Group 2 innate lymphoid cells and regulatory T cells: novel sources and targets of interleukin-4 in immunity to *Heligmosomoides polygyrus*

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Declaration

I, Victoria Sarah Pelly, declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

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Abstract

Intestinal helminth infections cause a significant global burden due to gaps in our translation of anti-helminth immunity. Immune responses to helminth infections are characterised by a balance between regulatory and effector cells, limiting immune-driven pathology in the host whilst enabling long-term survival of the parasite. Functional immunity to helminths is dependent on the activation of a type-2 immune cascade comprised of immune and non-immune cells that coordinate to promote the mechanistic expulsion of the parasite. The natural murine helminth *Heligmosomoides polygyrus* establishes chronic intestinal infections that can be expelled following secondary infection in drug-cured mice, and provides a good experimental model to test mechanisms of functional immunity. Immunity to *H. polygyrus* is dependent on the differentiation of IL-4 producing $CD4^+$ T helper 2 cells, however the exact mechanisms required for Th2 differentiation *in vivo* remain unclear.

In this study we have identified two novel mechanisms required for Th2 differentiation *in vivo.* Firstly, we identified that group 2 innate lymphoid cells (ILC2) are an important source of IL-4 for the differentiation of Th2 cells *in vitro* and *in vivo* during a chronic infection with *H. polygyrus.* Secondly, we have found that $Foxp3^+$ Treg cells convert to Th2 cells and contribute significantly to memory Th2 responses following a challenge infection with *Heligmosomoides polygyrus*. Using a fate-reporter mouse, we have deleted IL-4Rα on *Foxp3*-expressing cells to test whether IL-4 receptor signalling is required for the development of ex-Foxp3 Th2 cells and whether they contribute to functional immune responses. In summary, we have characterised ILC2s and Treg cells as novel sources and targets of IL-4 required for the differentiation of Th2 cells and propose a model whereby ILC2-derived IL-4 provides a source of IL-4 for the reprogramming of Treg cells in the tissue, ultimately supporting the expulsion of *H. polygyrus.*

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"For a scientist must indeed be freely imaginative and yet sceptical, creative and yet a critic. There is a sense in which he must be free, but another in which his thought must be very precisely regimented; there is poetry in science, but also a lot of bookkeeping". Sir Peter Medawar (Director of the NIMR, 1962-1971).

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List of abbreviations

Abbreviation	
1°/2°	Primary/secondary
AAMΦ	Alternatively activated macrophage
AID	Activation induced deaminase
AIRE	Autoimmune regulator
APC	Antigen-presenting cell
Arg1	Arginase 1
B cell	B lymphocyte
BCR	B cell receptor
CCR	Chemokine (C-C motif) receptor
C-type lectin	Calcium-binding lectin
CAMΦ	Classically activated macrophage
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CILP	Common innate lymphoid progenitor
CCR	C-C chemokine receptor
CD	Cluster of differentiation
Cre	Cre recombinase
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
Dnmt (*Dnmt*)	DNA (ctyosine-5)-methyltransferase 1
DP	Double positive
EAE	Experimental autoimmune encephalitis
et al	and others
FACS	Fluorescence activated cell sorting
Foxp3 (*Foxp3*)	Forkhead box P3
FP635	Fluorescent protein 635
GALT	Gut associated lymphoid tissue
GATA-3 (*Gata3*)	GATA binding protein 3
GFP	Green fluorescent protein
H & E	Hematoxylin & Eosin
HDM	House dust mite
H. polygyrus	*Heligmosomoides polygyrus*
HES	*H. polygyrus* excretory/secretory products

HSC	Haematopoietic stem cell
IEL	Intraepithelial lymphocytes
IFN (*Ifn*)	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2c	IL-2 complex
IL-4c	IL-4 complex
IL-4Rα (*Il4ra*)	IL-4 receptor alpha
ILC	Innate lymphoid cell
IRES	Internal ribosome entry site
IRS	Insulin receptor substrates
Jak	Janus Kinase
LP	Lamina propria
LPS	Lipopolysaccharide
LT	Leukotriene
MHC	Major histo-compatibility
miRNA	Micro-RNA
medLN	Mediastinal lymph node
MLN	Mesenteric lymph node
mRNA	Messenger RNA
mTEC	Medullary thymic epithelial cells
N. brasiliensis	*Nippostrongylus brasiliensis*
NFAT (*Nfat*)	Nuclear factor of activated T cells
OVA	Ovalbumin
P+I	PMA and ionomycin
PAMP	Pathogen associated molecular pattern
PAS	Period acid-Schiff
PG	Prostaglandin
PP	Peyer's patches
PRR	Pattern recognition receptor
Relmα (*Retnla*)	Resistin-like alpha
Relmβ (*Retnlb*)	Resistin-like beta
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RORγT (*Rorct*)	RAR-related orphan receptor C
RORα (*Rora*)	RAR-related orphan receptor A
S. mansoni	*Schistosoma mansoni*

STAT (*Stat*)	Signal transducer and activator of transcription
T cell	T lymphocyte
T-bet (*Tbx21*)	T-box transcription factor
T. muris	*Trichuris muris*
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
TGF-β	Transforming Growth Factor beta 1
Th	T helper
TLR	Toll-like receptor
Treg	T regulatory cell
TSLP (*Tslp*)	Thymic stromal lymphopoietin
VAT	Visceral adipose tissue
YFP	Yellow fluorescent protein
γc	Common gamma chain

Chapter 1: Introduction

1.1. The immune system

1.1.1. Key players in the immune system

The immune system is a highly evolved and tightly regulated system formed of diverse and specialised tissues and cells providing essential defence against invading pathogens. In steady-state conditions, cells of the immune system patrol the circulation and tissues integrating endogenous signals whilst maintaining the capacity to sense the presence of foreign antigen. The immune system can be broadly separated into the temporally distinct but inter-dependent innate and adaptive immune systems. Epithelial surfaces of the skin, lung and intestines are permanently exposed to environmental triggers and form the first line of defence against allergens and invading pathogens. Upon barrier breach by microorganisms, signals derived from damaged epithelial cells in combination with foreign antigen will activate tissue-resident innate immune cells. Following this, innate immune cells present foreign antigen to adaptive immune cells that are responsible for directing a specialised cascade of immune events. Central to this dichotomy is the ability of host immune cells to discern specific molecules present on the surface of pathogens without forming aberrant responses to self-antigens. The combination of innate and adaptive immune responses ultimately leads to the elimination of the foreign antigen whilst limiting host damage.

Immune cells originate either from the foetal yolk sac during embryonic development, or haematopoietic stem cell (HSC) progenitors in the bone marrow. Immune cells exit the bone marrow into the circulation from where they can migrate to vascularised tissues. Alternatively, they form part of the lymph and migrate through lymphatic vessels into lymphoid organs where they continue their maturation. An early event in the differentiation of HSCs is the development of common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) populations of cells (**Fig. 1.1**). CMP cells differentiate in the bone marrow into monocytes, dendritic cells (DCs),

granulocytes and mast cells. An important feature of CMP cells is their expression of specialised pattern recognition receptors (PRRs) that recognise highly specific pathogen associated molecular patterns (PAMPs). The largest family of PRRs are the Toll Like Receptors (TLRs) that are adapted for the recognition of a wide range of bacterial, viral and fungal antigens. For example TLR4 can recognise lipopolysaccharide (LPS) on the surface of gram-negative bacteria whereas intracellular TLR3 recognises double-stranded RNA, a replication intermediary for many viruses (Akira et al., 2006). In addition, innate cells express complement receptors and can recognise pathogen bound to complement enzymes. Granulocytes describe a functionally diverse group of cells (basophils, eosinophils and neutrophils) characterised by their cytoplasmic granules containing various enzymes, chemokines and growth factors. Upon infection or inflammation, granulocytes are recruited from the bone marrow through the circulation and into the tissue where they are activated, leading to their degranulation. For example, neutrophils are efficient phagocytic cells that kill intracellular viruses and bacteria through the release of inducible nitric oxide or anti-microbial peptides (reviewed in (Amulic et al., 2012)). In addition, activated neutrophils release neutrophil extracellular traps (NETs) recently shown to be required for limiting infections with larger pathogens such as the hyphal form of *Candida albicans* (Branzk et al., 2014). CMP cells can also differentiate into mast cell progenitors and mononuclear phagocytes. Mast cells progenitors are recruited out of the bone marrow through the circulation and into the tissue for their maturation. Mononuclear phagocytes are composed of monocytes and dendritic cells (DCs) (**Fig. 1.1**). Monocytes circulate in the bone marrow, blood stream and spleen taking up apoptotic cells and producing inflammatory cytokines (reviewed in (Geissmann et al., 2010)). From the circulation, monocytes get recruited to tissues and differentiate into macrophages. Macrophages respond to specific environmental cues to exert either inflammatory or regulatory functions. Classically activated macrophages (CAMΦ) recognise foreign antigens through toll like receptor activation to produce inflammatory cytokines as well as reactive oxygen species, driving a type-1 response and direct

killing of the bacteria. Conversely, alternatively activated macrophages (AAMΦ) differentiate and secrete molecules that promote wound healing and tissue-repair. In addition to monocyte-derived macrophages, a vast majority of tissue-resident macrophages originate from the foetal yolk sac during development. These cells are thought to be non-migratory and were recently shown to proliferate in the tissue (Jenkins et al., 2011). DCs are a specialised subset of mononuclear phagocytes otherwise defined as professional antigen-presenting cells. Immature pre-DCs exit the bone marrow into the circulation and migrate to the spleen or the intestine where they further differentiate into specialised subsets. In the tissue, invading pathogens are taken up by dendritic cells and processed into small peptides that are loaded onto major histo-compatibility (MHC) molecules and presented on the surface of DCs as peptide-MHC complexes. Pathogens that are endocytosed will be processed and presented on MHC Class II molecules present within the endosomal compartment of the cell whereas cytosolic antigens are cross-presented onto MHC Class I molecules. Presentation of peptide-MHC complexes is fundamental for T cell recognition of antigen as will be discussed later.

T- and B-lymphocytes develop from the CLP in the bone marrow and continue their maturation in primary lymphoid organs (**Fig. 1.1**). Immature T cell precursors migrate out of the bone marrow through the lymphatic system and into the thymus, a specialised and stratified primary lymphoid tissue that guides T cell maturation. B cells undergo the first stages of maturation in the bone marrow. Following this, B cell precursors migrate out of the bone marrow through the circulation and into the spleen. The maturation of T and B-lymphocytes requires the rearrangement of their antigen-specific T cell receptor (TCR) and B cell receptors (BCR). Following their maturation, T- and B-lymphocytes migrate through the lymphatic vessels into secondary lymphoid organs draining the tissue, where they will encounter antigen presented by dendritic cells. T lymphocytes are comprised of cluster of differentiation (CD) $CD4^+$ T helper and $CD8^+$ cytotoxic T cells that recognise antigen presented on DCs and produce characteristic cytokines. T helper cells are required for the amplification of immune

responses particularly by activating other cells of the immune systems such as B cells. B cells are also professional antigen-presenting cells and can take up and process antigen through the expression of antigen-specific B cell receptor (BCR) and present it on their surface a peptide-MHC complexes. Antigen-specific $CD4^+$ T cells recognise peptides presented on MHC Class II molecules on the surface of B cells and trigger their differentiation into antibody-secreting plasma B cells.

CLP cells were recently shown to differentiate into intermediate common innate lymphoid progenitor cells (CILP) in the fetal liver and the bone marrow. CILP give rise to all innate lymphoid cell subsets (ILC) including natural killer (NK) cells and lymphoid tissue inducer cells (LTi cells). NK cells are cytotoxic cells that mediate cell death through their secretion of pro-inflammatory cytokines and granules containing perforin and granzymes (reviewed in (Colucci et al., 2003)). It is thought that innate lymphoid cells exit the bone marrow as immature progenitors and complete their maturation in the periphery in response to tissue-derived cytokines, as will be discussed later.

1.2. αβ $CD4^+$ T cell development

1.2.1. Positive and negative selection in the thymus and lineage fate decisions

As common lymphoid progenitor cells enter the thymus they lose the ability to differentiate towards B cells and are termed double negative (DN) (no CD4 or CD8) cells. DN cells undergo 4 stages of early development (DN1-DN4) in the outer cortex of the thymus and can be identified by their expression of CD44 and CD25, diverting them away from TCRγδ or NK T cell lineages. $CD44^-CD25^+$ DN3 cells upregulate the surrogate T cell receptor alpha chain (pre-TCRα) (Germain, 2002). Following this, the TCRβ chain associates with pre-TCRα as well as cytoplasmic signalling molecules including the CD3 co-receptors (CD3-γ/δ/ε) and TRC-ζ to form a functional αβ TCR. Both TCRα and TCRβ loci are composed of interchangeable coding segments, namely the constant, (C), variable (V), joining (J) and diversity (D) (in the TCRβ loci only)

genes. Random rearrangements of V and J (for TCRα) and V, D and J (for TCRβ) form unique complementarity determining regions (CDRs) for the recognition of a huge diversity of peptides presented on MHC molecules (Davis and Bjorkman, 1988). VDJ recombination is dependent on recombination activating genes *Rag1* and *Rag2* (Dudley et al., 1994). *Rag1* and *Rag2*-deficient mice are therefore deficient in T cells and B cells, whose BCR immunoglobulin receptor undergoes similar rearrangement (Mombaerts et al., 1992; Shinkai et al., 1992; Shinkai et al., 1993). Activation of the TCR leads to an arrest in TCRβ rearrangement and the upregulation of both CD4 and CD8 co-receptors. In the thymus, T cells are educated to a repertoire of self-antigen and the fate of $CD4^+CD8^+$ double-positive (DP) cells is determined by their ability to discriminate self-antigen through their TCR presented in discrete environments within the thymus. The affinity of antigen recognition by the TCR correlates with 3 main developmental outcomes: death by neglect, positive selection or negative selection. Currently, affinity selection is a well-accepted model describing a graduated development of cells correlating with affinity for self (Klein et al., 2014). Ultimately, the purpose of affinity maturation is to select functional antigen-receptive cells that can favour recognition of foreign versus self-peptide (Vonboehmer et al., 1989). Cortical thymic epithelial cells play a crucial role in T cell selection by presenting self-peptide in the context of MHC Class I and II molecules. Most DP cells express non-functional TCRs that cannot recognise MHC molecules and results in 90% of DP precursors undergoing death by neglect. Upon peptide engagement of a functional TCR receptor, intracellular lymphocyte-specific protein-tyrosine kinase (Lck) bound to the TCR activates a cascade of signalling events resulting in the activation of genes required for the functional maturation of cells. Additionally, activated Lck associates with CD4 and CD8 co-receptors that can selectively recognise MHC Class II and MHC Class I molecules. Both instructive (through strength of signalling) and stochastic (random interactions) events are thought to contribute to the expression of either CD4 or CD8. Furthermore both transcriptional and epigenetic mechanisms can control CD4 versus CD8 fate. Indeed, the expression of TFs ThPOK (*Zbtb7b*) and Runt-related

transcription factor 3 (*Runx3*) is mutually exclusive and ultimately determines CD4 versus CD8 T cell fate (Reis et al., 2013). Furthermore, histone deacetylases 1 and 2 (HDAC1/2) were shown to maintain the CD4 lineage by limiting the expression of the Runx TF complexes (including Runx3) (Boucheron et al., 2014). Similarly, a specific region of the *Cd4* locus was specifically demethylated in $CD4^+$ cells and required for the maintenance of the CD4 lineage (Sellars et al., 2015).

1.2.2. Central and peripheral tolerance

Positively selected CD4 or CD8 lymphocytes upregulate the chemokine receptor CCR7 and migrate to the thymic medulla where they interact with medullary thymic epithelial cells (mTEC) expressing peripheral-tissue-specific antigens under the control of the AIRE gene (autoimmune regulator) (Liston et al., 2003). In addition, they interact with dendritic cells cross-presenting mTEC antigens and co-receptors. These interactions are essential for establishing central tolerance, either by clonal deletion or by clonal diversion. Following positive selection, cells continue to express TCRs with a broad range of affinities for self. Cells expressing very high-affinity TCR have the potential to develop into auto-reactive cells if selected into the periphery, and undergo clonal deletion, which describes the combined action of negative selection or cell death by apoptosis. At this stage, TCR signalling also drives the expression of the TF Forkhead box P3 (*Foxp3*) in developing $CD4^+$ T cells and the development of T regulatory cells (Treg) (Lee et al., 2012), as will be discussed later. As a result of TCR signalling in the thymus, $CD4^+$ T cells emerge into the peripheral lymphatic system with low affinity to self whereas Treg cells have a repertoire skewed towards self-recognition. Thymus-derived factors governing clonal deletion versus diversion are still not completely understood.

Despite a sophisticated process of affinity maturation and clonal selection, not all self-reactive cells are deleted and additional mechanisms are required to complement negative selection. A two-step model of $CD4^+$ T cell activation in the

periphery is essential for regulating the effector function of activated cells. Firstly, T cells encounter antigen in the context of peptide-MHC followed by stimulation with co-stimulatory signals expressed on antigen presenting cells. When self-reactive T cells encounter self-antigen in the absence of co-stimulation, for example on tolerogenic DCs, TCR signalling causes T cells to become anergic and functionally inactive. Additionally, immature DCs expressing CD86 recognise CTLA-4 (cytotoxic T lymphocyte antigen 4) on self-reactive naïve T cells, promote their anergy (Macian et al., 2004) and limit their migration to tissues (Walker et al., 2002). Consequently, mice deficient in CTLA-4 (Teft et al., 2006) and human patients with mutations in CTLA-4 (Schubert et al., 2014) exhibit profound immune dysregulation and the development of a range of autoimmune diseases. Upon infection, DCs upregulate higher levels of MHC and co-stimulatory molecules, which provide the necessary second signal for the activation of T cells and their differentiation into functional effector cells. However additional mechanisms of peripheral tolerance exist to limit recognition of self-antigens by 'escapee' $CD4^+$ thymocytes, including suppression by Treg cells, as will be discussed later.

1.2.3. $CD4^+$ T helper cells

Naïve $CD4^+$ T cells emerge from the thymus into the periphery and migrate through the lymphatic system to tissue-resident lymph nodes (LN), such as the lung-draining mediastinal LN (medLN) or gut-draining mesenteric LN (MLN). There are three fundamental signals required for T helper cell differentiation. Firstly, dendritic cells take up antigen in the tissue after infection and upregulate chemokine receptors, which will facilitate their migration to local LN. In the LN, DCs loaded with antigen enter the T-cell zone where they present antigen to T cells through the interaction of peptide-MHC complexes with the TCR. Upon functional TCR recognition of antigen presented on MHC Class II molecules, CD4 molecules expressed on T cells associate with the MHC:TCR complex and strengthen the interaction. Antigen recognition in the absence

of co-stimulation results in the inactivation of the cell. A second signal comes from T-cell associated CD28 interactions with CD80/86 receptors on the antigen presenting cell which initiates the activation and proliferation of the cell. Activated T cells rapidly produce IL-2 and further upregulate IL-2Rα on their surface, providing a positive feedback loop (Busse et al., 2010) and promoting their proliferation. DCs or other resident innate cells that have been primed by recognition of specific pathogens and tissue-derived alarmins provide the third signal, usually a cytokine signal, that dictate the differentiation of $CD4^+$ T cells into specialised subsets of cells called $CD4^+$ T helper (Th) cells. In addition, T cells express CTLA-4, which has a high affinity for CD80 and CD86, and therefore competes with CD28 to bind to DCs, indirectly limiting the proliferation or infiltration of T cells into the tissue (Yokosuka et al., 2010). Alternatively, CTLA-4-expressing T cells were shown to endocytose CD80/CD86, thereby limiting the availability of ligands for CD28 and indirectly inhibiting the activation of T cells (Qureshi et al., 2011).

Following cytokine signalling, the differentiation of T helper cells requires coordinated transcriptional and epigenetic changes, which facilitate heritable differentiation programs. T helper cell subsets develop through the coordinated expression of distinct signal transducer and activator of transcription (STAT) and transcription factors (TFs), ultimately leading to the secretion of characteristic cytokines such as interleukins (IL). Th1 cells express the TF T-box transcription factor (*Tbx21*) and secrete interferon gamma (IFN-γ) and are primarily required for the control of intracellular pathogens. Th2 cells express the TF GATA binding protein 3 (*Gata3)* and secrete interleukin 4 (IL-4), IL-5 and IL-13 required for anti-helminth defence but also responsible for driving allergic inflammation. Th17 cells express the TF RAR-related orphan receptor C (*Rorct)* and produce IL-17, which provides protection from bacterial and fungal infections. IL-9-producing Th9, IL-22-producing Th22 cells and T follicular helper cells are subsets of cells with polyvalent roles in infection and disease (**Fig. 1.1**).

1.2.3.1. T helper cell differentiation

Th1 and Th2 cells were originally described by Mosmann and Coffman as clonal populations of T cells capable of secreting distinct cytokines *in vitro* (Mosmann et al., 1986), later identified as IFN-γ and IL-4, respectively (Cherwinski et al., 1987). *In vivo,* the development of Th1 or Th2 cells was shown to correlate with susceptibility to *Leishmania* infection suggesting the cell types had distinct effector functions (Heinzel et al., 1989). In addition, the development of Th cells *in vivo* was strongly dictated by the nature of the infection suggesting distinct antigens and/or signalling molecules induced in response to specific pathogens could mediate the development of T cell subsets (O'Garra, 1998).

The study of T cell differentiation *in vitro* has been invaluable for our understanding of differentiation requirements and requirements for the maintenance of T helper subsets. Both TCR and CD28 engagement on T cells drive the expression of TFs such as nuclear factor of activated T cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which directly promotes *Il2* expression (Peng et al., 2001; Raab et al., 2001). IL-2 acts in an autocrine manner through STAT5 to initiate the proliferation of the cell (Northrop et al., 1994). In the context of an infection with intracellular pathogens, NK cells and APCs secrete IFN-γ and IL-27, which activate STAT1 phosphorylation and expression of the TF T-bet (*Tbx21*) (Szabo et al., 2000; Lighvani et al., 2001). Following this, *Tbx21* associates with co-factors such as H2.0-Like Homeobox (*Hlx)* and *Runx3* leading to the upregulation of surface IL-12Rβ2 (Afkarian et al., 2002). Consequently, macrophage- or APC-derived IL-12 (Hsieh et al., 1993) signals through the IL12Rβ2 to activate STAT3 and STAT4 (Jacobson et al., 1995), which cooperate with Runx3, HLX and T-bet to drive increased expression of IFN-γ (*Ifng*) (Mullen et al., 2002; Djuretic et al., 2007). Thus, secreted IFN-γ acts in an autocrine manner through phosphorylated STAT1 to reinforce *Tbx21* expression and stabilise IFN-γ production, thus promoting the maintenance of the Th1 lineage through a positive regulation loop (O'Garra, 1998). The differentiation of Th2 cells will be described in more detail later. Briefly, signalling downstream of the IL-4Rα

and Notch1 receptor lead to the indirect (via STAT6) or direct activation of *Gata3* and *Il4* (Amsen et al., 2007). Following this, GATA-3 promotes expression of the TF c-maf (*maf*) and together they further enhance the expression of *Il4, Il5* and *Il13* (Zhu, 2010). Autocrine IL-4 signalling and auto-activation of GATA-3 ensure stability of the Th2 lineage (Ouyang et al., 2000) (**Fig. 1.2**).

Following the differentiation of Th cells, the maintenance of Th lineages occurs through TF cross-regulation by active silencing of TFs. In Th1 cells, T-bet reduces the expression of *Gata3* (Usui et al., 2006) and cooperates with Runx3 to inhibit the expression of *Il4* (Djuretic et al., 2007), limiting the development of Th2 cells. Conversely, GATA-3 and STAT-6 bind to the *Ifng* promoter to inhibit IFN-γ secretion (Chang and Aune, 2007). Maintenance of T-bet and GATA-3 is therefore crucial for the stability of Th1 and Th2 subsets, respectively. As discussed later in the context of Treg development, methylation of DNA is an epigenetic modification of DNA that dictates its accessibility to TF binding, and correlates with stable gene expression (Wilson et al., 2009). GATA-3 and T-bet bind to the demethylated promoter regions of *Ifng* and *Il4* genes in order to promote their expression (Agarwal and Rao, 1998). Furthermore, the *Ifng* locus remains methylated in Th2 cells, and the *Gata3* locus methylated in Th1 cells, providing an additional mechanism of stability (Agarwal and Rao, 1998).

