P. A. Knight, H. R. P. Miller, D. R. Garrod, and R. K. Grencis. 2003. Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci USA* 100:7761-7766.
51. Shea-Donohue, T., L. Notari, J. Stiltz, R. Sun, K. B. Madden, J. F. Urban, and A. Zhao. 2010. Role of enteric nerves in immune-mediated changes in protease-activated receptor 2 effects on gut function. *Neurogastroenterol Motil* 22:1138-e1291.
52. Pearce, E. J., and A. S. MacDonald. 2002. The immunobiology of schistosomiasis. *Nat Rev Immunol* 2:499-511.
53. Cheever, A. W., M. E. Williams, T. A. Wynn, F. D. Finkelman, R. A. Seder, T. M. Cox, S. Hieny, P. Caspar, and A. Sher. 1994. Anti-IL-4 treatment of Schistosoma mansoni-

infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. *J Immunol* 153:753-759.
54. Wynn, T. A., I. Eltoum, A. W. Cheever, F. A. Lewis, W. C. Gause, and A. Sher. 1993. Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs of Schistosoma mansoni. *J Immunol* 151:1430-1440.
55. Yamashita, T., and D. L. Boros. 1992. IL-4 influences IL-2 production and granulomatous inflammation in murine schistosomiasis mansoni. *J Immunol* 149:3659-3664.
56. Wynn, T. A., R. W. Thompson, A. W. Cheever, and M. M. Mentink-Kane. 2004. Immunopathogenesis of schistosomiasis. *Immunol Rev* 201:156-167.
57. Brunet, L. R., F. D. Finkelman, A. W. Cheever, M. A. Kopf, and E. J. Pearce. 1997. IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J Immunol* 159:777-785.
58. Wynn, T. A., A. W. Cheever, M. E. Williams, S. Hieny, P. Caspar, R. Kühn, W. Müller, and A. Sher. 1998. IL-10 regulates liver pathology in acute murine Schistosomiasis mansoni but is not required for immune down-modulation of chronic disease. *J Immunol* 160:4473-4480.
59. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 182:18-32.
60. Shevach, E. M., R. A. DiPaolo, J. Andersson, D.-M. Zhao, G. L. Stephens, and A. M. Thornton. 2006. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev* 212:60-73.
61. Coombes, J. L., N. J. Robinson, K. J. Maloy, H. H. Uhlig, and F. Powrie. 2005. Regulatory T cells and intestinal homeostasis. *Immunol Rev* 204:184-194.
62. Xystrakis, E., S. E. Boswell, and C. M. Hawrylowicz. 2006. T regulatory cells and the control of allergic disease. *Expert Opin Biol Ther* 6:121-133.
63. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol* 7:875-888.
64. Rouse, B. T., P. P. Sarangi, and S. Suvas. 2006. Regulatory T cells in virus infections. *Immunol Rev* 212:272-286.
65. Kretschmer, K., I. Apostolou, E. Jaeckel, K. Khazaie, and H. von Boehmer. 2006. Making regulatory T cells with defined antigen specificity: role in autoimmunity and cancer. *Immunol Rev* 212:163-169.
66. Sakaguchi, S., M. Ono, R. Setoguchi, H. Yagi, S. Hori, Z. Fehervari, J. Shimizu, T. Takahashi, and T. Nomura. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212:8-27.
67. Zheng, Y., and A. Y. Rudensky. 2007. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol* 8:457-462.
68. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2:301-306.
69. Thornton, A. M., P. E. Korty, D. Q. Tran, E. A. Wohlfert, P. E. Murray, Y. Belkaid, and E. M. Shevach. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 184:3433-3441.
70. Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Cárcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 204:1757-1764.

71. Fantini, M. C., C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172:5149-5153.
72. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30:626-635.
73. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68-73.
74. Godfrey, V. L., J. E. Wilkinson, and L. B. Russell. 1991. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol* 138:1379-1387.
75. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.
76. Roncarolo, M. G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212:28-50.
77. Collison, L. W., V. Chaturvedi, A. L. Henderson, P. R. Giacomin, C. Guy, J. Bankoti, D. Finkelstein, K. Forbes, C. J. Workman, S. A. Brown, J. E. Rehg, M. L. Jones, H. Ni, D. Artis, M. J. Turk, and D. A. A. Vignali. 2010. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*.
78. Nakagawa, T., M. Tsuruoka, H. Ogura, Y. Okuyama, Y. Arima, T. Hirano, and M. Murakami. 2010. IL-6 positively regulates Foxp3+CD8+ T cells in vivo. *Int Immunol* 22:129-139.
79. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.
80. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 190:995-1004.
81. Rubtsov, Y. P., J. P. Rasmussen, E. Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W. R. Henderson, W. Muller, and A. Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28:546-558.
82. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* 420:502-507.
83. Kursar, M., M. Koch, H.-W. Mittrücker, G. Nouailles, K. Bonhagen, T. Kamradt, and S. H. E. Kaufmann. 2007. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. *J Immunol* 178:2661-2665.
84. Metenou, S., B. Dembele, S. Konate, H. Dolo, S. Y. Coulibaly, Y. I. Coulibaly, A. A. Diallo, L. Soumaoro, M. E. Coulibaly, D. Sanogo, S. S. Doumbia, S. F. Traoré, S. Mahanty, A. Klion, and T. B. Nutman. 2010. At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. *J Immunol* 184:5375-5382.
85. Baumgart, M., F. Tompkins, J. Leng, and M. Hesse. 2006. Naturally occurring CD4+Foxp3+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. *J Immunol* 176:5374-5387.
86. Beiting, D. P., L. F. Gagliardo, M. Hesse, S. K. Bliss, D. Meskill, and J. A. Appleton. 2007. Coordinated control of immunity to muscle stage Trichinella spiralis by IL-10, regulatory T cells, and TGF-beta. *J Immunol* 178:1039-1047.

87. Wilson, M. S., A. W. Cheever, S. D. White, R. W. Thompson, and T. A. Wynn. 2011. IL-10 blocks the development of resistance to re-infection with Schistosoma mansoni. *PLoS Pathog* 7:e1002171.
88. Wilson, M. S., M. D. Taylor, A. Balic, C. A. M. Finney, J. R. Lamb, and R. M. Maizels. 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med* 202:1199-1212.
89. Taylor, J. J., M. Mohrs, and E. J. Pearce. 2006. Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population. *J Immunol* 176:5839-5847.
90. Mitre, E., D. Chien, and T. B. Nutman. 2008. CD4(+) (and not CD25+) T cells are the predominant interleukin-10-producing cells in the circulation of filaria-infected patients. *J INFECT DIS* 197:94-101.
91. Piccirillo, C. A., J. J. Letterio, A. M. Thornton, R. S. McHugh, M. Mamura, H. Mizuhara, and E. M. Shevach. 2002. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 196:237-246.
92. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 194:629-644.
93. Li, M. O., Y. Y. Wan, and R. A. Flavell. 2007. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 26:579-591.
94. Green, E. A., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. *Proc Natl Acad Sci USA* 100:10878-10883.
95. Strauss, L., C. Bergmann, W. Gooding, J. T. Johnson, and T. L. Whiteside. 2007. The frequency and suppressor function of CD4+CD25highFoxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res* 13:6301-6311.
96. Hilchey, S. P., A. De, L. M. Rimsza, R. B. Bankert, and S. H. Bernstein. 2007. Follicular lymphoma intratumoral CD4+CD25+GITR+ regulatory T cells potently suppress CD3/CD28-costimulated autologous and allogeneic CD8+CD25- and CD4+CD25- T cells. *J Immunol* 178:4051-4061.
97. Walther, M., J. E. Tongren, L. Andrews, D. Korbel, E. King, H. Fletcher, R. F. Andersen, P. Bejon, F. Thompson, S. J. Dunachie, F. Edele, J. B. de Souza, R. E. Sinden, S. C. Gilbert, E. M. Riley, and A. V. S. Hill. 2005. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23:287-296.
98. Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450:566-569.
99. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson, and T. J. Ley. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21:589-601.
100. Gondek, D. C., L.-F. Lu, S. A. Quezada, S. Sakaguchi, and R. J. Noelle. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174:1783-1786.
101. Zhao, D.-M., A. M. Thornton, R. J. DiPaolo, and E. M. Shevach. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* 107:3925-3932.

102. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27:635-646.
103. Korten, S., M. Badusche, D. W. Büttner, A. Hoerauf, N. Brattig, and B. Fleischer. 2008. Natural death of adult Onchocerca volvulus and filaricidal effects of doxycycline induce local FOXP3+/CD4+ regulatory T cells and granzyme expression. *Microbes Infect* 10:313-324.
104. Hartmann, W., B. J. Marsland, B. Otto, J. Urny, B. Fleischer, and S. Korten. 2011. A novel and divergent role of granzyme A and B in resistance to helminth infection. *J Immunol* 186:2472-2481.
105. Pandiyan, P., L. Zheng, S. Ishihara, J. Reed, and M. J. Lenardo. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 8:1353-1362.
106. Borsellino, G., M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Höpner, D. Centonze, G. Bernardi, M. L. Dell'Acqua, P. M. Rossini, L. Battistini, O. Rötzschke, and K. Falk. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110:1225-1232.
107. Bopp, T., C. Becker, M. Klein, S. Klein-Hessling, A. Palmetshofer, E. Serfling, V. Heib, M. Becker, J. Kubach, S. Schmitt, S. Stoll, H. Schild, M. S. Staege, M. Stassen, H. Jonuleit, and E. Schmitt. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204:1303-1310.
108. Hewitson, J. P., Y. Harcus, J. Murray, M. van Agtmaal, K. J. Filbey, J. R. Grainger, S. Bridgett, M. L. Blaxter, P. D. Ashton, D. A. Ashford, R. S. Curwen, R. A. Wilson, A. A. Dowle, and R. M. Maizels. 2011. Proteomic analysis of secretory products from the model gastrointestinal nematode Heligmosomoides polygyrus reveals dominance of venom allergen-like (VAL) proteins. *J Proteomics* 74:1573-1594.
109. Fallarino, F., U. Grohmann, K. W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, M.-L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 4:1206-1212.
110. Mellor, A. L., and D. H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 4:762-774.
111. Cederbom, L., H. Hall, and F. Ivars. 2000. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur. J. Immunol.* 30:1538-1543.
112. Liang, B., C. Workman, J. Lee, C. Chew, B. M. Dale, L. Colonna, M. Flores, N. Li, E. Schweighoffer, S. Greenberg, V. Tybulewicz, D. Vignali, and R. Clynes. 2008. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* 180:5916-5926.
113. Sarris, M., K. G. Andersen, F. Randow, L. Mayr, and A. G. Betz. 2008. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* 28:402-413.
114. Tarbell, K. V., L. Petit, X. Zuo, P. Toy, X. Luo, A. Mqadmi, H. Yang, M. Suthanthiran, S. Mojsov, and R. M. Steinman. 2007. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J Exp Med* 204:191-201.
115. Hesse, M., C. A. Piccirillo, Y. Belkaid, J. Prufer, M. Mentink-Kane, M. Leusink, A. W. Cheever, E. M. Shevach, and T. A. Wynn. 2004. The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J Immunol* 172:3157-3166.
116. Suvas, S., A. K. Azkur, B. S. Kim, U. Kumaraguru, and B. T. Rouse. 2004. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* 172:4123-4132.

117. Montagnoli, C., A. Bacci, S. Bozza, R. Gaziano, P. Mosci, A. H. Sharpe, and L. Romani. 2002. B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to Candida albicans. *J Immunol* 169:6298-6308.
118. Ji, J., J. Masterson, J. Sun, and L. Soong. 2005. CD4+CD25+ regulatory T cells restrain pathogenic responses during Leishmania amazonensis infection. *J Immunol* 174:7147-7153.
119. Mendez, S., S. K. Reckling, C. A. Piccirillo, D. Sacks, and Y. Belkaid. 2004. Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. *J Exp Med* 200:201-210.
120. Hisaeda, H., Y. Maekawa, D. Iwakawa, H. Okada, K. Himeno, K. Kishihara, S.-i. Tsukumo, and K. Yasutomo. 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. *Nat Med* 10:29-30.
121. King, C. L., S. Mahanty, V. Kumaraswami, J. S. Abrams, J. Regunathan, K. Jayaraman, E. A. Ottesen, and T. B. Nutman. 1993. Cytokine control of parasite-specific anergy in human lymphatic filariasis. Preferential induction of a regulatory T helper type 2 lymphocyte subset. *J Clin Invest* 92:1667-1673.
122. Piessens, W. F., P. B. McGreevy, P. W. Piessens, M. McGreevy, I. Koiman, J. S. Saroso, and D. T. Dennis. 1980. Immune responses in human infections with Brugia malayi: specific cellular unresponsiveness to filarial antigens. *J Clin Invest* 65:172-179.
123. Yazdanbakhsh, M., W. A. Paxton, Y. C. Kruize, E. Sartono, A. Kurniawan, A. van het Wout, M. E. Selkirk, F. Partono, and R. M. Maizels. 1993. T cell responsiveness correlates differentially with antibody isotype levels in clinical and asymptomatic filariasis. *J INFECT DIS* 167:925-931.
124. Atmadja, A. K., R. Atkinson, E. Sartono, F. Partono, M. Yazdanbakhsh, and R. M. Maizels. 1995. Differential decline in filaria-specific IgG1, IgG4, and IgE antibodies in Brugia malayi-infected patients after diethylcarbamazine chemotherapy. *J INFECT DIS* 172:1567-1572.
125. Satoguina, J., T. Adjobimey, K. Arndts, J. Hoch, J. Oldenburg, L. Layland, and A. Hoerauf. 2008. Tr1 and naturally occurring regulatory T cells induce IgG4 in B cells through GITR/GITR-L interaction, IL-10 and TGF-beta. *Eur. J. Immunol.* 38:3101-3113.
126. Satoguina, J., E. Weyand, J. Larbi, and A. Hoerauf. 2005. T regulatory-1 cells induce IgG4 production by B cells: role of IL-10. *J Immunol* 174:4718-4726.
127. Babu, S., S. Q. Bhat, N. Pavan Kumar, A. B. Lipira, S. Kumar, C. Karthik, V. Kumaraswami, and T. B. Nutman. 2009. Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. *PLoS Negl Trop Dis* 3:e420.
128. Babu, S., C. P. Blauvelt, V. Kumaraswami, and T. B. Nutman. 2006. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176:3248-3256.
129. Wammes, L. J., F. Hamid, A. E. Wiria, H. Wibowo, E. Sartono, R. M. Maizels, H. H. Smits, T. Supali, and M. Yazdanbakhsh. 2012. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. *PLoS Negl Trop Dis* 6:e1655.
130. Satoguina, J., M. Mempel, J. Larbi, M. Badusche, C. Löliger, O. Adjei, G. Gachelin, B. Fleischer, and A. Hoerauf. 2002. Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). *Microbes Infect* 4:1291-1300.
131. García-Hernández, M. H., B. Alvarado-Sánchez, M. Z. Calvo-Turrubiartes, M. Salgado-Bustamante, C. Y. Rodríguez-Pinal, L. R. Gámez-López, R. González-Amaro, and D. P. Portales-Pérez. 2009. Regulatory T Cells in children with intestinal parasite infection. *Parasite Immunol* 31:597-603.

132. Turner, J. D., J. A. Jackson, H. Faulkner, J. Behnke, K. J. Else, J. Kamgno, M. Boussinesq, and J. E. Bradley. 2008. Intensity of intestinal infection with multiple worm species is related to regulatory cytokine output and immune hyporesponsiveness. *J INFECT DIS* 197:1204-1212.
133. Wammes, L. J., F. Hamid, A. E. Wiria, B. de Gier, E. Sartono, R. M. Maizels, A. J. Luty, Y. Fillié, G. T. Brice, T. Supali, H. H. Smits, and M. Yazdanbakhsh. 2010. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. *Eur. J. Immunol.* 40:437-442.
134. Elias, D., S. Britton, A. Aseffa, H. Engers, and H. Akuffo. 2008. Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* 26:3897-3902.
135. Cooper, P. J., M. Chico, C. Sandoval, I. Espinel, A. Guevara, M. M. Levine, G. E. Griffin, and T. B. Nutman. 2001. Human infection with Ascaris lumbricoides is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infect Immun* 69:1574-1580.
136. Grogan, J. L., P. G. Kremsner, A. M. Deelder, and M. Yazdanbakhsh. 1998. Antigen-specific proliferation and interferon-gamma and interleukin-5 production are down-regulated during Schistosoma haematobium infection. *J INFECT DIS* 177:1433-1437.
137. Watanabe, K., P. N. Mwinzi, C. L. Black, E. M. Muok, D. M. Karanja, W. E. Secor, and D. G. Colley. 2007. T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. *Am J Trop Med Hyg* 77:676-682.
138. Nausch, N., N. Midzi, T. Mduluza, R. M. Maizels, and F. Mutapi. 2011. Regulatory and activated T cells in human Schistosoma haematobium infections. *PLoS ONE* 6:e16860.
139. Gillan, V., and E. Devaney. 2005. Regulatory T cells modulate Th2 responses induced by Brugia pahangi third-stage larvae. *Infect Immun* 73:4034-4042.
140. McSorley, H. J., Y. M. Harcus, J. Murray, M. D. Taylor, and R. M. Maizels. 2008. Expansion of Foxp3+ regulatory T cells in mice infected with the filarial parasite Brugia malayi. *J Immunol* 181:6456-6466.
141. Dittrich, A. M., A. Erbacher, S. Specht, F. Diesner, M. Krokowski, A. Avagyan, P. Stock, B. Ahrens, W. H. Hoffmann, A. Hoerauf, and E. Hamelmann. 2008. Helminth infection with Litomosoides sigmodontis induces regulatory T cells and inhibits allergic sensitization, airway inflammation, and hyperreactivity in a murine asthma model. *J Immunol* 180:1792-1799.
142. Kitagaki, K., T. R. Businga, D. Racila, D. E. Elliott, J. V. Weinstock, and J. N. Kline. 2006. Intestinal helminths protect in a murine model of asthma. *J Immunol* 177:1628-1635.
143. Elliott, D. E., T. Setiawan, A. Metwali, A. Blum, J. F. Urban, and J. V. Weinstock. 2004. Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. *Eur. J. Immunol.* 34:2690-2698.
144. Grainger, J. R., K. A. Smith, J. P. Hewitson, H. J. McSorley, Y. Harcus, K. J. Filbey, C. A. Finney, E. J. Greenwood, D. P. Knox, M. S. Wilson, Y. Belkaid, A. Y. Rudensky, and R. M. Maizels. 2010. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-{beta} pathway. *J Exp Med*.
145. D'Elia, R., J. M. Behnke, J. E. Bradley, and K. J. Else. 2009. Regulatory T cells: a role in the control of helminth-driven intestinal pathology and worm survival. *J Immunol* 182:2340-2348.
146. Blankenhaus, B., U. Klemm, M.-L. Eschbach, T. Sparwasser, J. Huehn, A. A. Kühl, C. Loddenkemper, T. Jacobs, and M. Breloer. 2011. Strongyloides ratti infection induces expansion of Foxp3+ regulatory T cells that interfere with immune response and parasite clearance in BALB/c mice. *J Immunol* 186:4295-4305.

147. Layland, L. E., J. Mages, C. Loddenkemper, A. Hoerauf, H. Wagner, R. Lang, and C. U. P. da Costa. 2010. Pronounced phenotype in activated regulatory T cells during a chronic helminth infection. *J Immunol* 184:713-724.
148. Singh, K. P., H. C. Gerard, A. P. Hudson, T. R. Reddy, and D. L. Boros. 2005. Retroviral Foxp3 gene transfer ameliorates liver granuloma pathology in Schistosoma mansoni infected mice. *Immunology* 114:410-417.
149. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. *Nat Rev Immunol* 8:523-532.
150. Freeman, C. M., B.-c. Chiu, V. R. Stolberg, J. Hu, K. Zeibecoglou, N. W. Lukacs, S. A. Lira, S. L. Kunkel, and S. W. Chensue. 2005. CCR8 is expressed by antigen-elicited, IL-10-producing CD4+CD25+ T cells, which regulate Th2-mediated granuloma formation in mice. *J Immunol* 174:1962-1970.
151. Doetze, A., J. Satoguina, G. Burchard, T. Rau, C. Löliger, B. Fleischer, and A. Hoerauf. 2000. Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. *Int Immunol* 12:623-630.
152. Collison, L. W., V. Chaturvedi, A. L. Henderson, P. R. Giacomin, C. Guy, J. Bankoti, D. Finkelstein, K. Forbes, C. J. Workman, S. A. Brown, J. E. Rehg, M. L. Jones, H.-T. Ni, D. Artis, M. J. Turk, and D. A. A. Vignali. 2010. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*.
153. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 14:233-258.
154. Bour-Jordan, H., J. H. Esensten, M. Martinez-Llordella, C. Penaranda, M. Stumpf, and J. A. Bluestone. 2011. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunol Rev* 241:180-205.
155. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* 3:609-620.
156. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nature Reviews Immunology* 2:116-126.
157. Bour-Jordan, H., and J. A. Bluestone. 2009. Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. *Immunol Rev* 229:41-66.
158. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.
159. Ueda, H., J. M. M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D. B. Rainbow, K. M. D. Hunter, A. N. Smith, G. Di Genova, M. H. Herr, I. Dahlman, F. Payne, D. Smyth, C. Lowe, R. C. J. Twells, S. Howlett, B. Healy, S. Nutland, H. E. Rance, V. Everett, L. J. Smink, A. C. Lam, H. J. Cordell, N. M. Walker, C. Bordin, J. Hulme, C. Motzo, F. Cucca, J. F. Hess, M. L. Metzker, J. Rogers, S. Gregory, A. Allahabadia, R. Nithiyananthan, E. Tuomilehto-Wolf, J. Tuomilehto, P. Bingley, K. M. Gillespie, D. E. Undlien, K. S. Rønningen, C. Guja, C. Ionescu-Tîrgovişte, D. A. Savage, A. P. Maxwell, D. J. Carson, C. C. Patterson, J. A. Franklyn, D. G. Clayton, L. B. Peterson, L. S. Wicker, J. A. Todd, and S. C. L. Gough. 2003. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423:506-511.
160. Ying, H., L. Yang, G. Qiao, Z. Li, L. Zhang, F. Yin, D. Xie, and J. Zhang. 2010. Cutting edge: CTLA-4--B7 interaction suppresses Th17 cell differentiation. *J Immunol* 185:1375-1378.

161. Quezada, S. A., K. S. Peggs, T. R. Simpson, and J. P. Allison. 2011. Shifting the equilibrium in cancer immunoediting: from tumor tolerance to eradication. *Immunol Rev* 241:104-118.
162. Fife, B. T., I. Guleria, M. Gubbels Bupp, T. N. Eagar, Q. Tang, H. Bour-Jordan, H. Yagita, M. Azuma, M. H. Sayegh, and J. A. Bluestone. 2006. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J Exp Med* 203:2737-2747.
163. Riley, J. L. 2009. PD-1 signaling in primary T cells. *Immunol Rev* 229:114-125.
164. Zhang, L., T. F. Gajewski, and J. Kline. 2009. PD-1/PD-L1 interactions inhibit antitumor immune responses in a murine acute myeloid leukemia model. *Blood* 114:1545-1552.
165. Mumprecht, S., C. Schürch, J. Schwaller, M. Solenthaler, and A. F. Ochsenbein. 2009. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. *Blood* 114:1528-1536.
166. McGrath, M. M., and N. Najafian. 2012. The role of coinhibitory signaling pathways in transplantation and tolerance. *Front Immunol* 3:47.
167. Prasad, D. V. R., T. Nguyen, Z. Li, Y. Yang, J. Duong, Y. Wang, and C. Dong. 2004. Murine B7-H3 is a negative regulator of T cells. *J Immunol* 173:2500-2506.
168. Suh, W.-K., B. U. Gajewska, H. Okada, M. A. Gronski, E. M. Bertram, W. Dawicki, G. S. Duncan, J. Bukczynski, S. Plyte, A. Elia, A. Wakeham, A. Itie, S. Chung, J. Da Costa, S. Arya, T. Horan, P. Campbell, K. Gaida, P. S. Ohashi, T. H. Watts, S. K. Yoshinaga, M. R. Bray, M. Jordana, and T. W. Mak. 2003. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat Immunol* 4:899-906.
169. Wang, X., J. Hao, D. L. Metzger, A. Mui, Z. Ao, N. Akhoundsadegh, S. Langermann, L. Liu, L. Chen, D. Ou, C. B. Verchere, and G. L. Warnock. 2011. Early treatment of NOD mice with B7-H4 reduces the incidence of autoimmune diabetes. *Diabetes* 60:3246-3255.
170. Brusko, T. M., A. L. Putnam, and J. A. Bluestone. 2008. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev* 223:371-390.
171. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.
172. Tang, Q., K. J. Henriksen, M. Bi, E. B. Finger, G. Szot, J. Ye, E. L. Masteller, H. McDevitt, M. Bonyhadi, and J. A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199:1455-1465.
173. Tang, Q., K. J. Henriksen, E. K. Boden, A. J. Tooley, J. Ye, S. K. Subudhi, X. X. Zheng, T. B. Strom, and J. A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171:3348-3352.
174. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198:235-247.
175. Hippen, K. L., P. Harker-Murray, S. B. Porter, S. C. Merkel, A. Londer, D. K. Taylor, M. Bina, A. Panoskaltsis-Mortari, P. Rubinstein, N. van Rooijen, T. N. Golovina, M. M. Suhoski, J. S. Miller, J. E. Wagner, C. H. June, J. L. Riley, and B. R. Blazar. 2008. Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4-1BB expressed on artificial antigen-presenting cells. *Blood* 112:2847-2857.
176. Kroemer, A., X. Xiao, M. D. Vu, W. Gao, K. Minamimura, M. Chen, T. Maki, and X. C. Li. 2007. OX40 controls functionally different T cell subsets and their resistance to depletion therapy. *J Immunol* 179:5584-5591.
177. Lee, J., E. N. Lee, E. Y. Kim, H. J. Lee, H. J. Park, C. L. Sun, S. K. Lee, J. W. Joh, K. W. Lee, G. Y. Kwon, and S. J. Kim. 2005. 4-1BB promotes long-term survival in skin allografts treated