As well as coordinating the maintenance of T helper cell subsets, TF cross-regulation can also dictate the development of Th subsets. In particular, transforming growth factor beta 1 (TGF-β) was shown to promote the expression of *Rorct,* required for Th17 development, as well as the *Foxp3,* required for Treg development (Zhou et al., 2008). Furthermore, *Foxp3* expression was shown to directly bind to, and inhibit *Rorct* expression and Th17 differentiation (Zhou et al., 2008). However, in the presence of IL-6, phosphorylated STAT3 can directly inhibit *Foxp3* expression and instead, promote the expression of *Il21* (Veldhoen et al., 2006; Dardalhon et al., 2008). Following this, autocrine IL-21 drives further STAT3 phosphorylation and induces the expression of *Rorct*, *Il17a* and *IL-23R* (Gaffen et al., 2014). IL-23 signalling further stabilises the expression of *Il17a* and *Rorct* and promotes *Il17f* and *Il22* expression in

pathogenic Th17 cells (Gaffen et al., 2014). Similarly, although IL-4 alone drives Th2 differentiation, cells cultured in the presence of IL-4 and TGF-β were shown to lose the ability to produce IL-4 and secrete IL-9 (Veldhoen et al., 2008). Th9 cells are thought to share common transcriptional requirements with Th2 cells (*Stat6*, *Gata3* and *Irf4*) and express TFs such as *Spi1* (PU.1), *Batf* and *Bcl6,* important for their effector function *in vivo* (Schmitt et al., 2014). Finally, the cytokine IL-22 is part of the IL-10 family of cytokines and can be secreted both by Th17 cells and by Th22 cells in a STAT3-dependent manner, downstream of IL-23, IL-6 and IL-21 signalling (Ahlfors et al., 2014). Although *Rorct* is not sufficient to confer IL-22 capacity to T cells, *Roct*$^{-/-}$ cells have severely impaired IL-22 production (Ouyang et al., 2011).

Many cytokines typically associated with T helper cells are secreted by other immune subsets including γδ T cells and ILCs. Indeed, a high proportion of γδ T cells were shown to secrete IL-17 in naïve mice (Hirota et al., 2011). Furthermore, two recent studies showed ILCs were the predominant source of IL-22 (Ahlfors et al., 2014) and IL-9 (Turner et al., 2013) in the intestinal tract and lung tissue respectively. Furthermore, under some conditions such as infections with the intestinal helminth *N. brasiliensis*, ILC2s were shown to be the major IL-13 producing population (Neill et al., 2010). With emerging evidence of an effector role for ILCs, it will be important to address the relative contribution of T cell- and ILC-derived cytokines. Furthermore, ILCs secrete cytokines at steady state in the tissue, in the absence of an infection (Turner et al., 2013). This suggests they could present novel mechanisms of cell-intrinsic or extrinsic cytokine regulation.

1.2.3.2. Molecular mechanisms of Th2 differentiation

Both $CD4^+$ T cells and IL-4Rα expression on T cells are essential for protective immune responses to intestinal helminths (Allen and Sutherland, 2014) and therefore understanding the molecular mechanisms of Th2 differentiation *in vivo* is of significant importance. *In vivo,* Th2 cells develop in response to a diversity of antigens, ranging

from microscopic pollen allergens to macroscopic helminthic parasites. Th2 cells differentiate from naïve $CD4^+$ T cells in response to TCR stimulation and IL-4 signalling to secrete canonical cytokines including IL-4, IL-5, IL-13 and IL-9. The nature, strength and duration of TCR signalling following antigen recognition dictates the fate of naïve T cells differentiation towards distinct T helper subsets (van Panhuys et al., 2014) and low signal strength is thought to promote the differentiation of Th2 cells (Yamane and Paul, 2013). Concurrent to TCR engagement, IL- 4 binds to the high affinity IL-4Rα chain, which heterodimerises with the common gamma chain (γc) (a subunit of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Rochman et al., 2009)) to form the type-1 IL-4 receptor. The IL-4Rα chain can also heterodimerise with the IL-13Rα chain to form the type-2 IL-13 receptor, ubiquitously expressed on immune and non-immune cells (Junttila et al., 2008) including Th17 cells (Newcomb et al., 2009). Consequently, CD4-specific deletion of IL-4Rα impairs IL-4 and IL-13 signalling (Barner et al., 1998).

Following IL-4 engagement, cytoplasmic domains of the IL-4Rα chain are phosphorylated by activated kinases Janus Kinase 1 (Jak1) and Jak3 (Witthuhn et al., 1994). As a consequence, insulin receptor substrates 1 and 2 (IRS1/2) bound to the Tyr motifs on the cytoplasmic domains of the IL-4Rα are phosphorylated (Keegan et al., 1994). Following this, STAT6 which is bound to the SH2 motif on Tyr domains is phosphorylated, forms dimers and translocates to the nucleus where it binds specific DNA motifs essential for the differentiation as well as the proliferation of Th2 cells (Kaplan et al., 1996; Takeda et al., 1996; Nelms et al., 1999). For example STAT6 directly binds to, and promotes the expression of *Gata3* (Zhu et al., 2001). GATA-3 subsequently binds to the *Il4* locus to reinforce *Il4* expression (Zheng and Flavell, 1997). Indeed, enforced expression of both STAT6 (Kurata et al., 1999) and GATA-3 (Zhu et al., 2003) resulted in IL-4 secretion in $CD4^+$ Th1 cells. Furthermore, ectopic expression of GATA-3 could restore *Il4* expression in STAT6 deficient cells and IL-4 could directly induce GATA-3 expression in a STAT6-independent manner, suggesting that GATA-3 is sufficient to promote Th2 differentiation in the absence of STAT6

(Ouyang et al., 2000). Indeed, *Il4* activation and high levels of IL-4 production were shown to establish a positive feedback loop through the autoactivation of GATA-3, reinforcing the differentiation of Th2 cells (Ouyang et al., 2000). A potential mechanism of GATA-3-independent activation occurs through signalling downstream of Notch1 interactions with Jagged1 on APCs resulting in STAT-6 independent IL-4 production (Amsen et al., 2004). Mechanistically, both GATA-3 and STAT-6 were shown to facilitate long-range interactions forming between *Il4, Il5* and *Il13* loci required to maintain accessible conformation for TF binding and gene expression (Spilianakis and Flavell, 2004; Ansel et al., 2006). Consequently, GATA-3 was termed the 'master regulator' of Th2 cells (**Fig. 1.2**).

GATA-3 has since been shown to be expressed at low levels in naïve T cells and plays important roles in the development of many immune cells including $CD4^+$ T regulatory cells and ILC2s (Tindemans et al., 2014). *Gata3* deficiency is embryonically lethal and *Gata3*-deficient CD4 T cells do not develop in the thymus (Pai et al., 2003). Therefore the role of GATA-3 in $CD4^+$ T cells has been studied using mice where GATA-3 was specifically deleted in differentiated T cells, such as under the control of the distal Lck promoter expressed in late stage thymocytes or under the control of the OX40 promoter expressed in peripheral $CD4^+$ T cells (Tindemans et al., 2014). CD4-specific GATA-3 deficiency completely abrogated IL-5 and IL-13 production by T cells and partially inhibited IL-4 secretion *in vitro* (Zhu et al., 2004). Furthermore, IL-4 production was not significantly different in cells cultured for multiple rounds of *in vitro* polarisation suggesting that GATA-3 is required for *Il5* and *Il13* expression but that additional mechanisms promote the expression of *Il4* (Zhu et al., 2004). Indeed, the TFs c-maf (*Maf*) and JunB (*Junb)* were two TFs found to be highly expressed in Th2 cells. CD4-restricted deletion of *Maf* specifically impaired *Il4* expression (Kim et al., 1999) and c-maf transgenic mice showed enhanced Th2 differentiation *in vitro* (Ho et al., 1998). Furthermore, c-maf was shown to co-operate with JunB to promote IL-4 expression by directly binding to the *Il4* locus (Ansel et al., 2006). Another important transcription factor involved in Th2 differentiation is the family of NFAT transcription

factors (reviewed in (Ansel et al., 2006)). In particular, NFAT2 was shown to synergise with the TF interferon regulatory factor (Irf4*)* for optimal production of IL-4 (Rengarajan, 2002) and stabilise a complex containing GATA-3, STAT6 and c-maf at the *Il4* locus to promote *Il4* transcription (Kock et al., 2014).

A critical role for STAT6 and GATA-3 for the differentiation of Th2 cells *in vivo* has also been demonstrated. Th2 responses were impaired in the absence of IL-4-receptor or STAT6 following *N. brasiliensis* infection (Kopf et al., 1993) and *H. polygyrus* infection (Finkelman et al., 2000; Perona-Wright et al., 2010). Similarly, GATA-3 deficient peripheral $CD4^+$ T cells were impaired in their production of IL-4, IL-13 and IL-5 following *N. brasiliensis* infection, resulting in impaired class-switching and therefore reduced serum IgE levels, as well as impaired worm expulsion (Zhu et al., 2004). Transgenic GATA-3 dominant-negative mice that display attenuated levels of *Gata3* expression had reduced type-2 allergic inflammation after ovalbumin (OVA) immunisation and localised airway challenge (Zhang et al., 1999). Conversely, GATA-3 transgenic mice that overexpressed *Gata3* under the control of the *Lck* promoter displayed heightened IL-5 and IL-13 production by T cells and increased eosinophilia following *H. polygyrus* infection (Watanabe et al., 2003) and *N. brasiliensis* infection (Ozawa et al., 2005). Many other transcription factors are required for Th2 differentiation through promoting or stabilising the expression of GATA-3 and its associated TFs, including Nfκβ (Das et al., 2001) and Dec2 (Yang et al., 2009). Additionally, TFs such as GRAIL were shown to regulate Th2 differentiation through negative regulation of STAT6 (Sahoo et al., 2014).

As previously mentioned, both IL-4 and IL-2 signalling coordinate for the optimal differentiation of Th2 cells *in vitro* (Ben-Sasson et al., 1990; Le Gros et al., 1990; Zhu, 2010). The IL-2 receptor is composed of IL-2Rβ (CD122) and γc (CD132) readily found on naïve T cells. IL-2 is captured by IL-2Rα (CD25), which subsequently associates with IL-2Rβ and γc to form a functional receptor. IL-2 signalling through the IL-2 receptor leads to the phosphorylation of STAT5 and downstream gene activation, including positive regulation of *Il2ra* resulting in increased surface expression of CD25

and IL-2 signalling, required for the proliferation of the cells (Malek and Castro, 2010). Although early IL-4 signalling in naïve T cells could increase surface IL-4Rα expression independently of IL-2 (Perona-Wright et al., 2010), IL-2 secreted following TCR activation was shown to feed back on differentiating T cells to increase the expression of IL-4Rα (Liao et al., 2008) through direct binding of STAT5 to the *Il4ra* locus (Liao et al., 2008). Consequently, STAT5 deficiency or IL-2 neutralisation impaired IL-4 secretion *in vitro* and *in vivo* (Yamane et al., 2005). Mechanistically, IL-2 activated STAT5 to induce epigenetic changes at the *Il4* locus and cooperated with GATA-3 for *Il4* expression (Cote-Sierra et al., 2004). IL-2 is therefore fundamentally required for IL-4 production by Th2 cells.

Although the TFs required for cytokine production are well characterised, the differentiation of mature Th2 cells *in vivo* is likely to be far more complex, resulting in diverse and specialised subsets of Th2 cells. Indeed, transcriptional profiling of Th2 populations revealed significant gene expression variation between Th2 populations isolated from *in vitro* cultures, *H. polygyrus* infection or the inflamed lungs of house dust mite (HDM)-challenged mice (Okoye et al., 2014). Furthermore, Liang et al made use of cytokine reporter mice ($Il4^{KN2/4get}Il13^{Smart/+}$) expressing huCD2 under the control of the *Il4* promoter and huCD4 under the control of the *Il13* promoter to identify sub-populations of Th2 cells secreting either or both cytokines in the context of *N. brasiliensis* infection (Liang et al., 2012). One major finding was that a sub-population of $CD4^+$ T cells secreting IL-4 and not IL-13 were expressing markers characteristic with T follicular helper (Tfh) suggesting there are unique subsets of IL-4- and IL-13-secreting $CD4^+$ T cells (Liang et al., 2012). Furthermore, single-cell sequencing of a purified *Il13*-GFP^+ cells isolated from *N. brasiliensis* infected *Il13*-GFP reporter mice identified of a subpopulation of steroidogenic suppressor cells expressing the lipid enzyme Cyp11a1 (Mahata et al., 2014) highlighting the heterogeneity of Th2 cells *in vivo*. Thus, although we understand molecular mechanisms behind the differentiation of Th2 cells, these studies highlight the fascinating diversity of T effector cells *in vivo*,

suggesting there are many additional signals required for their effector function in diverse contexts.

The studies above describe a clear role for IL-4 in the differentiation of Th2 cells, however studies using *Il4*$^{-/-}$, *Il4ra*$^{-/-}$ and *Stat6*$^{-/-}$ mice have shown that T cells can differentiate into Th2 cells independently of IL-4 or IL-4 signalling, *in vitro* and *in vivo* (Bouchery et al., 2014). In the context of helminth infections, *Il4ra*$^{-/-}$ cells could both proliferate and secrete IL-4 in response to helminth antigens *ex-vivo* (Jankovic et al., 2000) and *Il4ra*$^{-/-}$ mice developed functional Th2 responses *in vivo* during *S. mansoni* infection (Jankovic et al., 1999). However Th2 responses in *Il4ra*- and *Stat6*-deficient mice were dramatically reduced in comparison to WT mice and insufficient to promote granuloma formation surrounding eggs in the liver (Jankovic et al., 1999). Furthermore, the differentiation and development of memory Th2 cells and functional immunity to *N. brasiliensis* was shown to be independent of STAT6 (van Panhuys et al., 2008; Van Panhuys et al., 2013). However this could be explained by the finding that TCR-signalling promotes GATA-3 expression in the absence of STAT6 leading to STAT6-independent IL-4 production (Ouyang et al., 2000). Alternatively, peptide activation of CD4^{+} naïve T cells *in vitro* induced sufficient concentrations of IL-4 to support Th2 differentiation of neighbouring T cells in the absence of an exogenous source of IL-4 (Noben-Trauth et al., 2000), suggesting T cells may require IL-4, however not from an exogenous source.

Taken together these studies suggest that IL-4 signalling is not always required for the differentiation of Th2 cells however it is required for the optimal development of a functional population of Th2 cells. Finally, IL-4 may not be required for the maintenance of Th2 cells *in vivo* following the observation that sustained activation of Th2 cells through the TCR results in the downregulation of IL4Rα (Perona-Wright et al., 2010).

1.3. $CD4^+$ $Foxp3^+$ Treg cell development and function

In addition to the mechanisms of central tolerance described above, the development of a specialised subset of $CD4^+Foxp3^+$ T regulatory cells (Treg) is required for the maintenance of peripheral tolerance and immune homeostasis in steady state and disease contexts.

T regulatory cells were first identified in the 1980's by several laboratories as a sub-population of $CD4^+$ lymphocytes expressing activation markers $CD5^+$ and $CD45RB^{low}$ and capable of limiting the development of autoimmune disease caused by the proliferation of self-reactive $CD4^+$ T cells (Sakaguchi et al., 2001). In a seminal series of experiments, the transfer of $CD45RB^{high}$ cells to lymphodeficient mice resulted in the development of spontaneous autoimmune disease in multiple organs and wasting, which could be prevented by the co-transfer of $CD45RB^{low}$ cells (Powrie et al., 1993). $CD45RB^{low}$ cells were further characterised as expressing the high affinity IL-2 receptor CD25 (IL2Rα) (Sakaguchi et al., 1995). $CD4^+$ cells depleted of $CD25^+$ cells (approximately 10% of the $CD4^+$ T cell pool) could also induce colitis in lymphodeficient recipients, which was prevented when $CD25^+CD4^+$ T cells were co-transferred. Additionally, $CD25^+CD4^+$ T cells were able to limit autoimmune development in thymectomised mice. Antibody-mediated depletion of $CD25^+$ cells in wild-type mice resulted in the development of a similar disease (Sakaguchi et al., 1995; Asano et al., 1996). These experiments demonstrated the existence of self-reactive T cells capable of mediating autoimmune disease that were controlled by a population of $CD25^+$ cells. The development of $CD25^+$ Treg cells was later shown to be dependent on the expression of the *Foxp3* (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Indeed, mutations in the *Foxp3* locus abolished the development of $CD25^+$ T cells resulting in a severe lymphoproliferative disorder in mice, called Scurfy disease (Brunkow et al., 2001; Fontenot et al., 2003) and in humans, called immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Bennett et al., 2001).

1.3.1 Treg cell development

It is currently widely accepted that Treg cells develop in response to instructive signals in the thymus (tTregs) and periphery (pTreg). tTregs develop in the thymus from $CD4^+$ SP precursors in response to high-affinity TCR recognition of self-peptide. The generation of tTreg cells was directly correlated to the affinity of the TCR for self-peptide, as shown using a panel of TCRs recognising OVA-peptide with different affinity (Lee et al., 2012). Additionally, the levels of self-antigen expressed in the thymus correlate with tTreg development, as shown using transgenic mice expressing varying amounts of HA mRNA in the thymus (Weissler and Caton, 2014). Combined, these studies suggested that the recognition of low abundance self-antigens in the thymus was required for Treg development. Indeed the Treg TCR repertoire was shown to have considerable overlap with the escapee self-reactive T cells (Hsieh et al., 2006). A two-step model has been proposed for the development of tTreg cells in the thymus. Firstly, high-affinity TCR signalling in $CD4^+$ SP cells leads to the upregulation of high levels of CD25 and binding of NFAT, AP1, CREB, ATF, Runx1 and Foxo family members Foxo1 and Foxo3 (Ouyang et al., 2012) to the *Foxp3* promoter and enhancer elements. Additionally NFκB signalling downstream of the TCR stimulates c-rel binding to *Foxp3*, enhancing Foxp3 expression (Long et al., 2009; Barbi et al., 2014). IL-2 signalling on the $CD25^+$ precursors drives phosphorylation of STAT5 and expression of *Foxp3* (Lio and Hsieh, 2008). Treg cells fail to develop in IL-2 and IL-2Rβ-deficient mice that develop systemic autoimmune disease, demonstrating a fundamental requirement for IL-2 signalling in combination with TCR signalling (Zorn et al., 2006; Burchill et al., 2007). Transfer of WT Treg cells to *IL2rb*$^{-/-}$ mice prevented the onset of autoimmune disease (Malek et al., 2002) Additionally, IL-2 was required for Treg maintenance in the periphery, as treatment with IL-2 neutralising antibody significantly reduced the population of Treg cells (Setoguchi et al., 2005), as well as the maintenance of *Foxp3* expression *in vivo* (Oldenhove et al., 2009). Importantly though, Treg cells do not secrete any IL-2, and IL-2 hypoproduction is absolutely essential for their suppressive function (Marson et al., 2007). Chromatin immuno-precipitation

(ChIP) revealed that as well as directly regulating *Il2* expression, Foxp3 targets genes of the TCR pathway, including cell surface molecules and signalling components such as *Zap70* (Marson et al., 2007), suggesting an early step in early differentiation of Treg cells is the suppression of excessive TCR signalling which could mediate cell death as well as initiate TCR-activation-dependent immune effector responses. Foxp3 therefore acts as an independent regulatory mechanism determining the outcome of TCR signalling and promoting cell survival and tTreg development over clonal deletion.

Naïve $CD4^+$ T cells can be induced to express *Foxp3 in vitro* (iTreg) in response to IL-2 and TGF-β signalling (Davidson et al., 2007). Additionally, the vitamin A metabolite retinoic acid (secreted in high levels in the intestine) was shown to enhance TGFβ signalling and *Foxp3* expression in iTregs (Liu et al., 2015). Evidence for the upregulation of Foxp3 *in vivo* was seen when T cells isolated from TCR transgenic Rag1-deficient mice deficient in Treg cells were able to upregulate *Foxp3 expression* after their adoptive transfer into WT mice (Apostolou and von Boehmer, 2004). Indeed $Foxp3^+$ cells can develop from conventional T cells in the periphery (pTregs) in response to antigens including microbial antigens. Recent work has shown intestinal commensal bacteria digest specific components in our diet and secrete immunogenic metabolites. One of these, butyrate, was shown to directly stimulate *Foxp3* expression and suppression (Arpaia et al., 2013; Furusawa et al., 2013). As described in the context of helminth infections, $Foxp3^+$ pTreg cells develop in response to foreign antigens and may play a different role to tTreg cells in the context of an infection. Additional stimuli were shown to support pTreg development, such as the vitamin a metabolite retinoic acid, highly expressed in gut-associated lymphoid tissues (Sun et al., 2007). Gene expression analysis revealed tTreg cells expressed unique genes differentiating them from other Treg populations (Sugimoto et al., 2006). Two molecules Helios (Thornton et al., 2010) and Neuropilin (Weiss et al., 2012; Yadav et al., 2012) were identified as markers preferentially expressed on thymic Treg cells, in an effort to distinguish tTreg cells in the periphery (**Fig. 1.1**).

1.3.2. Mechanisms of Treg suppression

As described above, CTLA-4 is essential for promoting anergy in self-reactive peripheral $CD4^+$ T cells after TCR stimulation. CTLA-4 is constitutively expressed on all Treg subsets and crucial for their suppressor function (Walker and Sansom, 2015). Treg cell-specific CTLA-4 deficiency impairs their suppressive function *in vitro* and *in vivo* (Wing et al., 2008) and blockade of CTLA-4 *in vivo* impairs suppressive activity of Treg cells leading to the development of disease (Read et al., 2006). Treg cells were shown to form long-lasting interactions with dendritic cells and suppress their function, indirectly resulting in limiting the differentiation of $CD4^+$ T helper cells (Onishi et al., 2008). One potential contact-dependent mechanism is the interaction of CTLA-4 with CD80/86 on APCs, triggering their secretion of IDO, which degrades tryptophan thereby limiting T cell growth (Fallarino et al., 2003). Conversely, depletion of DCs or DC-specific deletion of MHC Class II expression led to a loss of Treg cells, suggesting DCs promote Treg maintenance thereby enhancing the suppressive environment in a feedback regulatory loop and limiting the expansion of effector cells (Darrasse-Jeze et al., 2009). Other surface molecules shown to support Treg-mediated suppression include GITR (Shevach and Stephens, 2006), CD39, OX40 (Griseri et al., 2010) and CD73 (Morikawa and Sakaguchi, 2014) however deficiency in these molecules does not impair peripheral homeostasis as much as deficiencies in Foxp3 and CTLA-4. In the periphery, Treg cells form long-lasting interactions DCs, inhibiting their maturation (Onishi et al., 2008). Conversely, DCs were shown to maintain the Treg population, in a regulatory feedback loop (Darrasse-Jeze et al., 2009). IL-10 and TGF-β are immunosuppressive cytokines secreted by many cells of the innate and adaptive immune response including both $Foxp3^+$ and $Foxp3^-$Treg cells (Tr1), and a proportion of $Foxp3^+$ Treg cells. *Il10*$^{-/-}$ Treg cells were unable to limit the development of colitis after adoptive transfer of $CD45RB^{high}$ T cells into *Rag2*$^{-/-}$ mice (Asseman et al., 1999). Additionally, Treg-specific deletion of IL-10 leads to systemic autoimmunity and particularly severe colitis (Rubtsov et al., 2008). Furthermore, IL-10 signalling on Treg cells was required for Treg-mediated suppression of Th17 cells (Chaudhry et al., 2011)

and for limiting the development of colitis. In addition to cell-contact mechanisms, IL-10, TGFβ and IL-35 production by Foxp3^{+} Treg cells was shown to maintain DCs in an immature state, thus indirectly limiting T cell activation (Collison et al., 2007; Collison et al., 2010).