with anti-CD45RB and anti-CD40L monoclonal antibodies. *Transplant Proc* 37:123-125.
178. Niedbala, W., B. Cai, H. Liu, N. Pitman, L. Chang, and F. Y. Liew. 2007. Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25 T cells via p53, IL-2, and OX40. *Proc Natl Acad Sci USA* 104:15478-15483.
179. Chen, M., X. Xiao, G. Demirci, and X. C. Li. 2008. OX40 controls islet allograft tolerance in CD154 deficient mice by regulating FOXP3+ Tregs. *Transplantation* 85:1659-1662.
180. Choi, B. K., J. S. Bae, E. M. Choi, W. J. Kang, S. Sakaguchi, D. S. Vinay, and B. S. Kwon. 2004. 4-1BB-dependent inhibition of immunosuppression by activated CD4+CD25+ T cells. *J Leukoc Biol* 75:785-791.
181. Vu, M. D., X. Xiao, W. Gao, N. Degauque, M. Chen, A. Kroemer, N. Killeen, N. Ishii, and X. C. Li. 2007. OX40 costimulation turns off Foxp3+ Tregs. *Blood* 110:2501-2510.
182. Bour-Jordan, H., B. L. Salomon, H. L. Thompson, G. L. Szot, M. R. Bernhard, and J. A. Bluestone. 2004. Costimulation controls diabetes by altering the balance of pathogenic and regulatory T cells. *J Clin Invest* 114:979-987.
183. Kumanogoh, A., X. Wang, I. Lee, C. Watanabe, M. Kamanaka, W. Shi, K. Yoshida, T. Sato, S. Habu, M. Itoh, N. Sakaguchi, S. Sakaguchi, and H. Kikutani. 2001. Increased T cell autoreactivity in the absence of CD40-CD40 ligand interactions: a role of CD40 in regulatory T cell development. *J Immunol* 166:353-360.
184. Serra, P., A. Amrani, J. Yamanouchi, B. Han, S. Thiessen, T. Utsugi, J. Verdaguer, and P. Santamaria. 2003. CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells. *Immunity* 19:877-889.
185. Shevach, E. M., and G. L. Stephens. 2006. The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity? *Nat Rev Immunol* 6:613-618.
186. Mahesh, S. P., Z. Li, B. Liu, R. N. Fariss, and R. B. Nussenblatt. 2006. Expression of GITR ligand abrogates immunosuppressive function of ocular tissue and differentially modulates inflammatory cytokines and chemokines. *Eur. J. Immunol.* 36:2128-2138.
187. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263-266.
188. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.
189. McAdam, A. J., T. T. Chang, A. E. Lumelsky, E. A. Greenfield, V. A. Boussiotis, J. S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V. K. Kuchroo, V. Ling, M. Collins, A. H. Sharpe, and G. J. Freeman. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J Immunol* 165:5035-5040.
190. Shilling, R. A., B. S. Clay, A. G. Tesciuba, E. L. Berry, T. Lu, T. V. Moore, H. S. Bandukwala, J. Tong, J. V. Weinstock, R. A. Flavell, T. Horan, S. K. Yoshinaga, A. A. Welcher, J. L. Cannon, and A. I. Sperling. 2009. CD28 and ICOS play complementary non-overlapping roles in the development of Th2 immunity in vivo. *Cell Immunol* 259:177-184.
191. Aicher, A., M. Hayden-Ledbetter, W. A. Brady, A. Pezzutto, G. Richter, D. Magaletti, S. Buckwalter, J. A. Ledbetter, and E. A. Clark. 2000. Characterization of human inducible costimulator ligand expression and function. *J Immunol* 164:4689-4696.
192. Liang, L., E. M. Porter, and W. C. Sha. 2002. Constitutive expression of the B7h ligand for inducible costimulator on naive B cells is extinguished after activation by distinct B cell receptor and interleukin 4 receptor-mediated pathways and can be rescued by CD40 signaling. *J Exp Med* 196:97-108.

193. Wang, S., G. Zhu, A. I. Chapoval, H. Dong, K. Tamada, J. Ni, and L. Chen. 2000. Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* 96:2808-2813.
194. Zhou, Z., K. Hoebe, X. Du, Z. Jiang, L. Shamel, and B. Beutler. 2005. Antagonism between MyD88- and TRIF-dependent signals in B7RP-1 up-regulation. *Eur. J. Immunol.* 35:1918-1927.
195. Wahl, P., R. Schoop, G. Bilic, J. Neuweiler, M. Le Hir, S. K. Yoshinaga, and R. P. Wüthrich. 2002. Renal tubular epithelial expression of the costimulatory molecule B7RP-1 (inducible costimulator ligand). *J Am Soc Nephrol* 13:1517-1526.
196. Swallow, M. M., J. J. Wallin, and W. C. Sha. 1999. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. *Immunity* 11:423-432.
197. Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97-101.
198. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature* 409:102-105.
199. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, B. Odermatt, A. Ho, A. Itie, T. Horan, J. S. Whoriskey, T. Pawson, J. M. Penninger, P. S. Ohashi, and T. W. Mak. 2001. ICOS is essential for effective T-helper-cell responses. *Nature* 409:105-109.
200. de Jong, Y. P., S. T. Rietdijk, W. A. Faubion, A. C. Abadia-Molina, K. Clarke, E. Mizoguchi, J. Tian, T. Delaney, S. Manning, J.-C. Gutierrez-Ramos, A. K. Bhan, A. J. Coyle, and C. Terhorst. 2004. Blocking inducible co-stimulator in the absence of CD28 impairs Th1 and CD25+ regulatory T cells in murine colitis. *Int Immunol* 16:205-213.
201. Kopf, M., A. J. Coyle, N. Schmitz, M. Barner, A. Oxenius, A. Gallimore, J. C. Gutierrez-Ramos, and M. F. Bachmann. 2000. Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. *J Exp Med* 192:53-61.
202. Vidric, M., A. T. Bladt, U. Dianzani, and T. H. Watts. 2006. Role for inducible costimulator in control of Salmonella enterica serovar Typhimurium infection in mice. *Infect Immun* 74:1050-1061.
203. Wilson, E. H., C. Zaph, M. Mohrs, A. Welcher, J. Siu, D. Artis, and C. A. Hunter. 2006. B7RP-1-ICOS interactions are required for optimal infection-induced expansion of CD4+ Th1 and Th2 responses. *J Immunol* 177:2365-2372.
204. Bauquet, A., H. Jin, A. Paterson, M. Mitsdoerffer, I. Ho, A. Sharpe, and V. Kuchroo. 2008. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and T(H)-17 cells. *Nat Immunol*.
205. Akbari, O., G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff, and D. T. Umetsu. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 8:1024-1032.
206. Kohyama, M., D. Sugahara, S. Sugiyama, H. Yagita, K. Okumura, and N. Hozumi. 2004. Inducible costimulator-dependent IL-10 production by regulatory T cells specific for self-antigen. *Proc Natl Acad Sci USA* 101:4192-4197.
207. Burmeister, Y., T. Lischke, A. C. Dahler, H. W. Mages, K.-P. Lam, A. J. Coyle, R. A. Kroczek, and A. Hutloff. 2008. ICOS controls the pool size of effector-memory and regulatory T cells. *J Immunol* 180:774-782.
208. Kornete, M., E. Sgouroudis, and C. A. Piccirillo. 2012. ICOS-Dependent Homeostasis and Function of Foxp3+ Regulatory T Cells in Islets of Nonobese Diabetic Mice. *J Immunol* 188:1064-1074.