Many other mechanisms of suppression are likely to exist and remain unknown. Recently however, Treg cells were shown to secrete miRNA-containing exosomes, which could directly target Th1 cells *in vitro* and suppress their proliferation (Okoye et al., 2014). Both miRNA- and exosome-deficient Treg cells had impaired suppressive function *in vitro* and *in vivo* as assessed by their ability to limit the development of colitis when co-transferred with CD45RB^{high} cells (Okoye et al., 2014).

It remains unclear whether the ability of Treg cells to suppress auto-reactive T effector cells is dependent on the expression of similar self-antigen on the surface of APCs or if it is through bystander activation. Many studies show Treg suppression of T effector cells *in vitro* was not dependent on their expression of a TCR with the same affinity (Thornton and Shevach, 2000). *In vivo,* adoptively transferred *H. polygyrus*-primed Treg cells were able to suppress allergic airway inflammation (Wilson et al., 2005) and adoptive transfer of polyclonal but not not clonotypic Treg cells controlled the spontaneous development of arthritis in TS1 transgenic mice that express the S1 peptide (derived from a PR8 influenza strain) (Oh et al., 2012). Thus it seems likely that antigen-specificity is not required for their suppressor function, however recognition of self-antigen may be important in the context of Treg instability, as discussed later. Furthermore, Treg suppression mechanisms are likely to be context-dependent and increasing evidence shows suppressor function correlates with the expression of unique transcriptional profiles, as discussed later.

1.3.3. Transcriptional requirements for Treg development

Foxp3 expression is fundamentally required for Treg suppressor function, and has historically been described as the master regulator TF for Treg cells. Indeed,

ectopic expression of *Foxp3* is able to confer *in vivo* and *in vitro* suppressive activity to conventional T cells (Fontenot et al., 2003; Hori et al., 2003). An essential role for Foxp3 is to regulate the functional features of Treg cells through the simultaneous activation and repression of genes (Zheng and Rudensky, 2007). Experiments using *Foxp3*-GFP knockin mice highlighted the role of Foxp3 for Treg maintenance. Indeed, the IRES-GFP inserted into the *Foxp3 l*ocus was shown to disrupt Foxp3 function, inducing global transcriptional changes in their profile (Darce et al., 2012), resulting in their instability *in vivo* and the acquisition of effector cytokine production (Bettini et al., 2012). As described above, TCR signalling induces the expression of many genes, including the CD25 receptor. IL-2 signals through CD25 to phosphorylate STAT5 and activate *Foxp3* expression (Marson et al., 2007). Experiments in Foxp3-null mice with impaired *Foxp3* expression highlighted the role for Foxp3 in regulating the gene expression of key functional molecules such as CD25 and CTLA-4 whilst limiting the expression of *Il2* and pro-inflammatory cytokines such as *Il4* and *Ifng* (Gavin et al., 2007; Marson et al., 2007; Zheng et al., 2007). However these studies revealed many differentially expressed genes were not direct targets of Foxp3, suggesting other transcription factors were required for the *Foxp3*-dependent transcriptional profile. Indeed, two seminal studies have since characterised self-reinforcing transcriptional networks associated with Foxp3, and required for maintenance of *Foxp3* expression, defined as the 'Foxp3 interactome' (Fu et al., 2012; Rudra et al., 2012). Firstly, a proteomic approach characterised large protein complexes formed around Foxp3 and containing transcription factors as well as chromatin-remodelling complexes, all containing Foxp3 binding sites (Rudra et al., 2012). Using a computational approach, a 'quintet' of 5 transcription factors (*Eos, Gata1, Irf4, Lef1* and *Stab1*) was shown to co-operate with Foxp3 in a redundant manner to induce the Treg transcriptional program (Fu et al., 2012). Indeed, *Eos* had been previously shown to promote *Foxp3*-dependent gene silencing and maintain the stability of the Treg lineage (Pan et al., 2009). Both studies identified regulatory feedback loops and suggested the Treg interactome was required for the maintenance of a stable subset of Treg cells, which also suggests that

changes in the association of *Foxp3* and co-factors could impact the stability of the Treg cell. One example of an essential Foxp3 co-factor is the transcription factor GATA-3, typically associated with the differentiation of Th2 cells (Ouyang et al., 2000). Through genome-wide sequencing analysis, recent studies have shown GATA-3 forms a complex with Foxp3, through the mutual presence of binding sites on both genes (Rudra et al., 2012). Furthermore, Treg-specific deletion of GATA-3 impaired their suppressor function and homeostasis of the cells resulting in the spontaneous development of disease (Wang et al., 2011) and their inability to control colitis after adoptive transfer of $CD45RB^{high}$ cells into lymphodeficient mice (Wohlfert et al., 2011). Many more studies have highlighted the role of specific TFs for the maintenance of Treg function, including the recent description of *Bach2,* which stabilises iTreg development by directly repressing the expression of genes associated with T helper function such as *Irf4, Gata3* and *IL12Rb1* (Roychoudhuri et al., 2013).

1.3.4. Epigenetic regulation of Treg development and stability

Foxp3 expression has been shown to be transiently upregulated in both human and mouse CD4 T cells following TCR activation, however these cells lacked any other Treg features (Pillai et al., 2007; Wang et al., 2007; Miyao et al., 2012), suggesting that transient *Foxp3* expression is not sufficient for Treg development or function. Conversely, cells can possess Treg function without expressing *Foxp3*, as seen in Foxp3-null Treg cells (Barbi et al., 2014). Therefore the vast majority of studies agree that the sustained levels of Foxp3 are necessary for Treg function (Williams and Rudensky, 2007). Stability of gene expression is largely regulated by epigenetic modifications of the DNA that alter the accessibility of the gene and facilitate binding of transcription factors. Some examples of DNA modifications shown to play an important role in Treg development are histone modifications and DNA methylation, both heritable through cell divisions (Huehn and Beyer, 2015). Histones can be modified by acetylation, methylation and phosphorylation, all of which are reversible, indicating

epigenetic changes are dynamic. For example, TCR stimulation induced histone H3 lysine 4 dimethylation (H3-K4me2) and trimethylation (H3-K4me3) on the promoter regions of *Foxp3* and *Il2ra*, and further enhanced by TGF-β and RA signalling *in vitro* (Lu et al., 2011). In addition, recent work has described an essential role for the chromatin-remodelling methyltransferase enhancer of zeste homolog 2 (Ezh2) for the maintenance of Treg function and stability. Ezh2 functions to stabilise the Foxp3 transcriptional program by promoting the repression of its target genes (Arvey et al., 2014). Ezh2-deficiency in Foxp3-expressing cells therefore resulted in the development of systemic autoimmunity due to the uncontrolled expansion of T effector cells (DuPage et al., 2015). Furthermore, DNA methylation of cytosines is the predominant modification of mammalian DNA and occurs early in the development of a cell. 5'-CpG-3' dinucleotides are bound by factors such as DNA-methyl-binding domains (Mbd) and targeted for methylation through the recruitment of histone deacetylases (HDACs) and DNA methyl transferases (DNMTs) such as DNMT3a and DNMT3b (reviewed in (Smith and Meissner, 2013)). Most mammalian cytosines found at CpG dinucleotides are methylated, however CpG islands found at the promoter of many developmentally regulated genes remain hypomethylated, which confers accessibility to the gene locus for the binding of transcription factors, ultimately leading to the enhancement of transcription (Thurman et al., 2012). This is stably maintained throughout subsequent differentiation processes through DNMT1, which copies methylation patterns during cell division (Lee et al., 2001). Demethylation can occur passively via DNMT1, or actively through the modification of methylated cytosines (methylcytosine hydroxylation) by the ten-eleven translocation (TET) family of proteins (Ito et al., 2011). Thus, changes in methylation can be dynamically regulated throughout the lifetime of a cell, and lead to epigenetic reprogramming of a cell (Wu and Zhang, 2010). In the context of T helper cell differentiation, CpG methylation was shown to regulate the proliferation and differentiation of CD4 T helper cells by maintaining hypomethylation at specific cytokine gene loci. Indeed, methylation of the *Il4* locus is progressively lost during Th2 cell polarisation *in vitro,* correlating with increased *Il4* transcription (Lee et

al., 2002). Furthermore, *Dnmt*-deficient $CD4^+$ T cells were shown to simultaneously secrete multiple cytokines including IL-4 and IFN-γ, highlighting the importance of methylation for lineage specification (Lee et al., 2001). More recently, genome-wide analysis of 5'-methylcytosines revealed a unique pattern of methylation in both human and mouse Treg cells compared to conventional T cells (Floess et al., 2007; Wei et al., 2009; Ohkura et al., 2012). These studies identified Treg-specific demethylation regions (TSDRs) located in several key Treg-related genes such as *IL2ra, Tnfrsf18* (GITR), *Ikzf2* (Helios), *Ikzf4* (Eos) and *Foxp3* itself that were uniquely demethylated in Treg cells (Schmidl et al., 2009). Three conserved non-coding regions (CNS1, CNS2 and CNS3) were the primary targets of DNA methylation in the *Foxp3* locus and play important roles in Treg development and function (Zheng et al., 2010). Hypomethylation of CNS3 was required for *Foxp3* expression in the thymus whereas hypomethylation of CNS1 was required for *de novo* expression of *Foxp3* in peripheral $CD4^+$ T cells, through direct binding by TGF-β-elicited Smad2 and retinoic acid receptor (Zheng et al., 2007). In contrast, hypomethylation of CNS2, although not important for the induction of *Foxp3* expression, was crucial for the maintenance of Treg function (Zheng et al., 2010; Feng et al., 2014). Indeed, CNS2-deficient mice display reduced proportions of *Foxp3*-expressing cells (Zheng et al., 2010) correlating with an observed decrease in Treg suppressor function in mice with compromised CNS2 methylation (Mouly et al., 2010). Demethylation of the *Foxp3* locus and Treg enhancer regions occurred in $CD4^+CD25^+$ thymic Treg precursors upon TCR signalling and prior to their expression of *Foxp3* (Ohkura et al., 2012; Toker et al., 2013), thus establishing an epigenetic landscape accessible to the binding of *Foxp3* and its co-factors (Samstein et al., 2012). As a consequence, the methylation of the *Foxp3* locus gives a good indication of the stability of the Treg lineage. Interestingly, iTreg cells that develop *in vitro* in response to TFG- β and IL-2 express *Foxp3* but fail to induce hypomethylation at the *Foxp3* CNS2, correlating with the rapid loss of *Foxp3* expression upon re-stimulation in comparison to $CD25^+$ Treg cells isolated *ex vivo* (Floess et al., 2007). Conversely, tTreg cells have been shown to maintain a

methylated *Foxp3* CNS2 following TCR activation *in vitro* or after transfer into lymphodeficient hosts (Ohkura et al., 2012; Toker et al., 2013). *In vivo,* a small population of cells was shown to lose *Foxp3* expression but maintained a partially demethylated CNS2 locus (Miyao et al., 2012). These cells were able to readily re-express *Foxp3* upon TCR stimulation *in vitro* suggesting that CNS2 demethylation correlates with epigenetic memory of a Treg past, independently of *Foxp3* expression (Miyao et al., 2012). Conversely, another population of cells had also lost *Foxp3* expression and secreted T helper cell cytokines, which correlated with a methylated *Foxp3* CNS2, suggesting that functional reprogramming of Treg cells correlates with the re-methylation of the *Foxp3* locus (Miyao et al., 2012). Given that inflammatory cytokines can directly promote the re-methylation of *Foxp3* CNS2 (Feng et al., 2014), it is conceivable that these cells underwent *de novo* methylation of their *Foxp3* locus following cytokine signalling.

Together these studies demonstrate that methylation, and not *Foxp3* expression, correlates with Treg function. Indeed, Foxp3-null cells exhibited a very similar methylation pattern despite lacking Foxp3 protein (Gavin et al., 2007) highlighting the importance of TFs other than *Foxp3* for the function of Treg cells (Morikawa and Sakaguchi, 2014), as previously described. However, some genes are regulated by Foxp3 as well as by DNA methylation, such as *Il2ra, Ctla4 and Foxp3* itself. It is therefore likely that DNA methylation controls the expression of a limited number of genes, which in turn, drive the expression of a more diverse complex of TFs. This complex level of regulation may reflect an ancient evolutionary developmental pathway that enables maintenance and heritability of their Tregs and survival of the host.

1.3.5. Post-transcriptional and post-translational regulation of Treg differentiation and function

Post-transcriptional mechanisms of regulation such as micro-RNAs (miRNAs) also play an important part in the development and function of Treg cells. Treg cells

deficient in the miRNA-processing enzyme Dicer could develop in the thymus, however in the periphery, they lost *Foxp3* expression and started to produce T helper cell-related cytokines such as IL-4 and IFN-γ (Zhou et al., 2008). As a consequence, mice with a Treg-specific deletion of Dicer developed lymphoproliferative disease similar to that seen in *Foxp3*$^{-/-}$ mice (Zhou et al., 2008). Although many miRNAs were found to be highly upregulated in Treg cells, not much is known about the function of specific miRNAs. miR-155 is a well characterised miRNA which plays many important physiological functions including in immune cells (Vigorito et al., 2013). Foxp3-dependent miR-155 expression was required for the maintenance of Treg cells by heightening their sensitivity to IL-2 (Lu et al., 2009). In addition, miR-17-92 was critical for the accumulation of Treg cells and IL-10-mediated suppression of experimental autoimmune encephalitis (EAE) (de Kouchkovsky et al., 2013). Furthermore, miR-182 and miR-10a were required for the targeted suppression of Th2 and Th1 cells, respectively (Kelada et al., 2013). Combined, these results suggest that miRNAs contribute to the maintenance, specialisation and suppressor function of Treg cells, however more work need to be done to identify functions of unique miRNAs expressed in Treg cells.

Posttranslational mechanisms of regulation have received considerably less attention, however increasing evidence suggests they play an important role in regulation of Foxp3 and associated TFs required for Treg suppressor function (Barbi et al., 2014). For example, the Ubc13/Ikk axis was required for the expression of functional Treg-associated genes such as *Il10* and *Socs1* (Chang et al., 2012). More specifically, a recent study found that TCR signalling could induce the transcription of the protein kinase casein kinase 2 (CK2), which in turn phosphorylates up to 300 downstream target proteins including the inhibitory receptor ILT3, required Treg suppression of Th2 cells (Ulges et al., 2015). Thus, Treg-specific deletion of CK2 resulted in the spontaneous development of Th2 lymphoproliferative disease (Ulges et al., 2015). Additionally, Foxp3 stability can be positively and negatively regulated by acetylation of its lysine residues (van Loosdregt et al., 2010) and negatively regulated

through proteosomal degradation by ubiquitination (Barbi et al., 2014). For example, the E3 ubiquitin ligases TRAF-6 (Muto et al., 2013) and ITCH (Jin et al., 2013) were required for Treg suppression of Th2 cells. Similarly, Treg-specific deletion of the E3 ubiquitin ligase VHL (von Hippel-Landau) resulted in the loss of *Foxp3* expression in Treg cells and secretion of IFN-γ (Lee et al., 2015). Surprisingly, a single deubiquitinating enzyme (DUB) USP7 was sufficient to stabilise Foxp3 protein by inhibiting its upiquitination (van Loosdregt et al., 2013) and DUBs were also shown to stabilise GATA-3 protein, critical for Foxp3 maintenance (as described previously) (Barbi et al., 2014). Taken together, these studies suggest that post-translational and post-transcriptional mechanisms are required to maintain Treg function in the periphery, and confer Treg cells with the specific ability to suppress subsets of T effector cells.

1.3.6. Influence of the environment on Treg stability

In the periphery, Treg cells are exposed to a huge variety of triggers, including antigen, cytokines, metabolites and lipid mediators, all of which could potentially impact the stability and survival of the cell. TCR signalling plays an essential role for the development and function of Tregs. Although previous studies found that TCR signalling was only required early in Treg development (Thornton and Shevach, 2000), more recent evidence has found that continuous TCR signalling is essential for the effector function of Treg cells *in vivo* and *in vitro* (Levine et al., 2014), through the maintenance of a signature of genes required for Treg function such as *Irf4* (Ahyi et al., 2009; Cretney et al., 2011; Vasanthakumar et al., 2015). This suggests that any disruption in TCR signalling in the periphery could impact the stability of Treg cells. Similarly, cytokines play important roles in the development and maintenance of Treg cells. Indeed, IL-2 is critically important for the development and maintenance of Treg cells in the thymus (Fontenot et al., 2005). Furthermore, IL-10 acts on peripheral Tregs to increase their suppressor function and limit Th17 responses (Chaudhry et al., 2011).

Although cytokines can shape Treg function and enhance their ability to suppress T effector populations (as discussed later), in some contexts pro-inflammatory cytokines have been shown to impair Treg function. Indeed, levels of IL-6 can dictate the balance between the development of TGF-β-dependent Treg and Th17 cells (Veldhoen et al., 2006). Furthermore, IL-4 impairs Treg function by reducing *Foxp3* expression levels *in vitro* (Feng et al., 2014) and *in vivo* in the context of food allergy (Noval Rivas et al., 2015). In addition, Treg cells cultured with synovial fluid isolated from rheumatoid arthritis patients and containing high levels of TNFα, maintained *Foxp3* expression but lost their suppressive potential (Nie et al., 2013). More recently, IL-1β, TNFα and bacterial LPS were shown to reduce Foxp3 protein levels by stimulating increased expression of E3 ubiquitin ligase Stub1, resulting in the targeting of Foxp3 for proteosomal degradation (Chen et al., 2013). Collectively, this data suggests that TCR stimulation, combined with signalling from appropriate cytokines, are required for the development and maintenance of Treg cells, both of which can be disrupted in the context of inflammation and infection.

1.3.7. Treg cell specialisation

As discussed above, Treg cells are highly sensitive to changes in their environment. Tregs upregulate chemokine receptors and genes for their migration into tissues, where they express unique transcriptional signatures required for their maintenance and function (Feuerer et al., 2010) (**Fig. 1.5**). For example the transcription factor PPAR-γ was required for the accumulation of Tregs into the VAT of ageing mice and for limiting obesity-induced inflammation (Cipolletta et al., 2012). VAT-Treg cells were later shown to express *Batf* and *Irf4,* which could promote the expression of *Pparg* as well as high levels of *Il1rl1* (IL-33R) (Vasanthakumar et al., 2015). IL-33 signalling was shown to promote the proliferation and accumulation of Treg cells to the VAT (Vasanthakumar et al., 2015), as well enhance their expression of CD25 and suppressor function (Molofsky et al., 2015). IL-33R was also highly

expressed on colonic Treg cells where it was shown to be required for Treg-mediated control of intestinal inflammation (Schiering et al., 2014). As part of their adaptation to their microenvironment, both murine (Burzyn et al., 2013) and human (Duhen et al., 2012) Treg cells have been shown to up-regulate TFs associated with the development of T helper cell subsets, enabling them to specifically regulate immune responses. For example, Foxp3$^+$ cells isolated from the Peyer's patches expressed surface CXCR5 and the TF *Bcl6,* both of which are associated with Tfh development, and were shown to specifically suppress Tfh cells (Tfr) (Chung et al., 2011; Linterman et al., 2011). Tfr cells were essential to control late stage germinal centre reactions (Chung et al., 2011), thereby limiting the development of non-antigen-specific affinity matured B cells (Linterman et al., 2011). Furthermore, *Irf4,* which forms part of 'the quintet' of genes required for Foxp3 maintenance and is critically required for IL-4 production by Th2 cells (Rengarajan, 2002), was required for Treg suppression of Th2 cells. Indeed, *Irf4*-deficient Treg cells were selectively impaired in their ability to suppress Th2 cells, resulting in the development of an inflammatory disease characterised by elevated levels of serum IgG1 and IgE (Zheng et al., 2009). Furthermore, Treg cells up-regulated the TF *Tbx21* (T-bet) associated required for Th1 differentiation in response to IFN-γ stimulation (Koch et al., 2009). Consequently, *Tbx21*-deficient Treg cells failed to rescue Th1 cell-mediated inflammation in *scurfy* mice or limit type-1 responses during *Mycobacterium tuberculosis* infection (Koch et al., 2009). In addition, Treg cells were shown to upregulate T-bet and CXCR3 in response to IL-27 signalling, which was required for their migration to the intestine during infection with *Toxoplasma gondii* (Hall et al., 2012) as well as respond to T-cell derived IFN-γ and specifically suppress IFN-γ$^+$ Th1 cells in response to *Toxoplasma gondii* infection (Hall et al., 2012). In addition to responding to IFN-γ signalling, Foxp3$^+$ cells were shown to secrete IFN-γ in a model of graft versus host disease (GVHD) and during infection with *L. monocytogenes* (Koenecke et al., 2012) and *Ifng*$^{-/-}$ Treg cells were unable to prevent the development of GVHD (Koenecke et al., 2012). Similarly, Foxp3$^+$ Treg cells were shown to migrate to the LP and secrete IFN-γ in response to microbial stimuli and IL-12

signalling, leading to their suppression of intestinal inflammation (Feng et al., 2011). Lastly, Treg-specific STAT-3 deficiency resulted in a selective increase in pathogenic Th17 cells. *Stat3*$^{-/-}$ Treg cells were able to control T cell proliferation *in vitro* but unable to limit Th17 responses *in vivo,* resulting in the development of intestinal inflammation (Chaudhry et al., 2009). Taken together, these studies found that co-opting both TF and cytokine potential confers Treg cells with the specific ability to suppress T effector cells. In addition to expressing diverse TFs, Treg cells isolated from different infection settings expressed distinct miRNA profiles that can dictate their suppressor function. Indeed, miR-146a was shown to target STAT1 in Treg cells and was critical for Treg-mediated suppression of Th1 responses (Lu et al., 2010). In addition, IFN-γ-dependent miR-10a expression and IL-4-dependend miR-182a expression correlated with the ability of Tregs to suppress Th1 and Th2 responses in *L. major* and *S. mansoni* infections, respectively (Kelada et al., 2013). More recent studies have shown that bacterial metabolites could directly target Treg cells and maintain their suppressive potential in the small intestine (Murai et al., 2010; Arpaia et al., 2013) suggesting that immune and non-immune parameters are required to drive Treg specialisation.

Taken together, these studies suggest Treg populations are heterogeneous and can adapt to their local environment to enhance their suppressive specificity. Further research is required to better characterise the nature of local cytokines and tissue-derived molecules that can shape specialised Treg responses, in order to provide insight into directed Treg therapy. Preliminary evidence for cytokines and receptors involved in specialisation highlight the existence of control mechanisms ensuring the maintenance of the Treg phenotype in the context of a strong polarising environment, and inhibiting their conversion to T helper phenotypes.