209. Whitehead, G. S., R. H. Wilson, K. Nakano, L. H. Burch, H. Nakano, and D. N. Cook. 2012. IL-35 production by inducible costimulator (ICOS)-positive regulatory T cells reverses established IL-17-dependent allergic airways disease. *J Allergy Clin Immunol* 129:207-215.e205.
210. Quiroga, M. F., V. Pasquinelli, G. J. Martínez, J. O. Jurado, L. C. Zorrilla, R. M. Musella, E. Abbate, P. A. Sieling, and V. E. García. 2006. Inducible costimulator: a modulator of IFN-gamma production in human tuberculosis. *J Immunol* 176:5965-5974.
211. Bonhagen, K., O. Liesenfeld, M. J. Stadecker, A. Hutloff, K. Erb, A. J. Coyle, M. Lipp, R. A. Kroczek, and T. Kamradt. 2003. ICOS+ Th cells produce distinct cytokines in different mucosal immune responses. *Eur. J. Immunol.* 33:392-401.
212. Nurieva, R. I., J. Duong, H. Kishikawa, U. Dianzani, J. M. Rojo, I. c. Ho, R. A. Flavell, and C. Dong. 2003. Transcriptional regulation of th2 differentiation by inducible costimulator. *Immunity* 18:801-811.
213. Nurieva, R. I., S. Chuvpilo, E. D. Wieder, K. B. Elkon, R. Locksley, E. Serfling, and C. Dong. 2007. A costimulation-initiated signaling pathway regulates NFATc1 transcription in T lymphocytes. *J Immunol* 179:1096-1103.
214. Watanabe, M., S. Watanabe, Y. Hara, Y. Harada, M. Kubo, K. Tanabe, H. Toma, and R. Abe. 2005. ICOS-mediated costimulation on Th2 differentiation is achieved by the enhancement of IL-4 receptor-mediated signaling. *J Immunol* 174:1989-1996.
215. Clay, B. S., R. A. Shilling, H. S. Bandukwala, T. V. Moore, J. L. Cannon, A. A. Welcher, J. V. Weinstock, and A. I. Sperling. 2009. Inducible costimulator expression regulates the magnitude of Th2-mediated airway inflammation by regulating the number of Th2 cells. *PLoS ONE* 4:e7525.
216. Gonzalo, J. A., J. Tian, T. Delaney, J. Corcoran, J. B. Rottman, J. Lora, A. Al-garawi, R. Kroczek, J. C. Gutierrez-Ramos, and A. J. Coyle. 2001. ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. *Nat Immunol* 2:597-604.
217. Tesciuba, A. G., S. Subudhi, R. P. Rother, S. J. Faas, A. M. Frantz, D. Elliot, J. Weinstock, L. A. Matis, J. A. Bluestone, and A. I. Sperling. 2001. Inducible costimulator regulates Th2-mediated inflammation, but not Th2 differentiation, in a model of allergic airway disease. *J Immunol* 167:1996-2003.
218. Scales, H. E., M. X. Ierna, J.-C. Gutierrez-Ramos, A. J. Coyle, P. Garside, and C. E. Lawrence. 2004. Effect of inducible costimulator blockade on the pathological and protective immune responses induced by the gastrointestinal helminth Trichinella spiralis. *Eur. J. Immunol.* 34:2854-2862.
219. Neill, D. R., and A. N. McKenzie. 2011. Nuocytes and beyond: new insights into helminth expulsion. *Trends Parasitol* 27:214-221.
220. Barlow, J. L., A. Bellosi, C. S. Hardman, L. F. Drynan, S. H. Wong, J. P. Cruickshank, and A. N. J. Mckenzie. 2012. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol* 129:191-198.e191-194.
221. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345-350.
222. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.
223. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
224. Rottman, J. B., T. Smith, J. R. Tonra, K. Ganley, T. Bloom, R. Silva, B. Pierce, J. C. Gutierrez-Ramos, E. Ozkaynak, and A. J. Coyle. 2001. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat Immunol* 2:605-611.

225. Dong, C., U. A. Temann, and R. A. Flavell. 2001. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J Immunol* 166:3659-3662.
226. Grimbacher, B., A. Hutloff, M. Schlesier, E. Glocker, K. Warnatz, R. Dräger, H. Eibel, B. Fischer, A. A. Schäffer, H. W. Mages, R. A. Kroczek, and H. H. Peter. 2003. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* 4:261-268.
227. Bossaller, L., J. Burger, R. Draeger, B. Grimbacher, R. Knoth, A. Plebani, A. Durandy, U. Baumann, M. Schlesier, A. A. Welcher, H. H. Peter, and K. Warnatz. 2006. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* 177:4927-4932.
228. Vogelzang, A., H. M. McGuire, D. Yu, J. Sprent, C. R. Mackay, and C. King. 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29:127-137.
229. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y.-H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29:138-149.
230. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. *Immunity*.
231. Yu, D., A. H.-M. Tan, X. Hu, V. Athanasopoulos, N. Simpson, D. G. Silva, A. Hutloff, K. M. Giles, P. J. Leedman, K. P. Lam, C. C. Goodnow, and C. G. Vinuesa. 2007. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* 450:299-303.
232. Vinuesa, C. G., M. C. Cook, C. Angelucci, V. Athanasopoulos, L. Rui, K. M. Hill, D. Yu, H. Domaschenz, B. Whittle, T. Lambe, I. S. Roberts, R. R. Copley, J. I. Bell, R. J. Cornall, and C. C. Goodnow. 2005. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 435:452-458.
233. Zaretsky, A., J. Taylor, I. King, F. Marshall, M. Mohrs, and E. Pearce. 2009. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med*.
234. Ito, T., S. Hanabuchi, Y.-H. Wang, W. R. Park, K. Arima, L. Bover, F. X.-F. Qin, M. Gilliet, and Y.-J. Liu. 2008. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* 28:870-880.
235. Löhning, M., A. Hutloff, T. Kallinich, H. W. Mages, K. Bonhagen, A. Radbruch, E. Hamelmann, and R. A. Kroczek. 2003. Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. *J Exp Med* 197:181-193.
236. Ito, T., M. Yang, Y.-H. Wang, R. Lande, J. Gregorio, O. A. Perng, X.-F. Qin, Y.-J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* 204:105-115.
237. Herman, A. E., G. J. Freeman, D. Mathis, and C. Benoist. 2004. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med* 199:1479-1489.
238. Łuczyński, W., N. Wawrusiewicz-Kurylonek, A. Stasiak-Barmuta, R. Urban, E. Iłendo, M. Urban, M. Hryszko, A. Kretowski, and M. Górska. 2009. Diminished expression of ICOS, GITR and CTLA-4 at the mRNA level in T regulatory cells of children with newly diagnosed type 1 diabetes. *Acta Biochim Pol* 56:361-370.
239. Takahashi, N., K. Matsumoto, H. Saito, T. Nanki, N. Miyasaka, T. Kobata, M. Azuma, S.-K. Lee, S. Mizutani, and T. Morio. 2009. Impaired CD4 and CD8 effector function and decreased memory T cell populations in ICOS-deficient patients. *J Immunol* 182:5515-5527.

240. Marks, E., M. Verolin, A. Stensson, and N. Lycke. 2007. Differential CD28 and inducible costimulatory molecule signaling requirements for protective CD4+ T-cell-mediated immunity against genital tract Chlamydia trachomatis infection. *Infect Immun* 75:4638-4647.
241. Rutitzky, L. I., E. Ozkaynak, J. B. Rottman, and M. J. Stadecker. 2003. Disruption of the ICOS-B7RP-1 costimulatory pathway leads to enhanced hepatic immunopathology and increased gamma interferon production by CD4 T cells in murine schistosomiasis. *Infect Immun* 71:4040-4044.
242. Mahajan, S., A. Cervera, M. MacLeod, S. Fillatreau, G. Perona-Wright, S. Meek, A. Smith, A. MacDonald, and D. Gray. 2007. The role of ICOS in the development of CD4 T cell help and the reactivation of memory T cells. *Eur. J. Immunol.* 37:1796-1808.
243. Kamanaka, M., S. T. Kim, Y. Y. Wan, F. S. Sutterwala, M. Lara-Tejero, J. E. Galán, E. Harhaj, and R. A. Flavell. 2006. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity* 25:941-952.
244. Zaccone, P., O. Burton, S. E. Gibbs, N. Miller, F. Jones, G. Schramm, H. Haas, M. J. Doenhoff, D. Dunne, and A. Cooke. 2011. The S. mansoni glycoprotein ω-1 induces Foxp3 expression in NOD mouse $CD4^+$ T cells. *Eur. J. Immunol.* 41:2709-2718.
245. Griseri, T., M. Asquith, C. Thompson, and F. Powrie. 2010. OX40 is required for regulatory T cell-mediated control of colitis. *J Exp Med* 207:699-709.
246. Gottschalk, R. A., E. Corse, and J. P. Allison. 2011. Expression of Helios in Peripherally Induced Foxp3+ Regulatory T Cells. *J Immunol*.
247. Xiang, Y., J. Peng, N. Tai, C. Hu, Z. Zhou, F. S. Wong, and L. Wen. 2012. The Dual Effects of B Cell Depletion on Antigen-Specific T Cells in BDC2.5NOD Mice. *J Immunol* 188:4747-4758.
248. Santoro, M. G. 2000. Heat shock factors and the control of the stress response. *Biochem Pharmacol* 59:55-63.
249. Nussbaum, G., A. Zanin-Zhorov, F. Quintana, O. Lider, and I. R. Cohen. 2006. Peptide p277 of HSP60 signals T cells: inhibition of inflammatory chemotaxis. *Int Immunol* 18:1413-1419.
250. Zanin-Zhorov, A., R. Bruck, G. Tal, S. Oren, H. Aeed, R. Hershkoviz, I. R. Cohen, and O. Lider. 2005. Heat shock protein 60 inhibits Th1-mediated hepatitis model via innate regulation of Th1/Th2 transcription factors and cytokines. *J Immunol* 174:3227-3236.
251. Zanin-Zhorov, A., G. Nussbaum, S. Franitza, I. R. Cohen, and O. Lider. 2003. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB J* 17:1567-1569.
252. de Kleer, I., Y. Vercoulen, M. Klein, J. Meerding, S. Albani, R. van der Zee, B. Sawitzki, A. Hamann, W. Kuis, and B. Prakken. 2010. CD30 discriminates heat shock protein 60-induced FOXP3+ CD4+ T cells with a regulatory phenotype. *J Immunol* 185:2071-2079.
253. Zanin-Zhorov, A., L. Cahalon, G. Tal, R. Margalit, O. Lider, and I. R. Cohen. 2006. Heat shock protein 60 enhances CD4+ CD25+ regulatory T cell function via innate TLR2 signaling. *J Clin Invest* 116:2022-2032.
254. Scott-Browne, J. P., S. Shafiani, G. Tucker-Heard, K. Ishida-Tsubota, J. D. Fontenot, A. Y. Rudensky, M. J. Bevan, and K. B. Urdahl. 2007. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med* 204:2159-2169.
255. Vocanson, M., A. Rozieres, A. Hennino, G. Poyet, V. Gaillard, S. Renaudineau, A. Achachi, J. Benetiere, D. Kaiserlian, B. Dubois, and J. F. Nicolas. 2010. Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of TH17/TH1 and regulatory T cells. *J Allergy Clin Immunol* 126:280-289, 289.e281-287.
256. Guo, F., C. Iclozan, W. K. Suh, C. Anasetti, and X. Z. Yu. 2008. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. *J Immunol* 181:2285-2291.

257. Spadoni, I., I. D. Iliev, G. Rossi, and M. Rescigno. 2012. Dendritic cells produce TSLP that limits the differentiation of Th17 cells, fosters Treg development, and protects against colitis. *Mucosal Immunol* 5:184-193.
258. Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G. M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* 6:507-514.
259. Taylor, B. C., C. Zaph, A. E. Troy, Y. Du, K. J. Guild, M. R. Comeau, and D. Artis. 2009. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* 206:655-667.
260. Zaph, C., A. E. Troy, B. C. Taylor, L. D. Berman-Booty, K. J. Guild, Y. Du, E. A. Yost, A. D. Gruber, M. J. May, F. R. Greten, L. Eckmann, M. Karin, and D. Artis. 2007. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* 446:552-556.
261. Sun, C.-M., J. A. Hall, R. B. Blank, N. Bouladoux, M. Oukka, J. R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204:1775-1785.
262. Akimova, T., U. H. Beier, L. Wang, M. H. Levine, and W. W. Hancock. 2011. Helios expression is a marker of T cell activation and proliferation. *PLoS ONE* 6:e24226.
263. Serre, K., C. Bénézech, G. Desanti, S. Bobat, K.-M. Toellner, R. Bird, S. Chan, P. Kastner, A. F. Cunningham, I. C. M. Maclennan, and E. Mohr. 2011. Helios is associated with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of Foxp3 expression. *PLoS ONE* 6:e20731.
264. Izcue, A., J. L. Coombes, and F. Powrie. 2009. Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol* 27:313-338.
265. Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, T. Taniguchi, K. Takeda, S. Hori, I. I. Ivanov, Y. Umesaki, K. Itoh, and K. Honda. 2011. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science* 331:337-341.
266. Round, J. L., and S. K. Mazmanian. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences* 107:12204-12209.
267. John, B., T. H. Harris, E. D. Tait, E. H. Wilson, B. Gregg, L. G. Ng, P. Mrass, D. S. Roos, F. Dzierszinski, W. Weninger, and C. A. Hunter. 2009. Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii. *PLoS Pathog* 5:e1000505.
268. Stoll, S., J. Delon, T. M. Brotz, and R. N. Germain. 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 296:1873-1876.
269. Simonetta, F., A. Chiali, C. Cordier, A. Urrutia, I. Girault, S. Bloquet, C. Tanchot, and C. Bourgeois. 2010. Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells. *Eur. J. Immunol.* 40:2528-2538.
270. Totsuka, T., T. Kanai, R. Iiyama, K. Uraushihara, M. Yamazaki, R. Okamoto, T. Hibi, K. Tezuka, M. Azuma, H. Akiba, H. Yagita, K. Okumura, and M. Watanabe. 2003. Ameliorating effect of anti-inducible costimulator monoclonal antibody in a murine model of chronic colitis. *Gastroenterology* 124:410-421.
271. Akbari, O., P. Stock, E. H. Meyer, G. J. Freeman, A. H. Sharpe, D. T. Umetsu, and R. H. DeKruyff. 2008. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol* 180:5448-5456.
272. Huber, S., N. Gagliani, E. Esplugues, W. O'Connor, F. J. Huber, A. Chaudhry, M. Kamanaka, Y. Kobayashi, C. J. Booth, A. Y. Rudensky, M. G. Roncarolo, M. Battaglia, and R. A. Flavell. 2011. Th17 Cells Express Interleukin-10 Receptor and Are Controlled by