1.3.8. Therapeutic potential of Treg cells

As discussed, understanding the mechanisms regulating stability and suppression in Treg cells is of considerable therapeutic interest. Indeed, many groups

are currently investigating the use of adoptively transferred Treg cells to control the development of T-cell mediated inflammatory diseases such as rheumatoid arthritis (Miyara et al., 2014), and indeed, clinical trials are showing positive results for the control of type-1 diabetes by adoptively transferred Treg cells (Marek-Trzonkowska et al., 2014). Patient Treg cells can either be expanded or differentiated from conventional $CD4^+$ T cells into Treg cells *in vitro* before their transfer (Miyara et al., 2014), and therefore determining the stability of Treg cells in this context is important. Alternatively, Treg cells can be expanded *in vivo* by with recombinant IL-2 treatment, which is known to stabilise *Foxp3* expression in the context of inflammatory cytokines (Feng et al., 2014). The complex of IL-2 and anti-IL-2 antibody (clone JES61-A12) (IL-2c) stabilises the half-life of IL-2 and targets IL-2 to cells expressing high levels of CD25 (Boyman et al., 2006) and IL-2c treatment was shown to dramatically increase the proportion of *Foxp3*-expressing cells in naïve mice (Boyman et al., 2006) as well as reduce allergic lung inflammation (Wilson et al., 2008), allograft rejection and severity of EAE (Webster et al., 2009). However recent evidence has shown that IL-2 complex can also dramatically expand ILC2s (Roediger et al., 2013; Oliphant et al., 2014), which suggests IL-2c treatment may have undesired effects on other CD25-expressing cells. Indeed, IL-2c treatment was shown to drive eosinophilia and increased allergic symptoms (Van Gool et al., 2014), as well as increase the permeability of the vasculature through its effect of CD25-expressing endothelial cells, leading to internal bleeding (Krieg et al., 2010). A stable fusion of both IL-2 and anti-IL-2 antibody, the 'superkine' was shown to promote similar effects at a much lower concentration, which could be a safer application (Levin et al., 2012). Indeed, low-dose IL-2 treatment is already approved in the clinic for the treatment of cancer, however the impact of IL-2 on the stability of Treg cells has not been well studied in this setting.

The stability of $Foxp3^+$ Treg cells is critical for the maintenance of immune homeostasis and survival of the host. Which is why since their discovery 20 years ago, diverse cellular and molecular mechanisms have been identified to regulate their development and function. In addition, these studies have highlighted the impact of

environmental signals such as cytokines on the stability of the cells, in particular in the context of inflammation and infection. Therefore it remains critically important to understand how different immune contexts can impact the stability of the Treg cell and impact the outcome of disease.

1.4. Innate lymphoid cell (ILC) development and function

As mentioned previously, ILCs differentiate from the CLP in the bone marrow, and differentiate into a diverse family of cells predominantly located in mucosal tissues such as the lung and small intestine. Both through their developmental requirements as well as their cytokine profile, ILCs largely mirror $CD4^+$ T helper cell (Th) counterparts. They are distinguished from Th cells by their expression of the haematopoietic marker CD45 and the thymocyte marker Thy1.2 (CD90) as well as through their lack of expression of lineage markers commonly expressed on lymphocytes such as somatically rearranged antigen receptors. In addition, they can be identified by the co-expression of the high affinity subunit of the IL-2 receptor IL-2Rα (CD25) and IL-7Rα (CD127). Furthermore, ILC2s have been shown to express the IL-33 receptor (ST2) to varying levels depending on the tissue (Roediger and Weninger, 2015). The ILC family include group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3) innate lymphoid cells (which include lymphoid tissue inducer (LTi) cells) and conventional natural killer cells (NK). As their surface markers suggest, ILCs develop independently of recombination activating genes (*Rag1* or *Rag2*), and there is currently no evidence that ILCs express any antigen or pattern recognition receptors such as TLRs (**Fig. 1.3**).

1.4.1. Development of ILCs

All ILCs including conventional NK (cNK) cells and LTi cells develop from a common innate lymphoid progenitor (CILP) expressing the TF *E4bp4* (NFIL3) (Geiger et al., 2014) and the cell-surface integrin α4β7. In addition, the TF TOX (thymocytes selection-associated high-mobility group box protein), directly regulated by NFIL3 (Yu

et al., 2014), was required for the differentiation of all ILC subsets from the CILP (Seehus et al., 2015). Further downstream, the expression of *Gata3* (Yagi et al., 2014) and high levels of *Id2* (inhibitor of DNA binding) (Moro et al., 2010; Klose et al., 2014) gives rise to group 1, 2 and 3 ILCs, excluding cNK cells, although the upregulation of *Id2* was required at later stages for the development of cNK cells (Diefenbach et al., 2014). Further expression of the transcription factor (TF) *Plzf* (promyelocytic leukaemia zinc finger protein) in $Id2^+$ precursors was required for the development of group 1-3 ILCs excluding group 3 LTi cells (Constantinides et al., 2014; Di Santo, 2014). *Notch* expression was required for the additional lineage specification of *Rorct*-expressing ILCs (Possot et al., 2011). Unlike conventional natural killer cells (cNK) cells, ILCs developed independently of IL-15 and of the low affinity receptor shared between IL-2 and IL-15 (Moro et al., 2010) however their development was dependent on the expression of the common gamma chain (γc) and IL-7Rα, suggesting that IL-7 plays a crucial role for the development and function of ILCs and that IL-4, IL-9 or IL-21 may also be involved (Moro et al., 2010; Satoh-Takayama et al., 2010; Hoyler et al., 2012). Indeed, recombinant IL-7 was shown to maintain ILC populations *in vitro* (Neill et al., 2010) and recombinant IL-2 promotes enhanced cytokine production by ILC2s *in vitro* (Moro et al., 2010) (**Fig. 1.3**).

Similar to their $CD4^+$ Th cell counterparts, the final stage of development of ILCs results in their functional specialisation mediated by cytokine signalling and the expression of specific transcription factors (Robinette et al., 2015). IL-23, IL1β and aryl hydrocarbon receptor (AHR) ligands were shown to stimulate $ROR\gamma t^+$ ILC3s leading to their production of IL-22, IL-17A and GM-CSF. Furthermore, ILC3s were shown to play an important role in the maintenance of intestinal homeostasis and lymphoid tissue development (Cornelissen et al., 2011; Sawa et al., 2011). IL-12, IL-15 and IL-18 were shown to stimulate T-bet^+ ILC1s leading to their production of IFN-γ and TNFα. ILC1s play an important role in the control of intracellular bacteria and protozoa. Interestingly, both *Gata3* (Serafini et al., 2014) and *Tbx21* expression were also required for the

development of a population of intestinal RORγT$^+$ ILC3s, distinguished by the surface expression of NKP46 and secretion of IL-22 (Sciume et al., 2012). ILC2s can be distinguished from *Rorct*-expressing ILC3s by their expression of retinoic acid receptor alpha (*Rora*), which is required for their development (Halim et al., 2012; Wong et al., 2012), as will be discussed later (**Fig. 1.3**).

Due to their localisation in mucosal surfaces, ILCs are in close proximity to the epithelium and to commensal microbiota and many studies describe a strong influence of gut-derived signals on the function of ILCs (Sonnenberg and Artis, 2012; Kamada and Nunez, 2013; Philip and Artis, 2013). Recently, bacteria-derived metabolites were shown to directly stimulate intestinal Foxp3$^+$ Treg cells, and therefore they may also play a role in stimulating TLR-deficient ILC3s (Arpaia et al., 2013; Furusawa et al., 2013). Furthermore, diet-derived micronutrients were shown to directly impact ILC function (Veldhoen and Brucklacher-Waldert, 2012). For example vitamin A signalling influenced the ratio of ILC subsets in the small intestine in favour of ILC2 development, enhancing resistance to *T. muris* helminth infection (Spencer et al., 2014). Additionally, mice deficient in the aryl hydrocarbon receptor (AHR) of which one of the ligands is highly abundant in cruciferous vegetables, were deficient in IL-22 producing ILC3s (Lee et al., 2012). Finally, the indirect diet supplementation via maternal intake of retinoic acid was shown to influence foetal development of LTi cells (van de Pavert et al., 2014). Taken together, these studies demonstrate a diversity of signals are required for ILC function and highlight the need to identify unique upstream regulators to promote ILC function.

1.4.2. Differentiation of ILC2s

ILC2s were first described as natural helper cells, nuocytes or innate helper type 2 cells and were only later given the uniform nomenclature of group 2 innate lymphoid cells (ILC2s) (Spits et al., 2013) following the characterisation of the broader group of innate lymphoid cells. They were originally identified in the mesenteric fat, the

lungs and the small intestine (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010) and have now been described to populate tissue-draining lymph nodes, the dermis and visceral adipose tissue (Artis and Spits, 2015). As a result of their diverse localisations, ILC2s are likely to be heterogeneous cells and their developmental requirements and effector functions may vary from organ to organ. Differentiated ILC2s express the IL-25 receptor (*Il17rb*) and IL-33 receptor (ST2/*Il1rl1*) as well as high levels of the high affinity IL-2 receptor chain CD25 (*Il2ra*) (Moro et al., 2010). Other cell-surface markers have been used to characterise ILC2s, however their functional impact on ILC2s is less clear. Sca-1 and KLRG1 expression correlate with GATA-3 expression in ILC2s and have been used as surface markers to distinguish ILC2s (Hoyler et al., 2012). In addition, ILC2s express MHC Class II (Neill et al., 2010) and are thought to have antigen-presentation functions (Oliphant et al., 2014). Both *Rora* expression and (Wong et al., 2012) the maintenance of high levels of *Gata3* expression (Klein Wolterink et al., 2013) are required for the development and lineage specification of ILC2s (Spits and Di Santo, 2011; Hoyler et al., 2012; Mjosberg et al., 2012). Additional transcription factors have recently been shown to be required for ILC2 development, including factors required for T lymphocyte development such as T cell factor 1 (TCF-1; *Tcf7*) (Yang et al., 2013) which promotes *Il17rb* and *Il1rl1* expression, B cell lymphoma 11b (*Bcl11b*) (Walker et al., 2015; Yu et al., 2015) and GRF-interacting factor 1 (Gfi1; *Gfi1*) which regulates *Il1rl1, Il2ra* and *Il7ra* whilst suppressing the expression of *Rorct* and *Il17a* (Spooner et al., 2013), although it remains unclear how these TFs interact during the development of ILC2s (**Fig. 1.3**).

ILC2s can be distinguished from other ILCs by their secretion of canonical type-2 cytokines IL-13 and IL-5 (Moro et al., 2010). Furthermore, ILC2s are major producers of IL-9 in the lung (Wilhelm et al., 2011) and were shown to contribute to tissue-repair following *N. brasiliensis* infection (Turner et al., 2013). In addition, both murine and human ILC2s were shown to secrete IL-4 following PMA and ionomycin (P+I) stimulation (Moro et al., 2010) or stimulation with IL-33, IL-2, TSLP (Mjosberg et al., 2012) and IL-33 and IL-25 (Xue et al., 2014). ILC2s were also shown to secrete IL-2

following P+I stimulation in mouse ILC2s (Molofsky et al., 2013) or TLR4 stimulation in human ILC2s (Mjosberg et al., 2012) however the secretion of IL-4 and IL-2 has generally received less attention. Recently in-depth transcriptomics were performed on ILC populations isolated from the spleen, liver and small intestine (SI). SI lamina propria ILC2s were shown to express high levels of *Il5, Il13* and *Il9* similarly to previous transcriptional arrays of ILC2s isolated from the lung (Motomura et al., 2014) as well as *Il4* and *Il2* (Robinette et al., 2015). Interestingly, both TCF-1 and Gfi1 promote the expression of *Gata3*, which is known to regulate *Il5* and *Il13* expression in Th2 cells (Zhu et al., 2004) suggesting TFs such as c-maf may be required for ILC2-derived IL-4 secretion, similarly to Th2 cells (Kim et al., 1999).

Although signalling via the IL-25 receptor (*Il17rb*$^{-/-}$) or IL-33 receptor (*Il1rl1*$^{-/-}$) (Neill et al., 2010) was not required for the development of ILC2s, epithelial-cell derived alarmins IL-25 and IL-33 appear to be important for stimulating ILC2 effector function. Indeed, treatment of ILC2s with combinations of IL-25, IL-33 (Neill et al., 2010) and TSLP (Mjosberg et al., 2011; Kim et al., 2013; Molofsky et al., 2015) induced IL-5 and IL-13 secretion by ILC2s *in vitro.* In addition, IL-25 (Fallon et al., 2006) and IL-33 (Price et al., 2010) treatment *in vivo* promotes the proliferation of ILC2s and increased secretion of IL-5 and IL-13. Furthermore, IL-2 was shown to enhance cytokine production by ILC2s *in vitro* in the presence of IL-25 and IL-33 (Moro et al., 2010). In particular, IL-2 was required for optimal IL-9 secretion by ILC2s *in vitro* and *in vivo* (Wilhelm et al., 2011). Neither the low affinity IL-2 receptor (*Il2/Il15rb*$^{-/-}$) IL-4R (*Il4ra*$^{-/-}$) (Fort et al., 2001), *Il4*$^{-/-}$ (Kim et al., 2014) or *Il9r*$^{-/-}$ mice (Turner et al., 2013) were required for the development or function of ILC2s and for their expansion following IL-25 treatment (Fallon et al., 2006). Instead, these cytokines are increasingly thought to promote the effector function of ILC2s. Indeed, *Il9r*-deficient ILC2s secreted lower levels of IL-5 and IL-13 in the lung during *N. brasiliensis* infection (Turner et al., 2013) and culturing ILC2s with IL-9 *in vitro* led to increased cytokine production, suggesting autocrine IL-9 production maintains ILC2 function (Turner et al., 2013). In addition, basophil-derived IL-4 was shown to promote the effector function and proliferation of

ILC2s in the lungs (Kim et al., 2014) and skin (Kim et al., 2014; Motomura et al., 2014). Many more cytokines and growth factors are likely to play a role. For example the TNF superfamily member, TL1A, which binds to its receptor DR3 on ILC2s was shown to promote their effector function, proliferation and survival (Yu et al., 2014). Combined, these studies suggest cytokines play a major role for the development and function of ILC2s. Because most of these signals impact ILC2 populations in the lung and small intestine, identifying signals that are tissue-specific would enhance the design of immunotherapies targeting ILCs.

1.4.3. Effector function of ILC2s

Although the tools to specifically delete ILC2 cells are limited, a combination of genetic and cytokine/antibody treatments have been used to test the function of ILC2s *in vivo* in the context of allergic inflammation and anti-helminth immunity. Increasing evidence shows ILC2s play a key role in the initiation of immune responses to intestinal helminths. To date, the majority of studies have investigated the role of ILC2s in response to the mouse-adapted helminth *N. brasiliensis,* which has been extremely valuable for understanding the effector function of ILC2s. *Il25*$^{-/-}$ mice, deficient in ILC2s (then identified as IL-5, IL-13 and IL-4 producing non-B, non-T, c-kit^{+}, FCεR1^{-} cells) were impaired in their ability to expel *N. brasiliensis,* which is normally cleared from WT mice within 12 days (Fallon et al., 2006). In a separate approach, recombinant IL-25 treatment increased numbers of ILC2s correlating with a rapid clearance of *N. brasiliensis* in susceptible *Rag1*$^{-/-}$ mice (Price et al., 2010) but not in mice deficient in type-2 cytokines (*Il4*$^{-/-}$*Il5*$^{-/-}$*Il13*$^{-/-}$*Il9*$^{-/-}$) (Fallon et al., 2006) suggesting ILC2 production of type-2 cytokines was sufficient to mediate *N. brasiliensis* expulsion. Additionally, recombinant IL-25 treatment led to enhanced eosinophilia and serum IgG1 and IgE in WT but not *Il4ra*$^{-/-}$ mice (Fort et al., 2001), suggesting IL-13 was particularly important for driving effector responses downstream of ILC2s. Taken together these early experiments concluded that an innate, IL-25-responsive and *Rag*-dependent cell

produced type-2 cytokines and was sufficient to drive anti-helminth immunity (Fallon et al., 2006; Koyasu et al., 2010; Neill and McKenzie, 2011).

In addition to their role in promoting anti-helminth immunity, ILC2s have been shown to be major players in the development of allergic lung inflammation in multiple laboratory models (Halim et al., 2012; Pishdadian et al., 2012; Salmond et al., 2012; Scanlon and McKenzie, 2012) however currently this has not been shown in the context of asthma. Conversely, ILC2s have been implicated in mediating lung damage repair following viral or helminth infections, through their secretion of amphiregulin and IL-9, respectively (Monticelli et al., 2011; Turner et al., 2013). In addition, recent transcriptional profiling revealed ILC2s express many genes involved in lipid metabolism (Robinette et al., 2015). Indeed, ILC2s were shown to regulate tissue metabolism through their cooperation with eosinophils, resulting in the beiging of white adipose tissue (Brestoff et al., 2015; Lee et al., 2015). Furthermore, ILC2s are increasingly thought to cooperate with other innate and adaptive cells of the immune system, including AAMΦ and eosinophils (Molofsky et al., 2013; Nussbaum et al., 2013), γδ $CD4^+$ T cells and neutrophils (Van Dyken et al., 2014), and most recently Treg cells in the VAT (Molofsky et al., 2015).

1.4.4. ILC2-T cell crosstalk

Although some studies suggest ILC2s are sufficient to drive anti-helminth immunity, increasing evidence is pointing towards the cooperation between ILC2s and other immune cells for the development of optimal type-2 responses (**Fig. 1.3**). For example, ILC2-derived IL-5 was shown to maintain eosinophil populations in the adipose tissue (Molofsky et al., 2013). Furthermore, ILC2s were recently shown to promote the accumulation of Treg cells in the VAT through ICOSL-ICOS interactions (Molofsky et al., 2015). Similarly, ILC2s were an important source of IL-13 for the activation of alternatively activated macrophages in the adipose tissue (Nussbaum et al., 2013). Recent studies have focused on the co-operation between ILC2s and T

cells. Indeed, early experiments showed the adoptive transfer of WT and *Il13*$^{-/-}$ ILC2s to IL-25R-deficient mice infected with *N. brasiliensis* restored the ability of T cells to make IL-13 (Neill et al., 2010) suggesting ILC2s contribute to T cell differentiation in an IL-13-independent manner. The identification of ILC2-deficient mice has provided more definitive evidence of compromised immune responses in ILC2 deficiency. Indeed, ROR$\alpha^{sg/sg}$ mice were shown to be specifically deficient in ILC2s (Wong et al., 2012) and had dramatically reduced immune infiltrate in the bronchioalveolar spaces following papain challenge (Halim et al., 2012) suggesting type-2 responses were impaired in the absence of ILC2s. Following these observations, several studies have now shown that Th2 differentiation is impaired in ROR$\alpha^{sg/sg}$ mice, correlating with reduced type-2 inflammation in the skin and lungs after allergen challenge (Drake et al., 2014; Gold et al., 2014; Halim et al., 2014; Liu et al., 2015) and not due to a T cell intrinsic role for RORα (Yang et al., 2008; Gold et al., 2014). One proposed mechanism is that ILC2-derived IL-13 was required for the migration of DCs to draining lymph nodes for efficient T cell priming (Halim et al., 2014). To date, these observations have been mostly limited to models of allergic inflammation. However a recent study found Th2 differentiation was impaired in ROR$\alpha^{sg/sg}$ mice infected with *N. brasiliensis,* resulting in the delayed expulsion of the parasite (Oliphant et al., 2014), suggesting that although IL-25-expanded ILC2 cells are sufficient to promote immunity to *N. brasiliensis,* optimal immune responses require ILC2-T cell co-operation. Mechanistically, ILC2s were shown to stimulate T cell proliferation *in vitro* in an MHC Class II-dependent and contact-dependent manner (Mirchandani et al., 2014). Furthermore, transferred WT but not MHC Class II deficient ILC2s could promote expulsion of *N. brasiliensis* in *Il13*$^{-/-}$ mice (Oliphant et al., 2014). IL-2 is another essential cytokine for the development of Th2 cells (Cote-Sierra et al., 2004) and human ILC2s isolated form nasal polyps were shown to produce IL-2 in response to TLR stimulation (Crellin et al., 2010). Furthermore, a recent study suggests ILC3-derived IL-2 may support ILC2 function in the context of eosinophilic crystalline

pneumonia (ECP) (Roediger et al., 2015). Although the role of ILC2-derived IL-2 remains unclear, it is possible that it could act in a paracrine or autocrine manner to enhance the proliferation of cells. Together, these studies suggest that ILC2s may play a role in the priming and differentiation of Th2 cells, however a direct role of ILC2-derived cytokines in this process remains unclear.

Conversely, T cells may promote ILC2 function. Indeed, ILC2s numbers negatively correlated with the presence T and B cells when comparing WT and *Rag2*$^{-/-}$ mice (Wilhelm et al., 2011). In addition, ILC2 numbers in the MLN of *N. brasiliensis* infected mice were not maintained in *Rag2*$^{-/-}$ mice (Neill et al., 2010). In light of the *in vitro* data showing IL-2 promotes cytokine production (as discussed above), T-cell derived IL-2 was hypothesised to promote ILC2 function (Wilhelm and Stockinger, 2011). Although traditionally used for the expansion of CD25-expressing Treg cells, IL-2c treatment was shown to dramatically expand numbers of ILC2s in the mesenteric lymph nodes of *Rag*$^{-/-}$ mice, resulting in the expulsion of *N. brasiliensis* (Oliphant et al., 2014). Additionally, IL-2c expanded dermal ILC2s increased their secretion of IL-5, and IL-13, leading to increased skin inflammation and eosinophilia (Roediger et al., 2013). *In vivo* treatment of *Rag1*$^{-/-}$ mice with recombinant IL-2 restored lung IL-9 concentrations to normal levels (Wilhelm et al., 2011). Furthermore, blocking IL-2 following papain challenge reduced IL-9 concentrations in lung homogenates but not IL-4, IL-5 and IL-13, suggesting T-cell derived IL-2 may be required for the production of specific cytokines such as IL-9 (Wilhelm et al., 2011). Indeed, ILC-derived IL-9 was later shown to mediate lung damage repair following *N. brasiliensis* infection (Turner et al., 2013). These two studies suggest T cell derived IL-2 may play a specific role in driving IL-9 in the context of immune inflammation and damage, rather than in the initiation of immune responses. Given the similarity between ILC2s and their Th2 counterparts, more work is required to understand their relative contribution to pathogenic and protective type-2 immune responses such as anti-helminth immunity.

1.5. Immunity to helminth parasites

Type-2 immunity is a specialised immune response whose initiation is largely dependent on $CD4^+$ T cells secreting the cytokine interleukin-4 (IL-4) and driving a multi-cellular cascade of events combining immune and structural cells. Type-2 immune responses are evolutionarily ancient and are important both for protective responses to chronic infections with multi-cellular helminths and for the regulation of tissue repair following damage caused by invading helminths (Gause et al., 2013; Allen and Sutherland, 2014). Like all immune responses, the type-2 immune cascade is tightly regulated. Excessive type-2 responses can lead to inflammation as seen in the lungs of allergic individuals following sensitisation to allergens or in the liver of individuals chronically infected with the nematode *Schistosoma mansoni (S. mansoni)*.

1.5.1. Soil-transmitted helminths

Helminth infections form a large part of the 17 neglected tropical diseases (NTDs) and remain a huge global burden, with devastating economic and health impacts due to the infection of both animals and humans. The World Health Organisation (WHO) estimated that around 880 million pre-school or school-aged children from low socioeconomic backgrounds in tropical and sub-tropical countries were infected by one or more soil-transmitted helminths (STH) in 2013 (WHO, 2015). The most prevalent human species are the roundworm (*Ascaris lumbricoides*), the hookworms (*Necator americanus, Ancyslostoma duodenale*) and the whipworm (*Trichuris trichiura*). The morbidity associated with infection is directly correlated with the high burden and recurrence of infection due to limited access to treatment and poor hygiene. Hookworm infection alone caused a global loss of 3.2 million disability-adjusted life years (DALYs) in 2010 (Hotez et al., 2014). Hookworms feed on the intestinal mucosa, submucosa and blood, causing abdominal pain, blood and protein loss and growth retardation due to compromised nutrient absorption (Hotez et al., 2004). These can result in severe long-term consequences such as stunted growth and

impaired intellectual development (Hotez et al., 2004). Although antihelminthic treatment is relatively inexpensive, widely available and effective, only 35% of children in need of treatment received deworming treatment in 2013 (WHO, 2015). Vaccination is arguably the best solution to eradicate helminthiasis, however the mechanisms of natural or vaccine-associated immunity to human hookworm are relatively unclear and therefore major efforts are being made to understand how best to heighten protective responses. Combined progress in genomics, proteomics and transcriptomics are contributing to significant advances for the identification of helminth-derived molecules as both vaccine candidates and for use as novel anti-inflammatories. Recent sequencing of a wide array of human and lab-strains of nematode species (WormBase) including *N. Americanus* (Tang et al., 2014) will provide new insight into similarities between species and guide therapeutic strategies. In addition, proteomic studies are profiling the highly immunogenic helminth excretory/secretory proteins, or "secretome", that contain immunodulatory proteins involved in invasion (such as proteases) and evasion (Loukas et al., 2011). Phase 1 clinical trials are underway for a human hookworm vaccine directed against two adult *N. americanus* antigens thought to be involved in degradation of host hemaglobin, including the secreted Glutathione S-transferase Na-GST-1 (Hotez et al., 2013). Many lab-adapted strains of parasites exist for modelling helminth infections in mice, including the hookworm *Nippostrongylus brasiliensis,* which is a parasite of rats adapted for use in mice, the gastro-intestinal whipworm *Trichuris muris*, and the roundworm *Heligmosomoides polygyrus*.