Foxp3(-) and Foxp3(+) Regulatory CD4(+) T Cells in an Interleukin-10-Dependent Manner. *Immunity* 34:554-565.
273. Golovina, T. N., T. Mikheeva, M. M. Suhoski, N. A. Aqui, V. C. Tai, X. Shan, R. Liu, R. R. Balcarcel, N. Fisher, B. L. Levine, R. G. Carroll, N. Warner, B. R. Blazar, C. H. June, and J. L. Riley. 2008. CD28 costimulation is essential for human T regulatory expansion and function. *J Immunol* 181:2855-2868.
274. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.
275. Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17:167-178.
276. Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201:723-735.
277. Schorle, H., T. Holtschke, T. Hünig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 352:621-624.
278. Mor, F., and I. R. Cohen. 1996. IL-2 rescues antigen-specific T cells from radiation or dexamethasone-induced apoptosis. Correlation with induction of Bcl-2. *J Immunol* 156:515-522.
279. Mueller, D. L., S. Seiffert, W. Fang, and T. W. Behrens. 1996. Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells. A model for the long-term survival of memory cells. *J Immunol* 156:1764-1771.
280. Fos, C., A. Salles, V. Lang, F. Carrette, S. Audebert, S. Pastor, M. Ghiotto, D. Olive, G. Bismuth, and J. A. Nunès. 2008. ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. *J Immunol* 181:1969-1977.
281. Cheng, G., A. Yu, and T. R. Malek. 2011. T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells. *Immunol Rev* 241:63-76.
282. Tan, J. T., E. Dudl, E. LeRoy, R. Murray, J. Sprent, K. I. Weinberg, and C. D. Surh. 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci USA* 98:8732-8737.
283. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 180:1955-1960.
284. Seddon, B., P. Tomlinson, and R. Zamoyska. 2003. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol* 4:680-686.
285. von Freeden-Jeffry, U., P. Vieira, L. A. Lucian, T. McNeil, S. E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* 181:1519-1526.
286. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrançois. 2000. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nat Immunol* 1:426-432.
287. Xue, H.-H., P. E. Kovanen, C. A. Pise-Masison, M. Berg, M. F. Radovich, J. N. Brady, and W. J. Leonard. 2002. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. *Proc Natl Acad Sci USA* 99:13759-13764.
288. Leonard, W. J. 2002. TSLP: finally in the limelight. *Nat Immunol* 3:605-607.
289. Park, L. S., U. Martin, K. Garka, B. Gliniak, J. P. Di Santo, W. Muller, D. A. Largaespada, N. G. Copeland, N. A. Jenkins, A. G. Farr, S. F. Ziegler, P. J. Morrissey, R. Paxton, and J. E. Sims. 2000. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor:

Formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med* 192:659-670.
290. Peffault de Latour, R., H. C. Dujardin, F. Mishellany, O. Burlen-Defranoux, J. Zuber, R. Marques, J. Di Santo, A. Cumano, P. Vieira, and A. Bandeira. 2006. Ontogeny, function, and peripheral homeostasis of regulatory T cells in the absence of interleukin-7. *Blood* 108:2300-2306.
291. Perona-Wright, G., K. Mohrs, and M. Mohrs. 2010. Sustained signaling by canonical helper T cell cytokines throughout the reactive lymph node. *Nat Immunol*:1-8.
292. Shoemaker, J., M. Saraiva, and A. O'garra. 2006. GATA-3 directly remodels the IL-10 locus independently of IL-4 in CD4+ T cells. *J Immunol* 176:3470-3479.
293. Wohlfert, E. A., J. R. Grainger, N. Bouladoux, J. E. Konkel, G. Oldenhove, C. H. Ribeiro, J. A. Hall, R. Yagi, S. Naik, R. Bhairavabhotla, W. E. Paul, R. Bosselut, G. Wei, K. Zhao, M. Oukka, J. Zhu, and Y. Belkaid. 2011. GATA3 controls Foxp3$^+$ regulatory T cell fate during inflammation in mice. *J Clin Invest* 121:4503-4515.
294. Scheu, S., D. B. Stetson, R. L. Reinhardt, J. H. Leber, M. Mohrs, and R. M. Locksley. 2006. Activation of the integrated stress response during T helper cell differentiation. *Nat Immunol* 7:644-651.
295. Fernandez, J., I. Yaman, P. Sarnow, M. D. Snider, and M. Hatzoglou. 2002. Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2alpha. *J Biol Chem* 277:19198-19205.
296. Sonenberg, N., and A. G. Hinnebusch. 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 136:731-745.
297. Qin, X., and P. Sarnow. 2004. Preferential translation of internal ribosome entry site-containing mRNAs during the mitotic cycle in mammalian cells. *J Biol Chem* 279:13721-13728.
298. Feito, M. J., R. Vaschetto, G. Criado, A. Sánchez, A. Chiocchetti, A. Jiménez-Periáñez, U. Dianzani, P. Portoles, and J. M. Rojo. 2003. Mechanisms of H4/ICOS costimulation: effects on proximal TCR signals and MAP kinase pathways. *Eur. J. Immunol.* 33:204-214.
299. Stoecklin, G., S. A. Tenenbaum, T. Mayo, S. V. Chittur, A. D. George, T. E. Baroni, P. J. Blackshear, and P. Anderson. 2008. Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. *J Biol Chem* 283:11689-11699.
300. Tudor, C., F. P. Marchese, E. Hitti, A. Aubareda, L. Rawlinson, M. Gaestel, P. J. Blackshear, A. R. Clark, J. Saklatvala, and J. L. E. Dean. 2009. The p38 MAPK pathway inhibits tristetraprolin-directed decay of interleukin-10 and pro-inflammatory mediator mRNAs in murine macrophages. *FEBS Lett* 583:1933-1938.
301. Reinhardt, R. L., H.-E. Liang, and R. M. Locksley. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* 10:385-393.
302. Loke, P. n., X. Zang, L. Hsuan, R. Waitz, R. M. Locksley, J. E. Allen, and J. P. Allison. 2005. Inducible costimulator is required for type 2 antibody isotype switching but not T helper cell type 2 responses in chronic nematode infection. *Proc Natl Acad Sci USA* 102:9872-9877.
303. King, I. L., and M. Mohrs. 2009. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med*.
304. King, C., S. G. Tangye, and C. R. Mackay. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 26:741-766.
305. Kerfoot, S. M., G. Yaari, J. R. Patel, K. L. Johnson, D. G. Gonzalez, S. H. Kleinstein, and A. M. Haberman. 2011. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* 34:947-960.

306. Kitano, M., S. Moriyama, Y. Ando, M. Hikida, Y. Mori, T. Kurosaki, and T. Okada. 2011. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* 34:961-972.
307. Lee, S. K., R. J. Rigby, D. Zotos, L. M. Tsai, S. Kawamoto, J. L. Marshall, R. R. Ramiscal, T. D. Chan, D. Gatto, R. Brink, D. Yu, S. Fagarasan, D. M. Tarlinton, A. F. Cunningham, and C. G. Vinuesa. 2011. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med* 208:1377-1388.
308. Yusuf, I., R. Kageyama, L. Monticelli, R. J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* 185:190-202.
309. Bauquet, A. T., H. Jin, A. M. Paterson, M. Mitsdoerffer, I. C. Ho, A. H. Sharpe, and V. K. Kuchroo. 2008. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and T(H)-17 cells. *Nat Immunol*.
310. Linterman, M. A., L. Beaton, D. Yu, R. R. Ramiscal, M. Srivastava, J. J. Hogan, N. K. Verma, M. J. Smyth, R. J. Rigby, and C. G. Vinuesa. 2010. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* 207:353-363.
311. Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480-483.
312. Vijayanand, P., G. Seumois, L. J. Simpson, S. Abdul-Wajid, D. Baumjohann, M. Panduro, X. Huang, J. Interlandi, I. M. Djuretic, D. R. Brown, A. H. Sharpe, A. Rao, and K. M. Ansel. 2012. Interleukin-4 production by follicular helper T cells requires the conserved Il4 enhancer hypersensitivity site V. *Immunity* 36:175-187.
313. Bashir, M. E., P. Andersen, I. J. Fuss, H. N. Shi, and C. Nagler-Anderson. 2002. An enteric helminth infection protects against an allergic response to dietary antigen. *J Immunol* 169:3284-3292.
314. Helmby, H., and R. K. Grencis. 2003. Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur. J. Immunol.* 33:2382-2390.
315. Urban, J. F., N. Noben-Trauth, D. D. Donaldson, K. B. Madden, S. C. Morris, M. Collins, and F. D. Finkelman. 1998. IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. *Immunity* 8:255-264.
316. Angeli, V., C. Faveeuw, P. Delerive, J. Fontaine, Y. Barriera, N. Franchimont, B. Staels, M. Capron, and F. Trottein. 2001. Schistosoma mansoni induces the synthesis of IL-6 in pulmonary microvascular endothelial cells: role of IL-6 in the control of lung eosinophilia during infection. *Eur. J. Immunol.* 31:2751-2761.
317. Coulson, P. S. 1997. The radiation-attenuated vaccine against schistosomes in animal models: paradigm for a human vaccine? *Adv Parasitol* 39:271-336.
318. Mountford, A. P., P. S. Coulson, and R. A. Wilson. 1988. Antigen localization and the induction of resistance in mice vaccinated with irradiated cercariae of Schistosoma mansoni. *Parasitology* 97 ( Pt 1):11-25.
319. Coulson, P. S., and R. A. Wilson. 1993. Pulmonary T helper lymphocytes are CD44hi, CD45RB- effector/memory cells in mice vaccinated with attenuated cercariae of Schistosoma mansoni. *J Immunol* 151:3663-3671.
320. Smythies, L. E., R. M. Pemberton, P. S. Coulson, A. P. Mountford, and R. A. Wilson. 1992. T cell-derived cytokines associated with pulmonary immune mechanisms in mice vaccinated with irradiated cercariae of Schistosoma mansoni. *J Immunol* 148:1512-1518.
321. Crabtree, J. E., and R. A. Wilson. 1986. The role of pulmonary cellular reactions in the resistance of vaccinated mice to Schistosoma mansoni. *Parasite Immunol* 8:265-285.