1.5.2. *Heligmosomoides polygyrus:* a model of intestinal helminth infection

In contrast to other laboratory-adapted parasite models, the nematode *Heligmosomoides polygyrus* is a natural murine intestinal parasite which is commonly used in the laboratory to model chronic intestinal human hookworm infection in mice. The lifecycle of *H. polygyrus* is strictly enteric, making it a suitable model for studying the natural development of intestinal immune responses. Free-living L3 larval stages

are orally ingested, migrate to the small intestine and invade the submucosa and muscularis externa within 24 hours. There they undergo 2 developmental moults and emerge into the lumen as L5 larvae 8-10 days post-infection where they attach to the villi of the intestine and mature into adults. From approximately day 14 post-infection, sexually mature male and female worms mate and release eggs into the faeces that are consequently released into the environment. In favourable conditions, L1 larvae hatch from the eggs and develop into L2 larvae, then free-living L3 larvae. A new host will ingest L3 larvae in contaminated food or water, perpetuating the lifecycle (Johnston et al., 2015). Laboratory strains of mice have differing susceptibilities to the infection correlating with the strength of a type-2 immune response (Filbey et al., 2014). C57BL/6 mice are intermediate responders and infection can last 8-20 weeks. When a primary infection with *H. polygyrus* is cleared with a pyrantel-based anthelminthic drug, C57BL/6 mice develop highly protective immunity to secondary infections resulting in larval killing in the intestinal wall (Reynolds et al., 2012). *H. polygyrus* excretory/secretory (HES) products have been extensively characterised and most recently shown to contain both immunomodulatory proteins and vesicle-associated miRNAs thought to support parasite invasion (Buck et al., 2014). Characterisation of various lifecycle stages has revealed that larval and adult stages have distinct secretomes containing highly immunogenic components. Immunisation with both L4-derived HES and adult-derived HES was able to confer sterile immunity to re-infection with *H. polygyrus* (Hewitson et al., 2013). Furthermore, in-depth proteomics revealed that both HES fractions contained large amounts of acetylcholinesterase proteins, including ACE-1, which was strongly recognised by antibodies in the immunised animals, suggesting they may constitute promising vaccine candidates (Hewitson et al., 2013). Thus, the natural murine helminth *Helimgosomoides polygyrus,* which establishes chronic infections in inbred mice similar to its human counterpart, provides an appropriate experimental system to study co-evolved host-pathogen interactions (**Fig. 1.4**).

1.5.3. Type-2 immune responses to intestinal helminths

1.5.3.1. Intestinal epithelial cell secretion of alarmins following helminth infections

The gut-associated lymphoid tissue includes the Peyer's patches, lamina propria lymphocytes and intra-epithelial lymphocytes. Intestinal epithelial cells form the largest environmentally exposed surface in the body, composed of specialised cells providing both physical and anti-microbial defence mechanisms (Peterson and Artis, 2014). Intestinal epithelial cells are colonised with a large number of commensal microbes and have evolved to secrete a plethora of anti-microbial peptides such as defensins, C-type lectins and RNAses. This confers a natural chemical barrier limiting the interaction of pathogenic bacteria with epithelial cells (Gallo and Hooper, 2012). At steady state, tissue-resident dendritic cells extend trans-epithelial dendrites to sense luminal antigens and establish a tolerant relationship between commensal microbes and the immune system (Chieppa et al., 2006) through promoting the development and maturation of innate immune cells residing in the intestine (Hooper et al., 2012). However pathogens have evolved to disrupt barrier functions of the epithelium and can penetrate the epithelial barrier through direct receptor binding or by causing damage to epithelial cells, rendering them permissive to infection. Upon infection with an intestinal nematode, damaged intestinal epithelial cells secrete alarmins including the cytokines IL-33, IL-25 and TSLP that act on both innate and adaptive immune cells and are required for the initiation of type-2 immune responses (Saenz et al., 2008). As previously described, *Il25*$^{-/-}$ mice fail to expel *N. brasiliensis* due to the reduced activation of ILC2s (Fallon et al., 2006), and recombinant IL-25 treatment promoted type-2 immune responses and functional immunity to *Trichuris muris* (Owyang et al., 2006). In addition, IL-25 was shown to have a partial role in the development of type-2 responses to primary infection with *H. polygyrus* (Zaiss et al., 2013) and *Il33*$^{-/-}$ mice were susceptible to primary and secondary infection with *N. brasiliensis* (Hung et al., 2013). Furthermore, *Tslpr*$^{-/-}$ mice were unable to clear an infection with *T. muris*

(Siracusa et al., 2011) however functional immune responses to *H. polygyrus and N. brasiliensis* were not impaired (Massacand et al., 2009), suggesting their requirement may vary depending on the infection. Furthermore, epithelial-cell derived trefoil factor 2 (Tff2) promotes IL-33 production by lung epithelial cells following *N. brasiliensis* infection and consequently lung epithelial cells from *Tff2*$^{-/-}$ mice expressed lower levels of *Il33* resulting in impaired Th2 responses in the lung and delayed expulsion of *N.* brasiliensis (Wills-Karp et al., 2012). Additionally, epithelial-cell derived Act1 was shown to regulate IL-25 secretion by intestinal epithelial cells and consequently *K18*-Cre*Act1*$^{fl/fl}$ mice with a specific deletion in epithelial *Act1* had impaired Th2 responses in the mesenteric lymph nodes following *N. brasiliensis* infection and following IL-25 treatment, resulting in compromised worm expulsion (Kang et al., 2012). Taken together, these studies highlight the essential role of the intestinal epithelium for the initiation of type-2 immune responses following helminth infection.

1.5.3.2. Innate type-2 responses to helminths

Following infections with intestinal helminths, intestinal epithelial cell-derived IL-25, IL-33 and TSLP act on tissue-resident innate cells to enhance their function and promote type-2 responses, for example for the activation of ILC2s, as previously described (Saenz et al., 2013). In addition, DCs cultured *in vitro* with IL-25 and TSLP upregulated OX40L, which resulted in their ability to drive more proficient Th2 differentiation (Ito et al., 2005). Furthermore, IL-33 was shown to induce IL-13 production by bone marrow derived macrophages (BMDMs) and adoptive transfer of IL-33-activated BMDMs resulted in the expulsion of *H. polygyrus* following a primary infection (Yang et al., 2013). Innate cells themselves were shown to regulate IL-25 and IL-33 secretion and influence the development of the type 2 responses. For example, mast cells could promote IL-25, IL-33 and TSLP production in the small intestine, and consequently, mast-cell deficient mice had impaired Th2 responses to *H. polygyrus* (Hepworth et al., 2012). Conversely, the secretion of IL-1β from intestinal epithelial

cells and macrophages following *H. polygyrus* infection inhibited IL-25 and IL-33 secretion in the small intestine, resulting in decreased ILC2 activation (Zaiss et al., 2013). As a consequence *Il1b*$^{-/-}$ mice exhibited increased immunity to primary infection (Zaiss et al., 2013). Combined, these studies suggest IL-25 and IL-33 are crucial for activating cells of the innate immune system following *H. polygyrus* infection.

As described previously, innate phagocytic cells such as neutrophils and macrophages are generally associated with the control of intracellular pathogens such as bacteria and viruses. Due to their macroscopic size, helminth parasites evade phagocytic mechanisms and the precise role for neutrophils and macrophages in anti-helminth defence is unclear. However in the context of a helminth infection, monocytes get recruited to the tissue and differentiate towards AAMΦ in response to IL-4 and IL-13 (Kreider et al., 2007). Furthermore, IL-4 stimulated the proliferation of tissue-resident macrophages in the pleural cavity of mice infected with the helminth *Litosomoides sigmondontis* (Jenkins et al., 2013). Although Th2 cells were always described as an important source of IL-4 and IL-13 for AAMΦ activation, ILC2s are also major producers of these cytokines and therefore it is likely they play a major role in driving AAMΦ during a helminth infection given their tissue localisation. AAMΦ produce characteristic molecules such as arginase-1, resistin-like molecule alpha (Fizz1/*Relma*) and chitinase-like protein 3 (Ym1/*Chil3)* associated with functions such as wound healing and immune regulation (Allen and Sutherland, 2014). In addition, it is thought that AAMΦ contribute to the regulation of excessive type-2 responses by limiting the expansion of Th2 cells through the depletion of arginine sources (Pesce et al., 2009). Although AAMΦ play an important regulatory role, they were also shown to promote the larval killing of *H. polygyrus* following secondary challenge, however the mechanisms remain unclear (Anthony et al., 2006). There are many examples of neutrophils accumulating in the tissue surrounding helminths in the context of challenge infection. For example, neutrophils were rapidly recruited around the encysted larvae following *H. polygyrus* challenge infection (Morimoto et al., 2004). Furthermore, neutrophils accumulated in the lung tissue following secondary challenge

with *N. brasiliensis* and were shown to secrete IL-13 leading to the activation of AAMΦs (Chen et al., 2014). Consequently, depletion of neutrophils in a secondary but not primary inoculation with *N. brasiliensis* resulted in impaired AAMΦ differentiation and accumulation of the parasite in the lung (Chen et al., 2014). In addition, there is emerging evidence for novel roles for neutrophils and macrophages in the context of a type-2 response (Allen et al., 2015). Indeed, early recruitment of neutrophils to the lungs following *N. brasiliensis* infection was shown to be dependent on chitinase-like protein *Chil3* (Ym1) secreted by macrophages (Sutherland et al., 2014). Macrophage-dependent recruitment of neutrophils was shown to promote tissue damage and as a consequence, limit the establishment of the parasite (Sutherland et al., 2014). Taken together, this data suggests there is a strong cooperation between neutrophils and macrophages early in infection with intestinal helminths and that ILC2s may be an important source of type-2 cytokines for the development of early type-2 responses in the tissue.

1.5.3.3. Initiation of the adaptive immune response

As the evidence above suggests, innate cells can be sufficient to drive proficient type-2 responses to helminths independently of the adaptive immune system. However Th2 cells are fundamentally required for the amplification of immune responses to some intestinal helminths, such as *H. polygyrus* (Urban et al., 1991). For example, early type-2 responses to *H. polygyrus* including the differentiation of AAMΦ, occurs in the absence of $CD11c^+$ DCs, suggesting adaptive immune responses were not required (Smith et al., 2012). However the development of IL-4 and IL-13-producing $CD4^+$ Th2 cells, which are required for the functional expulsion of *H. polygyrus* (Urban et al., 1991) was severely compromised in the absence of $CD11c^+$ DCs (Smith et al., 2011). DCs play a crucial role for both the priming and maintenance of Th2 cells *in vivo* (Hammad et al., 2010; Phythian-Adams et al., 2010; Leon et al., 2012; Plantinga et al., 2013; Hussaarts et al., 2014). Both pathogen derived and epithelial cell-derived

molecules are thought to stimulate DC function in the tissue before they migrate to the local lymph node to prime the differentiation of Th2 cells. Unlike our understanding of DC recognition of specific bacterial and viral ligands, a role for TLR recognition of helminth-derived antigens is less clear. The *H. polygyrus* secretome contains glycoproteins and lipids that are likely to be recognised by TLRs (Hewitson et al., 2009). Indeed, the trematode *Schistosoma mansoni* secretome contains a phosphorylcholine (*S. mansoni* ES-62) that stimulated TLR4 signalling on DCs and impaired their ability to make IL-12p40 in response to LPS stimulation (Goodridge et al., 2004). Furthermore, immunity to *H. polygyrus* was shown to require MyD88 adaptor protein, part of the signalling pathway downstream of TLRs. Surprisingly, neither *Tlr2, Tlr4, Tlr5 or Tlr9*-deficient mice phenocopied the *Myd88*$^{-/-}$ mouse (Reynolds et al., 2014) suggesting that TLRs may signal redundantly to maintain *H. polygyrus* susceptibility (Reynolds et al., 2014). Other pattern recognition receptors are thought to recognise helminth-derived molecules, such as C-type lectin receptors (CLR) that were shown to recognise *S. mansoni* egg antigen (reviewed in (Everts et al., 2010)). Specific populations of DCs have been described to prime the development of Th2 cells in the lung and skin. CD103^{+} DCs and not CD11b^{high} DCs purified from cockroach or house dust mite challenged mice promoted the differentiation of Th2 cells *in vitro,* and deletion of CD103^{+} DCs using *Ccr7*$^{-/-}$ mice resulted in impaired Th2 differentiation (Nakano et al., 2012). Furthermore, two independent studies identified CD301b^{+} (*Mgl2*) DCs as important drivers of Type-2 immunity in the skin and lung respectively (Gao et al., 2013; Kumamoto et al., 2013). CD301b^{+} cells were shown to be *Irf4*-dependent and express PDL2, a marker associated with the regulation of Th2 differentiation. Deletion of CD301b^{+} DCs using conditional knockout mice (*Cd11c*^{cre}*Irf4*$^{fl/fl}$) or following the inducible deletion of *Mgl2* upon diphtheria toxin treatment (Mgl2DTR mice) resulted in a reduced proportion of Th2 cells in the lung draining lymph nodes of mice in response to both lung allergen challenge as well as *N. brasiliensis* infection (Gao et al., 2013; Kumamoto et al., 2013). Furthermore, the differentiation of Th2 cells during *H. polygyrus* infection was dependent on B-cell stimulated CXCL13 production, which

regulated the co-localisation of T cells with a population of $CXCR5^+$ DCs in B cells zones. This interaction was essential for the differentiation of Th2 cells (Leon et al., 2012). Taken together, this data suggests that DCs respond to helminth antigens to promote the differentiation of Th2 cells *in vivo*. Furthermore, this data highlights the essential role of IL-4 for Th2 differentiation *in vivo* following *H. polygyrus* infection and for the functional expulsion of *H. polygyrus* (Urban et al., 1991). However the source of IL-4 required for Th2 differentiation *in vivo* remains unclear.

1.5.3.4. Sources of IL-4 for the differentiation of Th2 *in vivo*

The study of bicistronic 4get (*Il4*-GFP) cytokine reporter mice has been invaluable for the characterisation of the cellular sources and regulation of *Il4* expression *in vivo* (Croxford and Buch, 2011). *Il4*-GFP mice were developed by targeting an IRES-eGFP (internal ribosome entry site-enhanced green fluorescent protein) construct to the 3'-UTR of the endogenous *Il4* locus resulting in the transcription of *Il4*-IRES-eGFP and translation of IL-4 and eGFP from the same locus (Mohrs et al., 2001). Furthermore, comparisons of *Il4*-GFP expression with *Il4* mRNA expression confirmed the knockin hadn't disrupted IL-4 expression and that IL-4 function was intact (Mohrs et al., 2001). In order to determine whether *Il4*-GFP expression was faithful to IL-4 protein secretion, *Il4*-GFP mice were crossed to KN2 mice, where exons 1 and 2 of the *Il4* locus contain a huCD2-encoding sequence (Mohrs et al., 2005). Analysis of these mice revealed that huCD2 expression on the surface of $CD4^+$ T cells faithfully reported IL-4 protein secretion rather than *Il4* expression, particularly in the MLN, Peyer's patches and lamina propria of mice infected with *H. polygyrus* (Mohrs et al., 2005). Following *H. polygyrus* infection, *Il4*-GFP^+ cells were systemically dispersed throughout lymphoid and non-lymphoid tissues, with the highest proportions in gut-draining lymph nodes (Mohrs et al., 2005). In addition, these mice revealed that a proportion of the *Il4*-GFP+ cells in draining lymph nodes during *H. polygyrus* infection and in SEA immunised mice co-localised to

the B cell follicles and expressed CXCR5 and PD-1, characteristic of Tfh cells (Glatman Zaretsky et al., 2009; King and Mohrs, 2009). Furthermore, IL-4-secreting Tfh cells were shown to contribute to the generation of mature, high-affinity IgG1 antibodies during *N. brasiliensis* infection (Reinhardt et al., 2009). This data suggested that many cell types have the capacity to secrete IL-4. Given that IL-4 is required for Th2 polarisation *in vitro* (as discussed), these results prompted a continuing debate on the cellular source of IL-4 required for the differentiation of Th2 cells *in vivo*. Indeed, several innate cells were shown to be capable of producing IL-4, such as mast cells, eosinophils and NK T cells (Voehringer et al., 2004; Gessner et al., 2005; Mohrs et al., 2005). TCR activation of NK T cells *in vitro* stimulated IL-4 production, which was maintained *in vivo* in the absence of IL4Rα signalling (Noben-Trauth et al., 1997) however the function of NK T cell-derived IL-4 is unclear. Several studies have described basophils as a dominant source of IL-4 (Min et al., 2004; Voehringer et al., 2006; van Panhuys et al., 2011) that can process and present antigen to $CD4^+$ T cells for their priming and differentiation into Th2 cells during allergic inflammation and helminth infections (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). However, experiments in basophil-deficient mice (*Mcpt8*$^{-/-}$ mast cell protease 8 deficient mice), basophil antibody-depleted mice (Phythian-Adams et al., 2010; Smith et al., 2012) and mice with a basophil-specific deletion in IL-4 revealed that basophils had a minimal role for the development of Th2 cells *in vivo* (Ohnmacht et al., 2010; Sullivan et al., 2011). In the context of *H. polygyrus* infection, basophils and eosinophils were shown to express and secrete IL-4 (Mohrs et al., 2005), however Th2 differentiation was not impaired in basophil-deficient mice, basophil-depleted mice or mice given anti-IL-5 to deplete eosinophils (Herbst et al., 2012; Smith et al., 2012; Schwartz et al., 2014). Instead, $CD4^+$ T cells were shown to stimulate IL-4 production by basophils *in vitro* (Sullivan et al., 2011) and *in vivo* (van Panhuys et al., 2011) following *N. brasiliensis* infection. Although dispensable for the priming of Th2 responses, basophils were shown to contribute to chronic type-2 responses such as allergic dermatitis, as well as proficient immunity to secondary infection with *N.*

brasiliensis (Ohnmacht et al., 2010) and *H. polygyrus* (Herbst et al., 2012), suggesting that they serve important functions other than their secretion IL-4. Taken together, this data suggests basophils play important roles for the development of chronic type-2 immune responses to *H. polygyrus*, but are redundant for the early differentiation of Th2 cells during *H. polygyrus* infection.

Both murine and human ILC2s have been shown to transcribe *Il4* (Fallon et al., 2006; Price et al., 2010; Molofsky et al., 2013; Robinette et al., 2015) and secrete IL-4 *in vitro* (Mjosberg et al., 2012; Doherty et al., 2013; Drake et al., 2014; Mirchandani et al., 2014). *In vivo*, increases in *Il4*-GFP^{+} ILC2s in the lung of *N. brasiliensis* infected mice preceded the major expansion of IL-4 producing CD4+ T cells suggesting they could provide the IL-4 required for Th2 differentiation (Fallon et al., 2006). However IL-4 was undetectable by ELISPOT assay following the re-stimulation of *Il4*-GFP-expressing ILC2s isolated from the MLN of *N. brasiliensis* infected mice (Fallon et al., 2006). Furthermore, following the infection of KN2 mice with *N. brasiliensis, Il4*-GFP^{+} lineage negative cells in the spleen, mediastinal lymph nodes and lung were negative for KN2 suggesting they were not secreting IL-4 *in vivo* (Price et al., 2010). Similarly, a proportion of ILC2s in the VAT were *Il4*-GFP^{+}KN2^{-} (Molofsky et al., 2013) suggesting ILC2s are poised for *Il4* secretion but may not secrete IL-4 *in vivo*. Early studies characterising the KN2 mice show that global *Il4*-GFP expression induced following *H. polygyrus* infection correlated the highest with KN2 expression in the gut associated lymphoid tissue (GALT) (MLN, PP, IEL and LP) highlighting the importance of looking at site-specific expression of cytokines in the context of different infections (Mohrs et al., 2005). IL-4 production by intestinal ILC2s has not yet been reported.

Although the signals required for IL-5 and IL-13 secretion by ILC2s are well characterised (as discussed above), the signals driving IL-4 secretion *in vivo* remain unclear. Basophil-derived IL-4 was shown to support ILC2 activation and proliferation in the skin (Kim et al., 2014) and ILC2 cytokine production in allergic lungs (Motomura et al., 2014) however it remains unclear whether basophil-derived IL-4 is required for ILC2-derived IL-4. Interestingly two members of the arachidonic pathway, PGD2 and

LTD_4, known to directly enhance human Th2 cytokine production (Xue et al., 2012), stimulated increased IL-4 secretion by human (Xue et al., 2014) and mouse ILC2s (Doherty et al., 2013) respectively. Furthermore, human ILC2s isolated from nasal polyps were shown to produce IL-4 in response to TSLP and IL-33 (Mjosberg et al., 2012). Still, the functional role of ILC2-derived IL-4 remains unclear. *In vitro,* one study reported reduced IL-5 and IL-13 measured in the supernatants following the co-culture of T cells with *Il4*$^{-/-}$ ILC2s compared to WT ILC2s (Drake et al., 2014) suggesting either ILC2-derived IL-4 was required for their own secretion of IL-5 and IL-13 or for T cells to secrete IL-5 and IL-13. Furthermore, blocking IL-4 *in vitro* reduced the total numbers of IL-13^{+} ILC2s and IL-13^{+} T cells, suggesting that either T cell or ILC2 derived IL-4 was required for the maintenance or activation of ILC2 cells (Oliphant et al., 2014). *In vivo,* one study showed that IL-4 was not required for the differentiation of Th2 cells in response to papain challenge in the lung, and concluded ILC2-derived IL-4 was not significant, however the role of IL-4 has not been tested in the context of an IL-4 dependent model (Halim et al., 2014).

Taken together, these studies suggest that the importance of any of these sources of IL-4 is most likely to depend on the tissue, and location of the infection, and highlight a gap in our knowledge on the function of ILC2-derived IL-4.

1.5.3.5. Type 2 effector responses downstream of type-2 cytokines

Following the initiation of the adaptive immune response to infection, Th2-derived IL-4, IL-13 and IL-5 promote a positive feedback loop by stimulating increased activation of the early intestinal immune and non-immune effector cells. For example, IL-4 and IL-13 serve to further amplify the alternative activation of macrophages, for example during memory responses to *H. polygyrus* when AAMΦ infiltrate the granuloma forming around the larvae resulting in larval trapping and killing (Anthony et al., 2006). Consequently, clodronate-dependent deletion of phagocytes (including macrophages) was shown to reduce immunity to *H. polygyrus* in the genetically

resistant strain of SJL mice (Filbey et al., 2014), highlighting the importance of effector responses for functional immunity to helminths. Indeed, the treatment of mice with IL-4 complex (IL-4c), known to prolong the *in vivo* half-life of IL-4 (Finkelman et al., 1993) was sufficient to drive expulsion of *N. brasilensis* in lymphodeficient SCID mice (Urban et al., 1995). Although IL-4 acts primarily on haematopoietic cells, both IL-4 and IL-13 can act on non-immune cells particularly intestinal epithelial cells expressing the IL-4Rα to promote the expulsion of helminths (Reinecker and Podolsky, 1995). Goblet cells are specialised epithelial cells synthesising mucin glycoproteins that assemble to form a protective mucus layer. Upon infection, goblet cells proliferate and diversify their composition of mucin proteins and type-2 cytokines are able to directly enhance this process. Indeed, IL-13 could directly increase the expression levels of *Muc2* and *Muc5ac* in the small intestine following *N. brasiliensis* infection (Hasnain et al., 2011). Consequently, expulsion of both *Trichuris muris* and *N. brasiliensis* was delayed in mice deficient in *Muc2* or *Muc5ac* (Hasnain et al., 2010; Hasnain et al., 2011). In addition to their ability to secrete mucus, goblet cells were shown to secrete resistin-like beta (Relmβ/*Retnlb*) in response to IL-13, which was essential for proficient immunity to *H. polygyrus* (Herbert et al., 2009). Treatment with recombinant Relmβ *in vitro* and *in vivo* had a direct impact on parasite fecundity and viability (Herbert et al., 2009) and consequently, *Retnlb*-deficient mice failed to expel *H. polygyrus* following secondary infection or IL-4c treatment of primary infected mice (Herbert et al., 2009).

In addition to promoting goblet cell proliferation, type-2 cytokines promote intestinal epithelial cell turnover and smooth muscle cell contractility, which co-ordinately mediate a ‘weep and sweep’ mechanism resulting in functional expulsion of the pathogen. IL-13 but not IL-4 were required for intestinal cell turnover during *Trichuris muris* infection (Cliffe et al., 2005) and both IL-4 and IL-13 were required for intestinal smooth muscle contractility during infection with *N. brasiliensis* (Zhao et al., 2003). Consequently, *Il4ra*-deficiency in smooth-muscle cells resulted in a delayed expulsion of *N. brasiliensis* (Horsnell et al., 2007; Horsnell et al., 2011). Furthermore, type-2 cytokines promote changes in epithelial cell function including increased

mucosal permeability, which is thought to participate in the expulsion of *H. polygyrus* (Shea-Donohue et al., 2001; Madden et al., 2002). Conversely, epithelial cell derived alarmins can directly stimulate Th2 cells and also amplify type 2 immune responses. Indeed, TSLP was shown to promote proliferation and survival of Th2 cells in allergic asthma (Kitajima et al., 2011) however its role in stimulating helminth-elicited Th2 cells has not been tested. Furthermore, IL-33 stimulates type-2 cytokine secretion from Th2 cells cultured *in vitro* (Schmitz et al., 2005) and IL-5 from memory Th2 cells *in vivo* (Endo et al., 2015).

An important role for Th2-derived cytokines in promoting functional effector responses is the stimulation of B cell class switching by IL-4. Indeed, IL-4 was required for B cell class switching to IgG1 and IgE-producing plasma cells (Kuhn et al., 1991) and increased type-2 responses correlated with an increase in polyclonal IgE and IgG1 in a primary infection with *H. polygyrus* (McCoy et al., 2008). Although antibodies do not play a role in limiting the establishment of a primary infection with *H. polygyrus*, passive transfer of polyclonal IgG antibodies impaired the fecundity of adult parasites (as measured by egg output) (McCoy et al., 2008). Furthermore, antigen-specific IgE and IgG1 antibodies directed against larval stages of the parasite were highly elevated in *H. polygyrus* secondary infection (McCoy et al., 2008). Consequently, μMT mice lacking B cells were unable to expel secondary infection with *H. polygyrus* (Wojciechowski et al., 2009) as were *Aicda*$^{-/-}$ mice (activation-induced cytodine deaminase) deficient in the production of class-switched antibodies (McCoy et al., 2008). Although B cells can feed-back on Th2 cells to support their activation (Wojciechowski et al., 2009), B-cell deficiency resulted in impaired immunity to *H. polygyrus* without affecting Th2 memory responses themselves, suggesting B-cell dependent mechanisms including antibody production are required rather than an indirect role for B cell help to T cells (Liu et al., 2010). Furthermore, two studies describe a new role for antibodies in promoting tissue repair after *H. polygyrus* secondary infection. Antigen-specific antibodies were shown to directly bind to and mediate the accumulation of macrophages in the granulomas around invading L4

larvae (Esser-von Bieren et al., 2013). This correlated with the ability of antibody to bind to FcRγ receptors expressed on macrophages and stimulate their secretion of arginase-1, leading to larval immobilisation *in vitro* (Esser-von Bieren et al., 2013). In addition, this interaction was found to promote would-healing around the invading larvae (Esser-von Bieren et al., 2015).

Although the secretion of IL-4, IL-13 and IL-5 by $CD4^+$ T cells is required for fully functional type-2 responses, emerging data suggests that many other T helper cell-associated cytokines play important roles for the control of helminth infections (Bouchery et al., 2014). For example, Th2 cells are an important source of IL-21, which was shown to act on macrophages to enhance their arginase activity (Pesce et al., 2006) as well as promote class-switching of B cells. Consequently, *Il21r*$^{-/-}$ mice displayed impaired Th2 responses during *H. polygyrus* infection including decreased eosinophilia, IgE and IgG1 antibody production and reduced antigen-specific Th2 cytokine production (Frohlich et al., 2007). Furthermore, both innate and adaptive cells secrete IL-9 during helminth infections including Th2 cells. IL-9 mRNA and protein were significantly increased during infection with *N. brasiliensis* and *Il9*$^{-/-}$ mice exhibited reduced type-2 responses (Licona-Limon et al., 2013) resulting in delayed worm expulsion (Turner et al., 2013). More surprisingly, Th17-derived IL-17 was shown to synergise with IL-13 and exacerbate lung inflammation following HDM challenge (Lajoie et al., 2010). Furthermore, IL-17 production by γδ T cells was hypothesised to mediate the recruitment of neutrophils to the lung following *N. brasiliensis* infection, leading to the production of Ym1 by macrophages required for lung damage repair and functional helminth expulsion (Sutherland et al., 2014).

In conclusion, structural cells of the small intestine secrete alarmins following helminth infections leading to the recruitment of innate cells, which secrete and respond to type-2 cytokines. Furthermore, antigen-presenting cells such as DCs deliver antigen to naïve T cells that differentiate into Th2 cells secreting characteristic IL-5, IL-13 and IL-4. Th2 cells are required to support and amplify innate mechanisms resulting in the development of a diversified type-2 immune response required for immunity to

the complex multi-cellular pathogens. Central to these responses are the cytokines IL-4 and IL-13 that are secreted by both innate and adaptive cells. The dominance of a certain cell type is likely to be tissue and context-dependent, and therefore deciphering the relevant sources of cytokines will be of huge importance for the design of effective and targeted therapeutic strategies.

1.5.4. Immunoregulation by intestinal helminths

Intestinal helminths have generated great interest as potential therapeutic tools to treat autoimmune and inflammatory diseases due to their potent immunoregulatory properties. For example, helminth-derived ES proteins were shown to directly induce the differentiation of $Foxp3^+$ Treg cells (Grainger et al., 2010) and *H. polygyrus*-elicited Treg cells were shown to suppress the development of allergic lung inflammation following HDM challenge (Wilson et al., 2005). Conversely, OVA-restricted Treg cells were expanded following *H. polygyrus* infection (Grainger et al., 2010) suggesting that Treg cells could efficiently suppress cells specific for a different antigen. Furthermore, the development of Th2 responses can suppress the development of inflammatory type-1 responses, due cross-regulatory nature of the $CD4^+$ Th2 and Th1 differentiation programs. Thus, parasite-derived antigens have been shown to limit the development of Th1-mediated autoimmune diseases (Harnett and Harnett, 2010). For example, *H. polygyrus* infection was shown to reduce the development of colitis in susceptible *Il10*$^{-/-}$ mice (Elliott et al., 2004). Furthermore, ingestion of *Trichuris suis* eggs can dramatically reduce intestinal inflammation in patients suffering from inflammatory bowel disease (IBD) (Summers et al., 2003) and Crohn's disease (Summers et al., 2005). However, helminth-induced inhibition of type-1 responses could also have detrimental effects on immune responses to viruses and bacteria. Indeed, epidemiological data suggests that helminth-elicited type-2 responses negatively impact protective immune responses to co-infections including malaria (Coomes et al., 2015), HIV (Cassol et al., 2009) and tuberculosis (Potian et al., 2011) (reviewed in

(Salgame et al., 2013)). Furthermore, increases in hygiene in developing countries as well as advances in medicine have had a consequence on the global distribution of the burden of helminth infections, correlating with a rise in asthma and allergy, indicative of the dominant regulatory environment established following helminth infection. The hygiene hypothesis proposes that reduced prevalence of intestinal helminths in developed countries has led to exacerbated type-2 immune responses normally kept in check by a regulatory environment in the context of helminth infections (Maizels, 2005). Excessive type 2 responses include the formation of obstructive granulomas in the liver of *Schistosoma mansoni* infected individuals (Gause et al., 2013) or hypersensitivity to food allergens causing severe vomiting and diarrhoea (Burton et al., 2014).

Tregs are thought to play a dominant regulatory role in the control of Th2 immune responses and immune-mediated pathology in response to helminth infections. Indeed, transient Treg depletion using DEREG mice preferentially expanded Th2 cells during *T. muris* infection (Sawant et al., 2014) and primary *H. polygyrus* infection (Rausch et al., 2009), suggesting that Treg cells were negatively regulating protective Th2 responses in these models. Similarly, concomitant antibody-mediated blockade of CD25-expressing cells and stimulation of $GITR^+$ cells following infection with *Litomosoides sigmondontis,* led to the expulsion of established worms and increased type-2 cytokine production by $CD4^+$ T cells *in vitro* after co-culture with APCs (Taylor et al., 2005), suggesting Treg cells limit functional expulsion of helminths. Conversely, Tregs limit the development of Th2-mediated pathology. For example, Treg cells were shown to accumulate in colonic granulomas surrounding *S. mansoni* eggs, and reduce the accumulation of immune cells and Th2-mediated fibrosis (Turner et al., 2011).

H. polygyrus induces an early increase in $Foxp3^+$ Treg cells in a primary infection (Finney et al., 2007; Rausch et al., 2008), in the face of a strong Th2 response (Grainger et al., 2010). Treatment of naïve $CD4^+$ T cells with HES, containing TGF-β homologs, results in the induction of *Foxp3* expression and proliferation of $Foxp3^+$ cells *in vitro* in a TGF-β-dependent way (Grainger et al., 2010). Furthermore, *in*

vivo blockade of TGF-β reduced worm burden in chronically infected mice (Grainger et al., 2010) demonstrating a role for parasite antigens in promoting a regulatory environment and limiting mechanisms of natural immunity. Another mechanism by which Treg cells may develop following *H. polygyrus* infection is through Treg stimulation by bacterial products such as LPS and lipoproteins that infiltrate the tissue following epithelial damage (Caramalho et al., 2003). Indeed, there is evidence that Treg cell numbers are impaired in the absence of TLR2 signalling (Reynolds et al., 2014) following *H. polygyrus* infection. In addition, a tolerogenic population of $CD11c^{low}$ DCs were shown to preferentially generate $Foxp3^{+}$ Treg cells *in vitro* in the presence of TGF-β, and depletion of $CD11c^{high}$ cells led to a reduction in effector but not Treg responses (Smith et al., 2011), suggesting that *H. polygyrus* could either directly or indirectly (via DCs) promote Treg differentiation. In addition, non-canonical cytokines have been shown to promote Treg cells. For example, IL-6 was shown to limit Th2 responses by promoting the expression of Foxp3, Helios and GATA-3 in $CD4^{+}$ T cells (Smith and Maizels, 2014). Thus $Il6^{-/-}$ mice display an altered Treg phenotype and heightened Th2 responses, resulting in the expulsion of a primary infection with *H. polygyrus* (Smith and Maizels, 2014). In addition, *H. polygyrus*-elicited IL-1β was shown to impair the development of Th2 responses and promote chronicity of infection and therefore IL-1β-deficient mice expelled primary infection with *H. polygyrus* (Zaiss et al., 2013).

Taken together, these studies highlight a diversity of mechanisms induced either directly or indirectly by intestinal helminths that result in the development of an immunoregulatory environment and the establishment of chronic infections.

1.6. T cell plasticity

1.6.1. T helper cell plasticity

Although differentiated T helper cells were historically described as stable cellular lineages, many studies have highlighted a significant degree of plasticity

between T helper cells *in vitro* and *in vivo* (Bluestone et al., 2009). As previously discussed, the expression of TFs in response to cytokine signalling is required for the cross-regulation of different T helper cell subsets and their development. What this implies is that the balance in the expression of certain TFs and cytokines can dictate cell fate and promote the reversibility of lineages *in vitro*. For example, Th1 or Th2 cells generated *in vitro* were shown to readily co-express the opposing cytokine when exposed to the appropriate polarising environment (Krawczyk et al., 2007). This system has widely been used to manipulate T cell fate and provided valuable knowledge on transcriptional networks required for cytokine production. However more definitive evidence for the flexibility of T helper cells has emerged from studies *in vivo.* Indeed, many studies have reported the existence of hybrid cells co-expressing one or more characteristic T helper cell marker. For example, IL-4$^+$IFN-γ$^+$ (Krawczyk et al., 2007) and T-bet$^+$Gata-3$^+$ (Peine et al., 2013) cells were identified *in vivo.* IL-4$^+$IFN-γ$^+$ cells were found to develop following infection with the Th1-driving LCMV (Lymphocytic Choriomeninigitis virus) in response to interferon and IL-12 signalling and could provide protection against viral persistence as well as immunity to challenge infection (Lohning et al., 2008; Hegazy et al., 2010). The flexibility of Th17 cells and their conversion to Th1 cells has been widely reported in the context of autoimmunity and inflammation. For example, *in vitro* generated Th17 cells were shown to upregulate IFN-γ in response to IL-12 stimulation and mediate colitis following adoptive transfer (Mukasa et al., 2010; Feng et al., 2011). A seminal study made use of fate-reporter mouse to track cells that have expressed IL-17 and demonstrated that the majority of CD4$^+$IFN-γ$^+$ cells isolated during EAE but not *Candida albicans* infected mice originated from IL-17-expressing cells (Hirota et al., 2011). Using this fate-reporter, Th17 were also shown to convert to T follicular helper cells in the Peyer's patches following adoptive transfer, and contribute to the generation of class-switched mucosal IgA (Hirota et al., 2013). The characterisation of cytokine reporter mice has been valuable for increasing our understanding of the migration and fate of T helper cells. Indeed, *in vitro* generated Th2 cells (CD4$^+$*Il4*-GFP$^+$) cells were shown to convert to functional CD4$^+$*Ifng*-YFP$^+$ cells

and secrete IFN-γ during an infection with *Plasmodium chabaudi,* thereby controlling parasitemia levels (Coomes et al., 2015). Furthermore, although *Ifng*-YFP$^+$ ex-*Il4*-GFP$^+$ cells were transcriptionally re-wired, they were still able to produce IL-5 and IL-13 following stimulation *ex vivo,* suggesting they maintained epigenetic memory of their Th2 past. It remains to be determined whether the flexible relationships described *in vitro* are functionally relevant *in vivo.* Furthermore, the extent of T helper cell plasticity in the context of infection and co-infection remains relatively unexplored. In this latter study, TCR engagement, IFN-γ and IL-12 signalling were required for Th2 to Th1 conversion, which correlated with impaired Th2 responses and impaired immunity to *H. polygyrus* in mice co-infected with *P. chabaudi* (Coomes et al., 2015) suggesting Th conversion could be detrimental in the context of a co-infection with pathogens promoting polarised immune responses.

It is interesting to speculate that the flexibility of T helper cells is evolutionarily advantageous in the context of co-infections with pathogens whose elimination is dependent on opposing T helper cell responses. In addition, emerging data suggests that protective immune responses to helminths are associated with multiple T helper cell lineages such as IL-4 and IL-17 suggesting certain infections may promote mixed T effector profiles.

1.6.2. Treg plasticity

As previously described, Treg cells develop independently to naïve T cells, and have always been thought to maintain stable expression of *Foxp3.* Although many mechanisms exist for the maintenance of Treg cells *in vivo*, recent studies have identified that both mouse and human Treg cells are unstable *in vitro* and *in vivo* in the context of inflammation and can lose *Foxp3* expression to acquire pathogenic properties (Zhou et al., 2009; McClymont et al., 2011). Given that the TCR repertoire of Treg cells is so similar to auto-reactive T cells, Treg instability was shown to result in their differentiation into auto-reactive T cells that have the potential to contribute to the

development of disease. Understanding diverse disease and infection settings where Treg instability occurs is of significant therapeutic relevance as Treg-based therapies move closer to the clinic.

1.6.2.1. Treg plasticity *in vitro*

As previously discussed, *Foxp3* mRNA and Foxp3 protein are maintained through tightly regulated and diverse stabilising mechanisms including through cytokine signalling with IL-2 (Fontenot et al., 2005) and IL-10 (Chaudhry et al., 2011). Indeed, the IL-2/STAT5 axis was shown to play an important role in the maintenance of *Foxp3* expression in the presence of inflammatory cytokines (Feng et al., 2014) suggesting that Treg cells have control mechanisms limiting loss of *Foxp3* in response to cytokines, and that any change in CD25 expression or IL-2 levels have a significant impact on the stability of Treg cells. Therefore, recent work has focused on elucidating the impact of inflammatory cytokines on the stability of Treg cells, as purified Treg cells stimulated with inflammatory cytokines *in vitro* were shown to readily lose *Foxp3* expression (Feng et al., 2014). As previously described, a cross-regulatory relationship exists between Th17 and Treg cells during their development. Indeed, Treg cells cultured in the absence of TGF-β, which is required for their maintenance *in vitro* (Xu et al., 2007), and in combination with pro-inflammatory cytokines such as IL-6 were shown to lose *Foxp3* expression (Veldhoen et al., 2006) and convert to IL-17-secreting cells (Yang et al., 2008). Furthermore, *in vitro* stimulation of Treg cells with both IL-6 and IL-4 reduced *Foxp3* expression and promoted type-2 cytokine secretion by $Foxp3^{+}CD25^{-}$ cells (Komatsu et al., 2009). Furthermore, Treg-specific ablation of Ubc13 ubiquitin-conjugating enzyme resulted in their production of IL-17 and IFN-γ (Chang et al., 2012). In addition, *Bach2*-deficient Treg cells were able to secrete T helper cell cytokines *in vitro* (Roychoudhuri et al., 2013). Together, these studies support the fact that cytokines can promote Treg instability as well as their conversion to cytokine-secreting T helper cells.

1.6.2.2. Treg plasticity *in vivo*

Evidence for instability of *Foxp3* expression *in vivo* stems from recent observations demonstrating that the transfer of purified Foxp3$^+$ Treg cells into a lymphodeficient host resulted in the loss of *Foxp3* expression and acquisition of T effector cytokine production. Indeed, up to 90% of cells lost *Foxp3* expression and started to produce effector cytokines including IL-4, IFN-γ and IL-2 following adoptive transfer into T-cell deficient or *Rag2*-deficient mice (Duarte et al., 2009; Komatsu et al., 2009). Additionally, adoptively transferred Treg cells were shown to lose *Foxp3* expression and convert to Tfh cells in the Peyer's patches (Tsuji et al., 2009) although it remains unclear whether this has any functional relevance, such as driving antibody class-switching (Hirota et al., 2013). In one previously discussed study, Foxp3$^+$ Treg cells were shown to migrate to the LP and secrete IFN-γ whilst maintaining their suppressor function (Feng et al., 2011). Furthermore, these specialised Foxp3$^+$IFN-γ$^+$ Treg cells were later shown to lose *Foxp3* expression and contribute to the pathogenic Th1 pool driving intestinal inflammation (Feng et al., 2011). This study suggests there may be a tipping point between the specialisation of Treg cells and their conversion to cytokine-producing T effector cells (**Fig. 1.5**).

Taken together, these studies provided evidence that Treg cells had the potential to lose *Foxp3* expression, previously thought to be a stable and heritable Treg marker. However there are caveats associated with Treg adoptive transfer models, including the risk of transferring small proportions of Foxp3$^-$ contaminants that may preferentially expand and differentiate into T effector cells. Additionally, the environment in lymphodeficient hosts is vastly different to that of WT mice, in particular due to the low levels of circulating IL-2 (normally secreted by proliferating T effector cells), required for *Foxp3* maintenance (Fontenot et al., 2005). Indeed, the co-transfer of *Foxp3*$^-$ T cells with *Foxp3*$^+$ Treg was shown to reduce the proportion of cells that lost

Foxp3 expression (Komatsu et al., 2009) suggesting the lymphodeficient environment is promoting exaggerated *Foxp3* loss.

Support for the functional loss of *Foxp3* expression *in vivo* stemmed from studies performed in the context of infection and inflammation where the dramatic reduction in Treg numbers correlated with increased T-cell mediated disease. For example, the frequencies of islet-cell resident Treg cells were shown to decline during the development of Type 1 diabetes in favour of increased pathogenic T effector cells (Tang et al., 2008). Thus, IL-2 treatment was employed to maintain a functional Treg population and control of pathogenic islet-cell reactive CD4+ T cells (Liston et al., 2007). Similarly, frequencies of Foxp3^{+} Treg cells were dramatically decreased during an infection with *Toxoplasma gondii,* correlating with increased proportions of Th1 cells and reduced availability of IL-2 (Oldenhove et al., 2009). Furthermore, this correlated with increased expression of *Tbx21* and secretion of IFN-γ by the remaining T cells, suggesting a proportion of the Treg population had lost *Foxp3* expression and acquired a Th1 phenotype (Oldenhove et al., 2009). Indeed, the rescue of Treg numbers using IL-2c resulted in reduced immune-mediated pathology and disease (Oldenhove et al., 2009). Taken together, these studies described environments where the proportion and absolute numbers of Treg cells reduce significantly to give way to a strong T effector population, and suggested that ex-*Foxp3* cells may be contributing to the pathogenic T effector population.

One way to test these observations and identify ex-Foxp3 cells *in vivo* is to use a mouse fate-mapping approach. Recent studies have taken advantage of fate-reporter mice to track the fate of *Foxp3*-expressing cells in various disease settings and to identify cells that have lost *Foxp3* expression *in vivo* (ex-Foxp3 cells). For example, one study generated Foxp3 fate-reporter mice by crossing knockin BAC transgenic mice expressing GFP-Cre under the control of the *Foxp3* promoter with ROSA-LSL-YFP mice (Zhou et al., 2009). Using these mice, the authors were able to determine that 10-15% of peripheral CD4+ T cells were GFP^{-}YFP^{+} suggesting they had lost *Foxp3* expression (Zhou et al., 2009). The proportion of ex-*Foxp3* cells secreting IFN-γ

and IL-17A was increased in the pancreatic lymph nodes of diabetic mice. Additionally, ex-*Foxp3* cells were pathogenic after adoptive transfer (Zhou et al., 2009), suggesting that ex-*Foxp3* cells were functional T effector cells. Similarly, Treg cells were shown to lose *Foxp3* expression during the development of EAE (Bailey-Bucktrout et al., 2013). Ex-*Foxp3* cells isolated from the spinal fluid of diseased mice had a demethylated *Foxp3* CNS2 locus suggesting they had derived from bona-fide Treg cells. Furthermore, ex-Foxp3 cells were reactive to myelin oligodendricyte glycoprotein (MOG), secreted IFN-γ and were able to induce EAE in recipient mice following adoptive transfer (Bailey-Bucktrout et al., 2013). In a separate study, ex-*Foxp3* cells were shown to accumulate in the synovial fluid of the joints during autoimmune arthritis and develop into IL-17-secreting cells (Komatsu et al., 2014). In contrast to the previous study, ex-Foxp3 cells displayed a methylated *Foxp3* CNS2 locus suggesting there may have been *de novo* methylation of the *Foxp3* locus. Functionally, IL-17^{+} ex-*Foxp3* cells were shown to promote osteoclatogenesis following adoptive transfer suggesting they may contribute to the pathogenesis of disease (Komatsu et al., 2014). Taken together these studies demonstrate that ex-Foxp3 cells can develop *in vivo* and that their proportion increases in the context of disease. Furthermore, these studies found that ex-*Foxp3* T cells were pathogenic and contributed to the development of disease. Together, these *in vitro* and *in vivo* studies suggest that inflammatory cytokines could be promoting the loss of *Foxp3,* however the role of cytokines on Treg instability has not been investigated in these studies (**Fig. 1.5**).