322. Hoffmann, K. F., S. L. James, A. W. Cheever, and T. A. Wynn. 1999. Studies with double cytokine-deficient mice reveal that highly polarized Th1- and Th2-type cytokine and antibody responses contribute equally to vaccine-induced immunity to Schistosoma mansoni. *J Immunol* 163:927-938.
323. Wilson, M. S., M. M. Mentink-Kane, J. T. Pesce, T. R. Ramalingam, R. Thompson, and T. A. Wynn. 2007. Immunopathology of schistosomiasis. *Immunol Cell Biol* 85:148-154.
324. Smythies, L. E., P. S. Coulson, and R. A. Wilson. 1993. Immunity to Schistosoma mansoni in mice vaccinated with irradiated cercariae: cytokine interactions in the pulmonary protective response. *Ann Trop Med Parasitol* 87:653-657.
325. Maizels, R. M., N. Gomez-Escobar, W. F. Gregory, J. Murray, and X. Zang. 2001. Immune evasion genes from filarial nematodes. *Int J Parasitol* 31:889-898.
326. McLaren, D. J., and R. J. Terry. 1982. The protective role of acquired host antigens during schistosome maturation. *Parasite Immunol* 4:129-148.
327. Sher, A., B. F. Hall, and M. A. Vadas. 1978. Acquisition of murine major histocompatibility complex gene products by schistosomula of Schistosoma mansoni. *J Exp Med* 148:46-57.
328. Sher, A., and D. Benno. 1982. Decreasing immunogenicity of developing schistosome larvae. *Parasite Immunol* 4:101-107.
329. Wynn, T. A., A. Reynolds, S. James, A. W. Cheever, P. Caspar, S. Hieny, D. Jankovic, M. Strand, and A. Sher. 1996. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. *J Immunol* 157:4068-4078.
330. Falcone, F. H., C. A. Dahinden, B. F. Gibbs, T. Noll, U. Amon, H. Hebestreit, O. Abrahamsen, J. Klaucke, M. Schlaak, and H. Haas. 1996. Human basophils release interleukin-4 after stimulation with Schistosoma mansoni egg antigen. *Eur. J. Immunol.* 26:1147-1155.
331. Williams, M. E., M. C. Kullberg, S. Barbieri, P. Caspar, J. A. Berzofsky, R. A. Seder, and A. Sher. 1993. Fc epsilon receptor-positive cells are a major source of antigen-induced interleukin-4 in spleens of mice infected with Schistosoma mansoni. *Eur. J. Immunol.* 23:1910-1916.
332. Arimura, Y., H. Kato, U. Dianzani, T. Okamoto, S. Kamekura, D. Buonfiglio, T. Miyoshi-Akiyama, T. Uchiyama, and J. Yagi. 2002. A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. *Int Immunol* 14:555-566.
333. Linterman, M. A., R. J. Rigby, R. Wong, D. Silva, D. Withers, G. Anderson, N. K. Verma, R. Brink, A. Hutloff, C. C. Goodnow, and C. G. Vinuesa. 2009. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. *Immunity* 30:228-241.
334. Maizels, R. M. 2005. Infections and allergy - helminths, hygiene and host immune regulation. *Curr Opin Immunol* 17:656-661.
335. Chaudhry, A., R. M. Samstein, P. Treuting, Y. Liang, M. C. Pils, J.-M. Heinrich, R. S. Jack, F. T. Wunderlich, J. C. Brüning, W. Müller, and A. Y. Rudensky. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of th17 cell-mediated inflammation. *Immunity* 34:566-578.
336. Gu, Y., J. Yang, X. Ouyang, W. Liu, H. Li, J. Yang, J. Bromberg, S.-H. Chen, L. Mayer, J. C. Unkeless, and H. Xiong. 2008. Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *Eur. J. Immunol.* 38:1807-1813.
337. Li, M. O., and R. A. Flavell. 2008. Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* 28:468-476.

338. McGeachy, M. J., K. S. Bak-Jensen, Y. Chen, C. M. Tato, W. Blumenschein, T. McClanahan, and D. J. Cua. 2007. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 8:1390-1397.
339. Ivanov, I. I., K. Atarashi, N. Manel, E. L. Brodie, T. Shima, U. Karaoz, D. Wei, K. C. Goldfarb, C. A. Santee, S. V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D. R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485-498.
340. Guo, F., C. Iclozan, W.-K. Suh, C. Anasetti, and X.-Z. Yu. 2008. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. *J Immunol* 181:2285-2291.
341. Frick, J. S., N. Zahir, M. Müller, F. Kahl, O. Bechtold, M. B. Lutz, C. J. Kirschning, J. Reimann, B. Jilge, E. Bohn, and I. B. Autenrieth. 2006. Colitogenic and non-colitogenic commensal bacteria differentially trigger DC maturation and Th cell polarization: an important role for IL-6. *Eur. J. Immunol.* 36:1537-1547.
342. Brunner, S. M., G. Schiechl, W. Falk, H. J. Schlitt, E. K. Geissler, and S. Fichtner-Feigl. 2011. Interleukin-33 prolongs allograft survival during chronic cardiac rejection. *Transpl Int* 24:1027-1039.
343. Pace, L., C. Pioli, and G. Doria. 2005. IL-4 modulation of CD4+CD25+ T regulatory cell-mediated suppression. *J Immunol* 174:7645-7653.
344. Zheng, S. G., J. D. Gray, K. Ohtsuka, S. Yamagiwa, and D. A. Horwitz. 2002. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. *J Immunol* 169:4183-4189.
345. Zheng, S. G., J. H. Wang, M. N. Koss, F. Quismorio, J. D. Gray, and D. A. Horwitz. 2004. CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol* 172:1531-1539.
346. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29:138-149.
347. Asseman, C., S. Read, and F. Powrie. 2003. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10. *J Immunol* 171:971-978.
348. Uhlig, H. H., J. Coombes, C. Mottet, A. Izcue, C. Thompson, A. Fanger, A. Tannapfel, J. D. Fontenot, F. Ramsdell, and F. Powrie. 2006. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol* 177:5852-5860.
349. Glocker, E.-O., D. Kotlarz, K. Boztug, E. M. Gertz, A. A. Schäffer, F. Noyan, M. Perro, J. Diestelhorst, A. Allroth, D. Murugan, N. Hätscher, D. Pfeifer, K.-W. Sykora, M. Sauer, H. Kreipe, M. Lacher, R. Nustede, C. Woellner, U. Baumann, U. Salzer, S. Koletzko, N. Shah, A. W. Segal, A. Sauerbrey, S. Buderus, S. B. Snapper, B. Grimbacher, and C. Klein. 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 361:2033-2045.
350. Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 6:152-162.
351. Brunstein, C. G., J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, P. B. McGlave, B. R. Blazar, and J. E. Wagner. 2011. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117:1061-1070.