There have been conflicting reports on Treg stability using fate-reporter mice. Another study generated fate-reporter mice by inserting a GFP-Cre-ERT2 vector into the *Foxp3* locus and crossing the mice to ROSA-LSL-YFP reporter mice. This system allowed for the temporal expression of ROSA-LSL-YFP following tamoxifen treatment allowing the authors to track the loss of *Foxp3* in mature Treg cells rather than throughout their development (Rubtsov et al., 2010). In contrast to previous strategies, the authors found that very few cells lost *Foxp3* expression (around 5%) either at steady state or in the context of autoimmune inflammation or infection with *Listeria*

monocytogenes (Rubtsov et al., 2010) suggesting that *Foxp3* cells do not lose *Foxp3* expression following their development. In a third study, a GFP-Cre vector was inserted into the endogenous *Foxp3* locus and the mice were crossed to ROSA-LSL-RFP mice. The authors describe the accumulation of ex-*Foxp3* cells throughout life that had lost Treg-associated markers and were secreting inflammatory cytokines (Miyao et al., 2012). Further characterisation and lineage tracking of these cells revealed they were a small proportion of the total Treg pool expressing low levels of the fate-mark RFP and low levels of CD25, correlating with a methylated *Foxp3* locus. Together, these results suggested that a proportion of newly developed *Foxp3*$^{+}$ thymocytes were transiently expressing *Foxp3* (Miyao et al., 2012). Indeed, this population was also identified in a separate study where the authors found that a fraction of peripheral cells lost *Foxp3* expression but maintained a demethylated *Foxp3* locus, termed 'epigenome+ cells'. Together, these studies suggest that the demethylation of the *Foxp3* locus rather than *Foxp3* expression itself correlated with a Treg phenotype, highlighting the separate nature of these events and the redundancy of *Foxp3* expression (Ohkura et al., 2012). To rectify many of the above discrepancies, the authors defined a 'heterogeneity model' of Treg development. In this model, it was proposed that a proportion of peripheral CD4^{+} T cells activate *Foxp3* in response to TCR signalling without remodelling the CNS2 region of the *Foxp3* locus or other Treg specific demethylated regions (Ohkura et al., 2012). These transiently expressing ex-*Foxp3* cells are later thought to differentiate into cytokine-producing effector cells. Importantly, this model also suggests that a proportion of the peripheral cells have truly lost *Foxp3* expression *in vivo.* One caveat of these studies is the insertion of fusion proteins such as GFP-Cre into the *Foxp3* locus, which was previously shown to disrupt *Foxp3* function and its genomic signature, including the stability of *Foxp3* expression (Bettini et al., 2012; Darce et al., 2012). Despite this, fate-mapping tools have provided a sophisticated method to identify *Foxp3* instability *in vivo,* which will be important to test in diverse settings of immunity and infection.

1.6.3. Plasticity between Treg and Th2 cells

Treg cells are important for the control of pathogenic Th2 responses in the context of allergic disease (Fyhrquist et al., 2012). In particular, extra-thymically derived Treg cells are thought to be important suppressors of Th2 responses and limit commensal-induced development of mucosal allergic disease (Josefowicz et al., 2012). Furthermore, helminth infections can promote *de novo* peripheral Treg differentiation and suppress the early differentiation of Th2 responses (Taylor et al., 2005; Taylor et al., 2009) and consequently Treg deletion during a primary infection restores Th2 responses (Sawant et al., 2014). Recent work has described specific transcription factors associated with *Foxp3* that are required for the regulation of Th2 responses by Treg cells (Zheng et al., 2009; Sawant et al., 2012). In addition, many studies describe Treg cells that have reduced or lost *Foxp3* expression or are deficient in a co-factor associated with Foxp3 can preferentially acquire a Th2 effector phenotype (Wan and Flavell, 2007; Wang et al., 2010), indicating that there is a cross-inhibitory relationship between Treg and Th2 cells. Indeed, many studies have reported cross-regulation between Foxp3 and IL-4 *in vitro* (Pace et al., 2005; Skapenko et al., 2005; Mantel et al., 2007; Wei et al., 2007; Takaki et al., 2008; Hadjur et al., 2009; Pillemer et al., 2009; Chapoval et al., 2010; Tran et al., 2012), however the role of IL-4 signalling on $Foxp3^{+}$ Treg cells *in vivo* is less clear.

1.6.3.1. Cross-regulation between Treg and Th2 cells

As mentioned above, the cross regulation between Treg and Th2 cells has been well documented *in vitro*. Mechanistically, *Foxp3* was shown to regulate IL-4 expression by inhibiting the translocation of NfκB to the nucleus as well as modifying the chromatin structure and accessibility of the *Il4* locus (Kwon et al., 2008). Furthermore, TGF-β signalling was shown to activate the adaptors Ndfip1 and TF *JunB* and inhibit IL-4 expression, thereby promoting iTreg differentiation (Beal et al., 2012). Similarly, the TF *Sox4*, expressed upon TGF-β signalling, was shown to directly bind to

Gata3 and the *Il5* promoter, thereby inhibiting Th2 function through two independent mechanisms (Kuwahara et al., 2012). Conversely, both the Th1 and Th2 differentiation programs were shown to inhibit iTreg formation *in vitro.* Indeed, IL-4 could directly inhibit the differentiation of Treg cells through STAT6 activation (Wei et al., 2007). Furthermore, enforced GATA-3 expression in naïve T cells rendered the cells resistant to *Foxp3* expression following their culture in iTreg conditions (Wei et al., 2007). Indeed, GATA-3 was shown to directly bind to the *Foxp3* promoter and reduce iTreg frequency *in vitro* (Mantel et al., 2007). More recently, IL-4 induced pSTAT6 was shown to directly bind *Dnmt1* DNA methyltransferases, leading to reduced mRNA expression of Foxp3 *in vitro,* which suggests that IL-4 signalling can directly interfere with the epigenetic status of the of the *Foxp3* locus (Feng et al., 2014). Indeed, IL-4-elicited STAT6 was shown to compete with IL-2 elicited STAT6 for *Foxp3* binding (Feng et al., 2014). Evidence *in vivo* stems from a correlative study showing that treatment with IL-4c resulted in reduced numbers of *Foxp3*-expressing cells, suggesting that IL-4 directly regulates the Treg population (Mantel et al., 2007). In addition, STAT6-deficient mice had elevated frequencies of $Foxp3^+$ cells after OVA challenge suggesting IL-4 acts through STAT6 and GATA3 to limit the expansion of Treg cells (Chapoval et al., 2011). Furthermore, the development of type-2 immune responses was shown to impair Treg function. Indeed, Th2-induced IgE was shown to promote Treg tolerance to oral allergens resulting in exacerbated disease (Burton et al., 2014). Furthermore, transgenic mice expressing a mutant IL-4Rα chain with increased signalling activity (*IL4ra*^{F709}) showed impaired iTreg formation and impaired suppressor function in the context of OVA sensitisation (Noval Rivas et al., 2015). In summary, the differentiation of Treg or Th2 cells is tightly controlled by the cross-regulation of TFs downstream of cytokine signalling. Consequently, changes in the cytokine environment and activation of TFs have the potential to skew the balance towards either of the two populations.

1.6.3.2. Conversion of Treg to Th2 cells

In the aforementioned study, increased IL-4 signalling in transgenic *IL4ra*^{F709} Treg cells was shown to promote Treg reprogramming to Th2 effector cells, correlating with the development of enhanced allergic disease following oral challenge (Noval Rivas et al., 2015). This study provides evidence of a direct role for IL-4 inhibition of Treg stability and function *in vivo.* This may be surprising given the observation that Treg cells increase dramatically despite the development of a polarised Th2 response following infections with diverse intestinal helminths (Taylor et al., 2009; Grainger et al., 2010). However, it has been shown that the proportion of Treg cells was markedly reduced in the context of even stronger Th2 responses, for example following secondary infection with *H. polygyrus* (Liu et al., 2010), suggesting that high levels of IL-4 may be inhibiting Treg formation in the context of intestinal helminth infections. Furthermore, it is conceivable that Treg cells could be reprogramming to Th2 cells and contributing to memory Th2 cells, similarly to the observed conversion of Treg to Th1 cells during *Toxoplasma gondii* infection (Oldenhove et al., 2009). Indeed, there is some evidence that Treg cells can convert to Th2 cells *in vivo. FILIG* (Foxp3-IRES-luciferase-IRES-eGFP) mice were generated by knocking in IRES-eGFP cassette into the 3'-untranslated region of the endogenous *Foxp3* locus, leading to *Foxp3* mRNA destabilisation. Consequently, although *FILIG* mice displayed normal proportions of Treg cells, Foxp3^{+} cells had attenuated levels of *Foxp3* expression and a reduced suppressive function, which resulted in the development of spontaneous autoimmune disease (Wan and Flavell, 2007). Interestingly, cells that exhibited reduced *Foxp3* levels were shown to secrete functional levels of the canonical Th2 cytokines IL-4, IL-13 and IL-10 (Wan and Flavell, 2007) that could promote the differentiation of naïve T cells into Th2 cells *in vitro* (Wang et al., 2010). Th2 cytokine production by *FILIG*$^{+}$ cells occurred independently of IL-4/STAT6 signalling but was dependent on GATA-3 (Wang et al., 2010). As previously described, Th2 differentiation can occur in a STAT6 independent manner and similar mechanisms may be at play here (Wang et al., 2010). Similarly, a population of human CD45Ra^{-} Treg cells was shown to readily lose *Foxp3*

expression *in vitro*, correlating with increased expression of *Gata3* and the secretion of IL-4 and IL-13 (Hansmann et al., 2012). More recently, mice with a Treg-specific deficiency in *Itch* were shown to develop systemic inflammation and increased death due to dysregulation of Th2 responses (Jin et al., 2013). *Itch*-deficient Treg cells were shown to secrete IL-4, IL-5 and IL-13 and induce type-2 inflammation in response to OVA challenge (Jin et al., 2013). In addition, *Bcl6*-deficient Treg cells expressed higher levels of GATA-3 and Th2-related genes, indicating *Bcl6* acts to suppress the development of a Th2 program in Treg cells (Sawant et al., 2012). Furthermore, *Traf6*-deficient mice were shown to be prone to Type-2 autoimmune disease and *Traf6*-deficient Treg cells were shown to lose *Foxp3* expression in lymphopenic conditions and secrete IL-4 (Muto et al., 2013). Finally, Treg-specific deletion in the protein kinase Ck2 led to dysregulated Th2 responses and the development of allergic disease (Ulges et al., 2015).

In summary, these studies demonstrate a close link between Treg and Th2 cells and suggest that discrete transcriptional, post-transcriptional and post-translational mechanisms limit Treg to Th2 conversion. This suggests that Treg to Th2 conversion could readily be disrupted *in vivo,* and furthermore, could be manipulated to promote the development of type-2 responses in the context of helminth infections.

1.7. Thesis aims

IL-4 producing $CD4^+$ Th2 cells are required for functional immunity to the natural murine intestinal helminth *H. polygyrus* (Urban et al., 1991; Urban et al., 1991), however the cellular sources of IL-4 required for Th2 differentiation *in vivo* remain unclear. ILC2s have been shown to secrete type-2 cytokines in response to tissue-derived alarmins or inflammatory mediators following intestinal helminth infections, including IL-5, IL-13, IL-4 and IL-2. Although ILC2s have been shown to be sufficient for the expulsion of some helminths, it is increasingly thought that they support the development of downstream type-2 responses, including the activation of T cells,

however the role for ILC2-derived cytokines for Th2 differentiation is unclear. In this thesis, we hope to characterise a role for ILC2-derived IL-4 in driving the differentiation of Th2 cells *in vitro* and *in vivo* during a chronic infection with *H. polygyrus.* Furthermore, ILC2s secrete IL-2 and are responsive to IL-2 signalling *in vitro* and *in vivo,* however the role of autocrine or paracrine IL-2 for the effector function of ILC2s has not been well characterised. In this thesis, we investigate the role of IL-2, including ILC2-derived IL-2 on the development and effector function of ILC2s. We hypothesise that ILC2s are an important source of IL-4 required for Th2 differentiation and immunity to *H. polygyrus.*

Intestinal helminth infections promote a strong regulatory environment through the induction of $CD4^{+}Foxp3^{+}$ Treg cells, which limit the development of Th2 cells and proficient immunity. Functional expulsion of *H. polygyrus* occurs following secondary challenge, and correlates with a shift in the ratio of Treg to Th2 cells, resulting in the activation of a cascade of effector cells required for immunity. Diverse regulatory mechanisms control the maintenance of Treg cells and consequently maintain immune homeostasis and host survival. However in the context of inflammatory diseases, $Foxp3^{+}$ Treg cells have been shown to lose *Foxp3* expression and convert to pathogenic T helper cells. Whether Treg cells can convert to protective T helper cells and participate in pathogen clearance is currently unknown. In the second part of this thesis, we aim to test whether Treg cells can convert to Th2 cells and contribute to immunity to *H. polygyrus.* Furthermore, we aim to test the requirement of IL-4 signalling for the development and plasticity of Treg cells. Lastly, we would like to test the impact of Treg conversion to Th2 cells on functional immune responses.

By studying the function of ILC2-derived IL-4 and the role of IL-4 signalling for Treg to Th2 conversion, we hope we can uncover two new pathways required for the differentiation of Th2 cells *in vivo* and functional immunity to *H. polygyrus*.

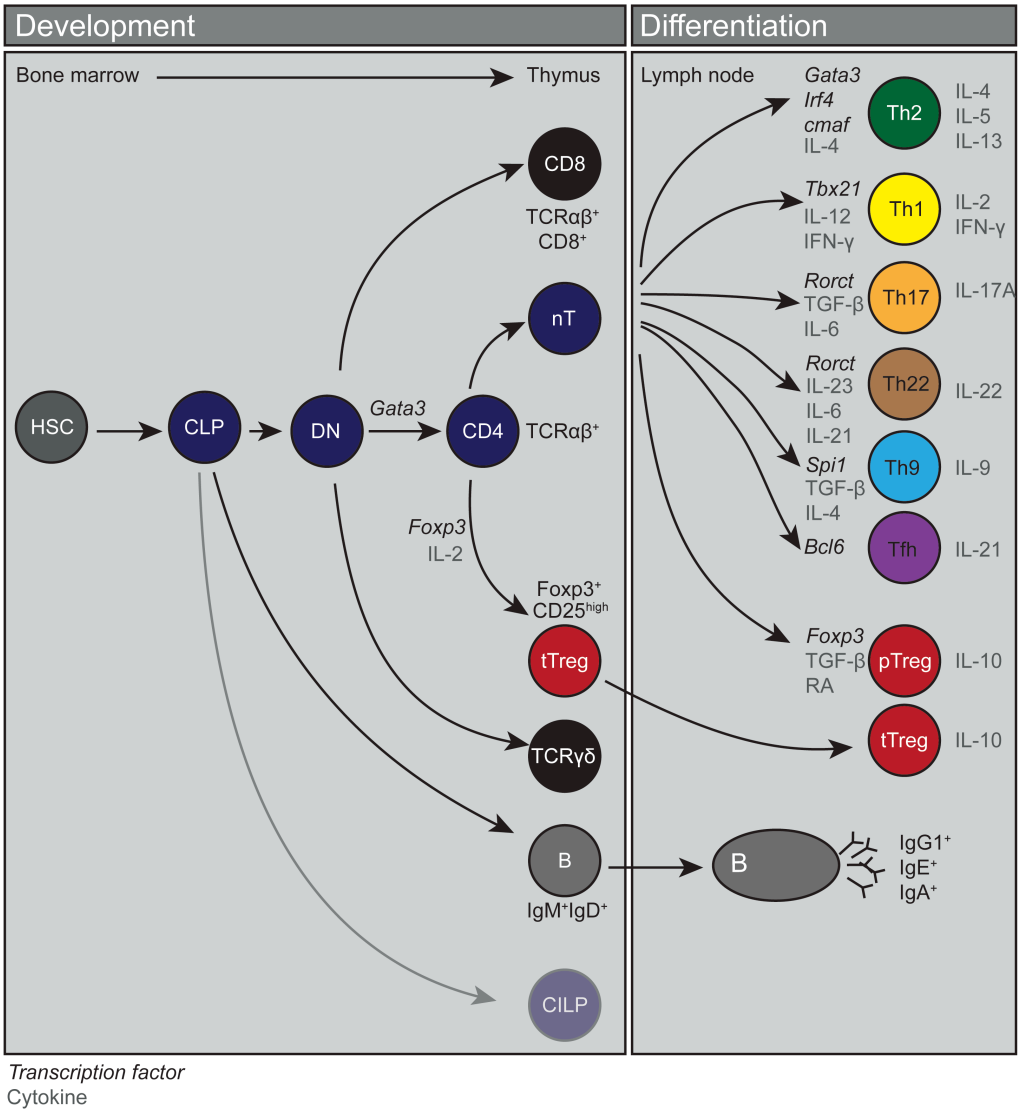


Figure 1.1. B- and T-lymphocyte development and differentiation. Schematic diagram of $CD4^+$ T cell development and differentiation. Abbreviations: HSC (haematopoietic stem cell), CLP (common lymphoid progenitor), CILP (common innate lymphoid progenitor), DN (double negative), Treg (T regulatory cell), Th (T helper). CD4+ T cells, CD8+ T cells, B cells and CILP develop from the CLP in the bone marrow. DN T cells migrate to the thymus where they differentiate into γδ T cells or further differentiate into TCRαβ+ CD4- or CD8-expressing mature T cells. Immature B cells migrate to the spleen and lymph nodes where they undergo class switching upon activation to produce a diverse library of antibodies. In the thymus, T cells then undergo positive selection, negative selection or death by neglect, depending on the affinity of the TCR for self-antigen. Positively selected naive CD4+ T cells exit into the periphery where they migrate through the lymphatics to tissue-draining lymph nodes. In the thymus, CD4+TCRαβ+ with a strong recognition for self can further differentiate into $CD25^{high}Foxp3^+$ Treg cells. In the context of infection and inflammation, the presence of specific cytokines will drive the differentiation of naïve CD4+ T cells into Th1, Th2, Th17, Th22, Th9, Tfh or pTreg cells that secrete characteristic cytokine required for the development of proficient immune responses.

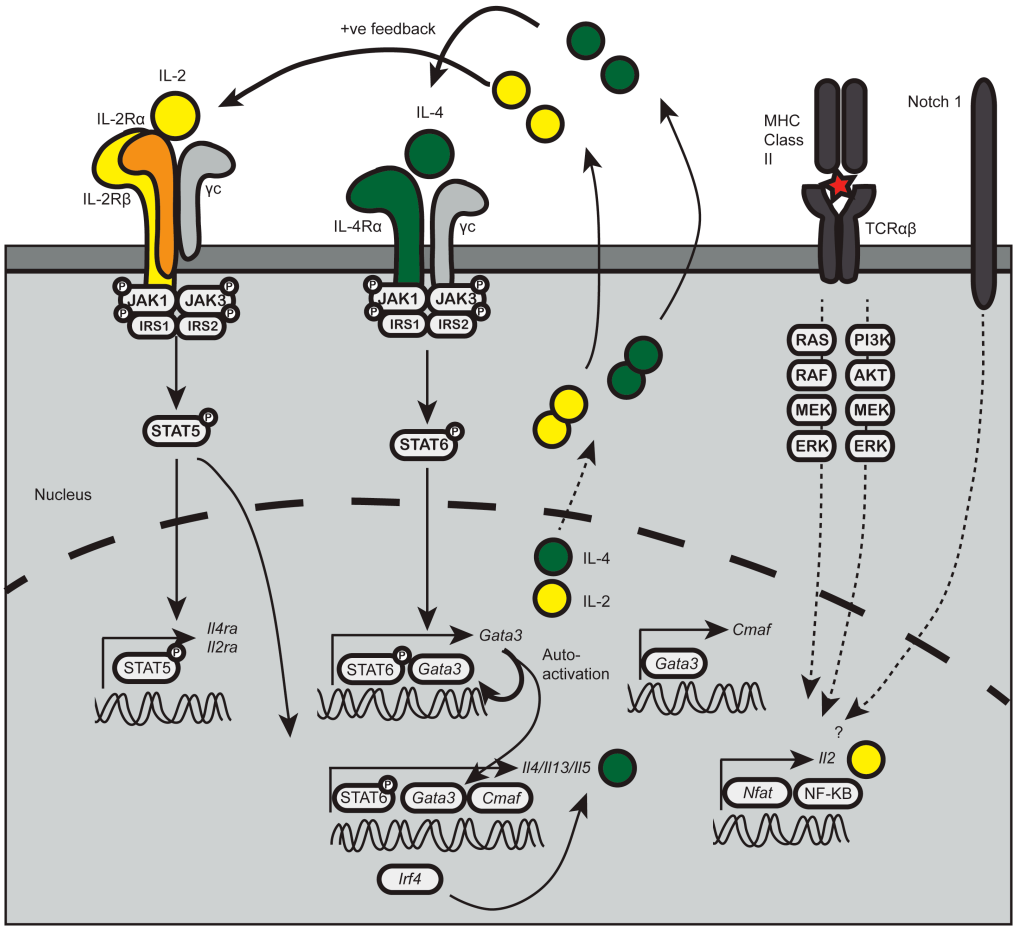


Figure 1.2. Molecular mechanisms of Th2 differentiation. Schematic diagram of the signalling pathways required for the differentiation of $CD4^+$ Th2 cells. Both IL-4 and IL-2 signalling (through the Type-1 IL-4R and IL-2R, respectively) are required for the differentiation of Th2 cells. Following TCR engagement and the activation of NFAT and NF-κB, IL-2 is secreted from activated cells and signals through STAT5 to activate *Il4* as well as increase the expression of *Il2ra* and *Il4ra*. Following this, IL-4 signals through STAT6 to promote the expression of *Gata3*. Both STAT6 and GATA-3 can bind to the Th2 locus, comprised of *Il4, Il13* and *Il5* loci and enhance their expression. Furthermore, Gata3 establishes a positive feedback loop by binding to its own promoter. Although Gata3 is fundamental for *Il5* and *Il13* expression, the TF c-maf is required for optimal expression of *Il4*, and occurs following *Gata3* expression. In addition, the TF *Irf4* stabilises the complex of STAT6, GATA-3 and c-maf. Thus, GATA-3 is fundamentally important for the differentiation and maintenance of Th2 cells.

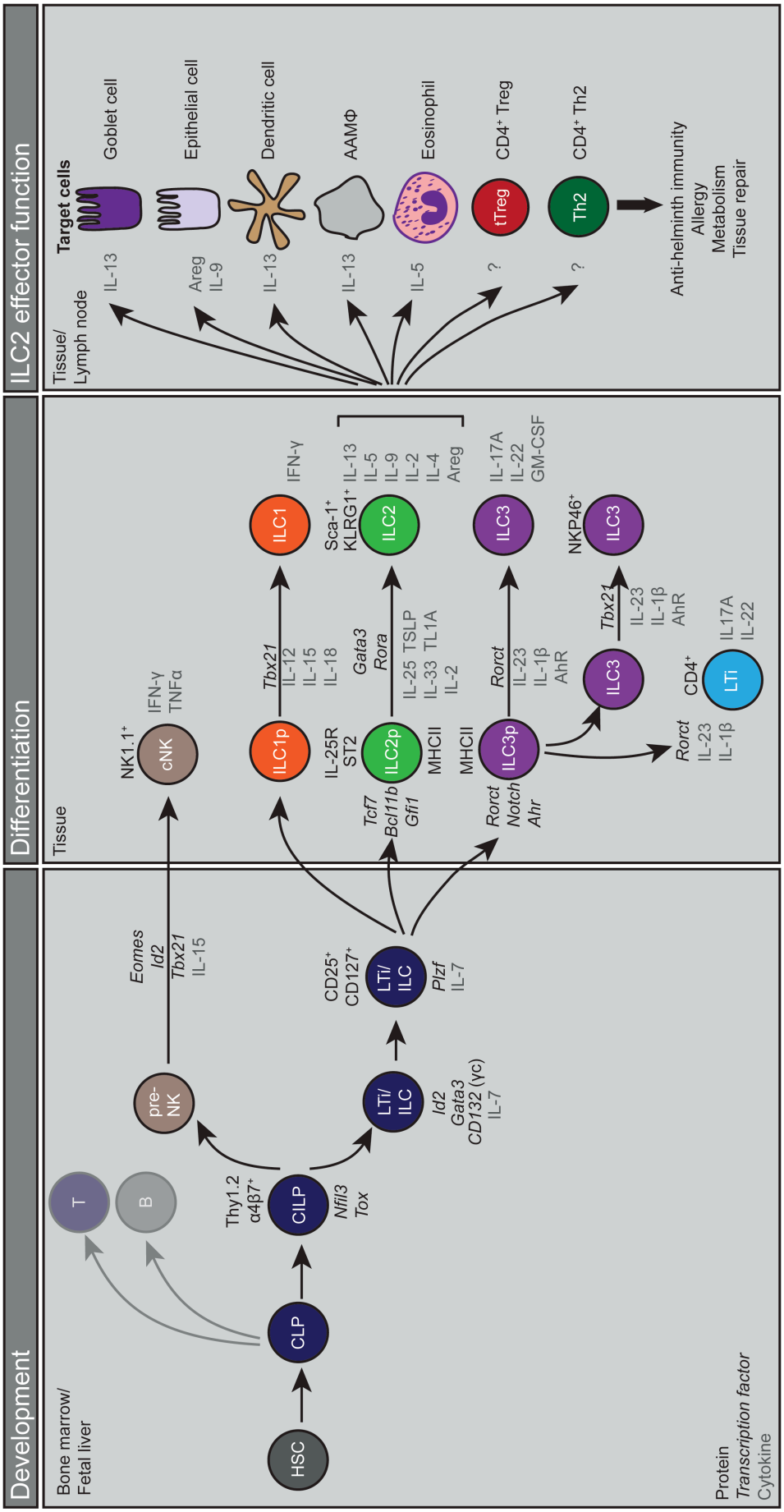

Development
Differentiation
ILC2 effector function
Bone marrow/
Fetal liver
Tissue
Tissue/
Lymph node
Target cells
HSC
CLP
CILP
T
B
pre-
NK
LTi/
ILC
LTi/
ILC
CD25+
CD127+
Thy1.2
α4β7+
Nfil3
Tox
Id2
Gata3
CD132 (γc)
IL-7
Eomes
Id2
Tbx21
IL-15
NK1.1+
cNK
IFN-γ
TNFα
ILC1p
ILC1
Tbx21
IL-12
IL-15
IL-18
IL-25R
ST2
IL-25 TSLP
IL-33 TL1A
IL-2
ILC2p
ILC2
Gata3
Rora
Tcf7
Bcl11b
Gfi1
MHCII
ILC3p
ILC3
Rorct
IL-23
IL-1β
AhR
Rorct
Notch
Ahr
Plzf
IL-7
IFN-γ
Sca-1+
KLRG1+
IL-13
IL-5
IL-9
IL-2
IL-4
Areg
IL-17A
IL-22
GM-CSF
NKP46+
Tbx21
IL-23
IL-1β
AhR
CD4+
LTi
Rorct
IL-23
IL-1β
IL17A
IL-22
Protein
Transcription factor
Cytokine
IL-13
Areg
IL-9
IL-13
IL-13
IL-5
?
?
Goblet cell
Epithelial cell
Dendritic cell
AAMΦ
Eosinophil
CD4+ Treg
tTreg
CD4+ Th2
Th2
Anti-helminth immunity
Allergy
Metabolism
Tissue repair

Figure 1.3. Development and effector function of ILC2s. Schematic diagram of ILC2 development and effector function. Abbreviations: HSC (haematopoietic stem cell), CLP (common lymphoid progenitor), CILP (common innate lymphoid progenitor), NK (natural killer), LTi (lymphoid tissue inducer), ILC (innate lymphoid cell), AAMΦ (alternatively activated macrophage), DC (dendritic cell). ILCs develop from the CILP in response to IL-7 signalling, and further mature in peripheral tissues. Group 2 ILCs (ILC2s) form part of the wider ILC family, which includes NK cells, group 1 (ILC1) and group 3 (ILC3) ILCs. ILC2s develop from CD25-, Sca-1-, CD127-expressing progenitor cells in the bone marrow, and are dependent on the expression of *Gata3, Rora, Tcf7, Bcl11b* and *Gfi1*. In the tissue, mature ILC2s express IL-25R, ST2 (IL-33R) and KLRG1 and secrete characteristic cytokines such as IL-13 and IL-5 in response to IL-25 and IL-33 signals. Furthermore, ILC2s secrete IL-9, IL-4, IL-2 and amphiregulin. ILC2s form functional relationships with other immune and non-immune cells through their cytokine production and expression of MHC Class II molecules. Thus, ILC2s have been implicated in the development of allergic disease as well as promoting wound healing and worm expulsion following infections with intestinal helminths.

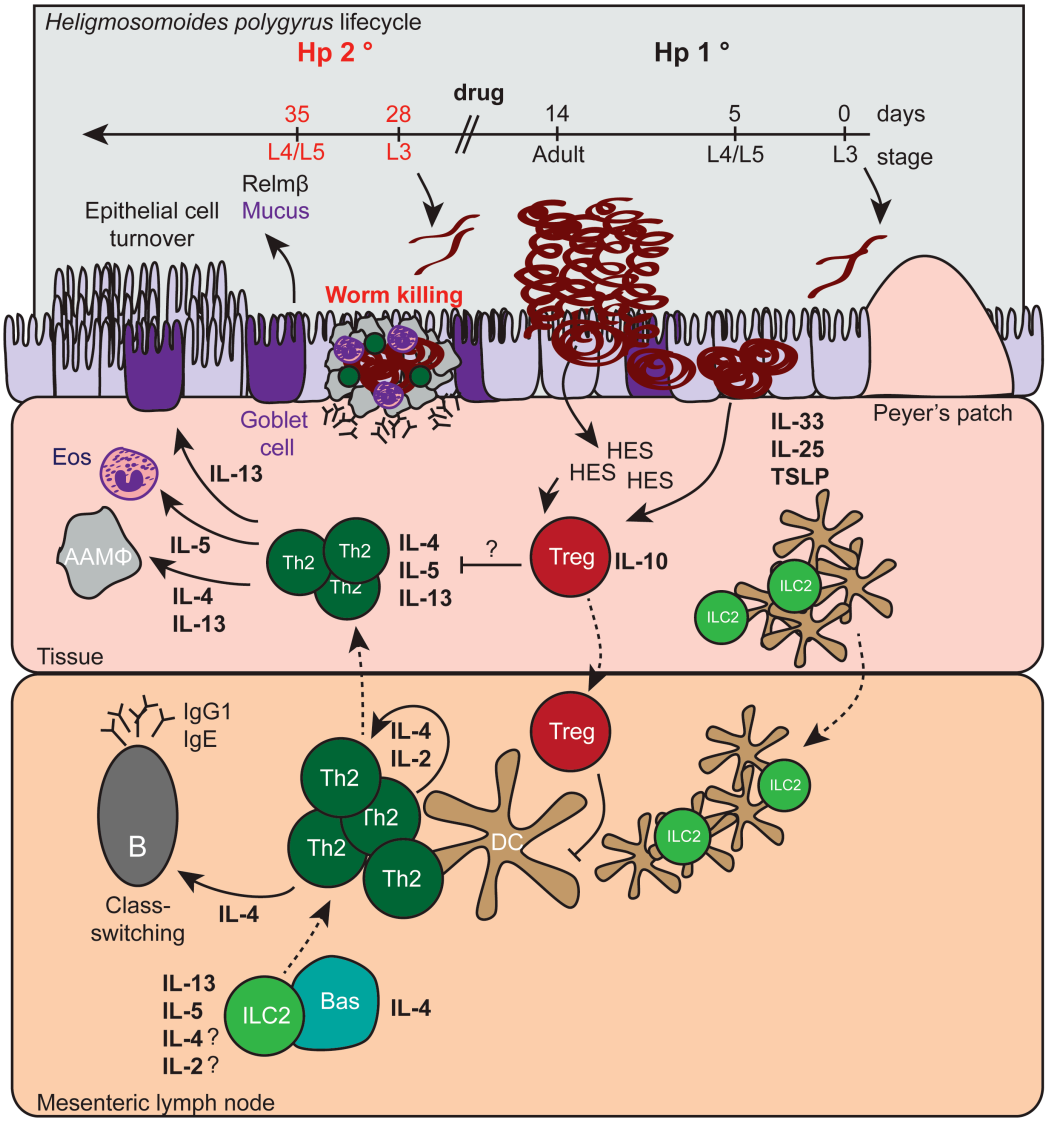


Figure 1.4. Type-2 immune responses to the intestinal helminth *Heligmosomoides. polygyrus*. Schematic diagram of immune responses to *Heligmosomoides polygyrus.* Abbreviations: Hp (*Heligmosomoides polygyrus*), 1°/2° (primary/secondary), L3/4/5 (larval stages) HES (Hp excretory/secretory proteins), AAMΦ (alternatively activated macrophage), TSLP (thymic stromal lymphopoietin), Bas (basophil), Eos (eosinophil). Upon a 1° infection with Hp, activated and antigen-loaded DCs, migrate from the small intestine to the mesenteric lymph nodes and in the presence of IL-4, stimulate the differentiation of $CD4^+$ Th2 cells secreting the characteristic cytokines IL-4, IL-13 and IL-5. Th2-derived IL-4 promotes class-switching of B cell to IgE and IgG1-secreting plasma cells. Th2 cells migrate back to the tissue and activate a variety of immune and non-immune effector cells. After challenge infection (2°) in drug-cured mice, a large granuloma composed of AAMΦ, DCs, eosinophils and Th2 cells forms around the invading larvae, creating a distinct granulomatous structure ultimately killing Hp larvae before their maturation. Extensive research has identified key immune pathways required for functional immunity to *H. polygyrus*, however the initial pathogen-derived triggers and the early events crucial for the development of $CD4^+$ Th2 cells are still incompletely understood, including the role of ILC2s for the priming of Th2 cells in the MLN.

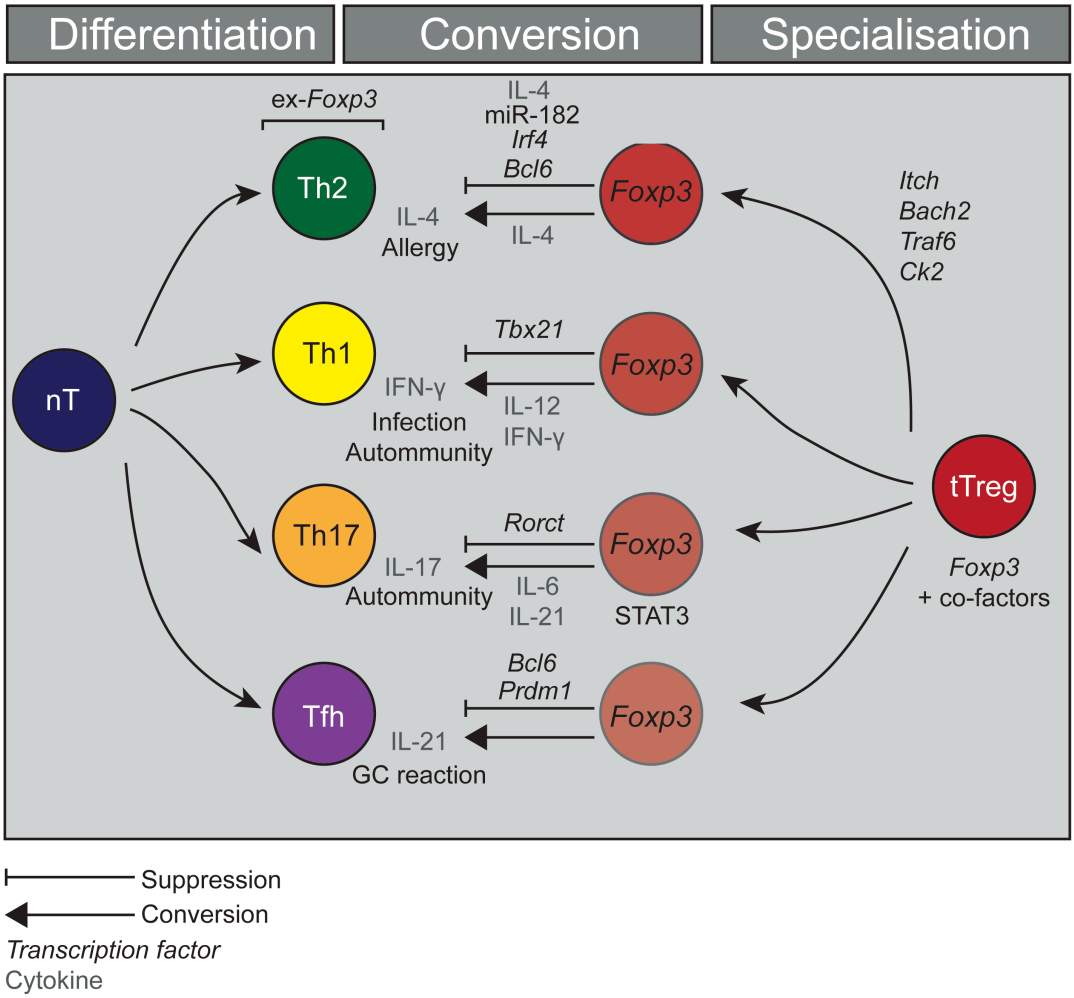


Figure 1.5. Treg specialisation and plasticity. Schematic diagram of Treg specialisation and plasticity. Foxp3$^+$ Treg cells express TFs that are required for the stability of Foxp3 expression. For example, upon Treg-specific deletion of *Itch, Bach2, Traf6 and Ck2* expression, Treg cells lose *Foxp3* expression fail to limit the development of type-2 inflammatory disease. Furthermore, Treg cells can upregulate TFs associated with the development and differentiation of T helper cell subsets, enabling more specialised suppression. For example, IL-4 stimulated the expression of miR-182, which was required for their suppression of Th2 cells. Finally, cytokines can also promote loss of *Foxp3* expression and conversion of Treg to T helper cells. For example, IL-4 was shown to stimulate the loss of *Foxp3* expression *in vitro* and *in vivo* and result in the reprogramming of Foxp3$^+$ cells to IL-4-expressing Th2 cells in the context of oral allergy.

Chapter 2: Materials and Methods

2.1. Animals

All mice used in this study were bred and maintained under specific pathogen-free conditions at the Mill Hill Laboratory, Francis Crick Institute. Strains used include: C57BL/6, B6/CD45.1, C57BL/6 *Rag2*$^{-/-}$(Shinkai et al., 1992), SJL *Rag2*$^{-/-}$*Il2*$^{-/-}$ crossed with C57BL/6 at the Mill Hill Laboratory, *Tcra*$^{-/-}$, TRE-IL-7R.BrtTA.C^{huCD2}*Il7r*$^{-/-}$ (Buentke et al., 2006) and DEREG (Lahl et al., 2007) mice. Double-reporter mice (*Il4*-GFP*Foxp3*-RFP) were established by crossing *Il4*-GFP (4get) (Mohrs et al., 2001) mice with *Foxp3*-RFP (FIR) (Wan and Flavell, 2005) mice. Fate-reporter mice (*Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635}) were established by crossing *Foxp3*$^{YFP\text{-}Cre}$ (Rubtsov et al., 2008) mice with *Il4*-GFP (4get) (Mohrs et al., 2001) mice and *R26R*^{FP635} (Coomes et al., 2015) mice. *Il4ra*$^{-/-}$ fate-reporter mice (*Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635}*Il4ra*$^{fl/fl}$) were established by crossing *Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635} fate-reporter mice with C57BL/6 *Il4ra*$^{flox/flox}$ (Herbert et al., 2004) mice (backcrossed onto the C57BL/6 background by Judi Allen, University of Edinburgh). Bone marrow from the following strains were used for the generation of bone marrow chimeras: C57BL/6 *Il4ra*$^{-/-}$ (Jenkins et al., 2013), C57BL/6 *Il4*$^{-/-}$ (backcrossed onto the C57BL/6 background by Judi Allen, University of Edinburgh), *Foxp3*$^{-/-}$ (Fontenot et al., 2003) and ROR$\alpha^{sg/sg}$ (Sidman et al., 1962). Animal experiments were performed according to institutional guidelines and following UK Home Office regulations (project license 80/2506) and were approved by the UK National Institute for Medical Research Ethical Review Panel. Mouse strains were genotyped by PCR analysis of tail DNA and using Transnetyx Automated Genotyping.

2.2. *Heligmosomoides polygyrus* infection

The *Heligmosomoides polygyrus* lifecycle was maintained at Mill Hill Laboratory, The Francis Crick Institute. C57BL/6 lifecycle mice were infected with 200 *H. polygyrus* larvae. Faeces from mice with established infections (day 14-30 post-

infection) were spread on to damp filter paper and covered with activated charcoal. The filter paper was placed into a petri dish containing Milli-Q purified water and in a damp box protected from light exposure. From around day 7-10, infective third stage larvae (L3) accumulated in the purified water. The larvae were collected into 50ml tubes, filtered through 100µm filters (Fisher), spun at 200g for 5 minutes and adjusted to the appropriate concentration before use. Experimental mice were infected with 200 *H. polygyrus* L3 larvae by oral gavage. For some experiments, mice were treated with 2.5mg/mL Pyrantel Embonate (Pfizer) on day 14 and 15 post-infection and subsequently given a challenge infection with 200 *H. polygyrus* larvae on day 28.

2.3. Worm counts

The small intestine was isolated from infected mice and opened longitudinally in PBS. The dissected small intestine was laid out lumen side up and adult worms were removed one at a time and counted using a stereoscopic microscope (SMZ-2B; Nikon). For worm counts at d9, the intestine was laid out lumen down in a petri dish and L5 larvae were counted in the outer wall of the intestine before counting luminal worms as described above.

2.4. IL-2 complex and antibody treatments

Where indicated, mice were treated with IL-2 complex (IL-2c) i.p. IL-2c was formed of recombinant IL-2 (R&D) and anti-IL-2 antibody (clone JES6-1A12; BioXcell). They were prepared at a 1:10 ratio of IL-2:anti-IL-2 in sterile PBS for i.p delivery. 3 doses were used in this study, IL-2c^{high} (5µg:50µg), IL-2c^{med} (2.5µg:25µg) and IL-2c^{low} (0.5µg:5µg). For each experiment, 3 treatments are delivered to the mice every other day, on days 0, day 2 and day 4 (as specified in figure legends). For depletion of ILCs, *Rag2*$^{-/-}$ mice were injected with 300µg of anti-Thy1.2 antibody (clone 30H12; BioXCell) every other day for 5-6 treatments (as detailed in the specific figure legends) starting

one day before infection with *H. polygyrus*. Control mice received 300µg of isotype control antibody (clone LTF-2; BioXCell) at the same time-points.

2.5. Preparation of single-cell suspensions

2.5.1. Isolation of cells from the spleen, mesenteric lymph nodes and Peyer's patches

The spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP) were dissected from mice and kept on ice in cIMDM (complete Iscove's Modified Dulbecco's Medium (Gibco) containing 1% FBS, 1mM EDTA, 100 U/mL Penicillin and 100 µg/mL Streptomycin (Gibco), 8 mM L-glutamine (Gibco), and 0.05 mM 2-mercaptoethanol (Gibco)). Single cell suspensions were prepared by gently mashing the organ through a 40µM filter (ThermoScientific) using a 5mL syringe plug. Cells were washed using PBS and centrifuged the cells at 1500pm (450g for 5min). Splenic red blood cells were lysed using ACK lysis buffer (Life Technologies; 1mL per spleen for 2min), washed with 10mL PBS and centrifuged (1500prm, 5min). Cells were resuspended in cIMDM and prepared for Fluorescence Activated Cell Sorting (FACS) analysis or FACS sorting.

2.5.2. Isolation of lamina propria cells

For the isolation of lamina propria cells, the small intestine (SI) was dissected from mice making sure to remove all the mesenteric fat and PP. In a petri dish placed on ice, intestines were opened up longitudinally and the faecal contents were shaken out in PBS, twice. Intestines were cut into approximately 2-inch segments. Using a scalpel blade, the mucus layer was gently scraped off the lumen of the intestine avoiding damage to the lamina propria. Intestines were washed in PBS, resuspended in PBS containing 5% FBS and 25mM HEPES (Lonza) and centrifuged (1500rpm, 3min). The IEL fraction was separated by incubating the intestines in PBS containing 10% FBS, 15mM HEPES, 5mM EDTA (Life Technologies) and 1mM Dithiothreitol

(Sigma) for 25min in a bacterial shaker at 37°C. IEL fractions were discarded through a wide mesh into a beaker. To obtain the lamina propria fraction, the tissue was washed 2-3 times in PBS containing 5% FBS and 25mM HEPES, centrifuged (1500rpm, 3min) and incubated in cIMDM containing 0.5mg/ml Liberase TL (Roche) and 60μg/ml DNAse (Roche) for 25-30min in a 37°C shaker. The digested tissue was passed through a 100μm filter and centrifuged (1500rpm, 5min). To separate lymphocytes from the digested tissue, the cells were layered onto 40% isotonic Percoll (GE Healthcare) in cIMDM and centrifuged (2000rpm, 20min with applied breaks). Cells were resuspended in cIMDM and prepared for FACS analysis or sorting.

2.5.3. Cell counting

Aliquots of single-cell suspensions were diluted in cIMDM (1:5, 1:10, 1:20) and mixed at a 1:1 ratio in Trypan blue (Sigma). Live cells were counted using a cell counting haemocytometer (Hawksley) and a LED inverted light microscope (Leica).

2.5.4. Cytospins

FACS purified cells were fixed onto a slide using a Cytospin 4 cytocentrifuge (Thermo Scientific) and stained with a modified Giemsa stain (Sigma). Slides were scanned using a scanning light microscope. Pictures were taken at 60X with oil immersion using an Olympus IX71 inverted microscope (Olympus), captured with a Camera QIClick colour CCD camera (QImaging) and processed using Image Pro-Plus software (Media Cybernetics). Scanned images were analysed using Fiji (Open Source).

2.6. Cell sorting and Flow Cytometry

Cell sorting was performed using a FACS Aria II (BD Biosciences) or Influx (BD Biosciences). When sorting $CD4^+$ T cells, CD4-expressing cells were first enriched

using MACS CD4 (L3T4) positive selection Microbeads and a MACS column (Miltenyi Biotech) according to the manufacturer's instructions. Single cell suspensions were incubated on ice for 15 minutes with CD4 Microbeads in sterile PBS containing 0.5% FBS and 0.5mM EDTA. Cells were passed through magnetic selection columns and magnetically labelled cells were flushed into a 15mL flacon tube and centrifuged (1500rpm, 5min). When sorting ILCs from the MLN or lamina propria, single cell suspensions were obtained (as described above) and stained directly. Antibodies were made up to concentrations as indicated in Table 1 in PBS containing 2% FBS. Cells were stained in antibody mix for 25 min at 4°C, washed, centrifuged (1500rpm, 5min) and diluted in phenol-red free cIMDM for sorting. Sorted fractions were collected into phenol-red free cIMDM containing 20% FBS. FACS analysis was performed using a LSR II (BD Biosciences) or LSR Fortessa (BD Bioscience) FACS analyser. For FACS analysis, antibodies were made up to concentrations as indicated in Table 1 in PBS containing 2% FBS. 2-6x10^6 cells were stained in 100µl of antibody mix for 25 min at 4°C, washed, centrifuged (1500rpm, 5min) and resuspended in PBS containing 2% FBS. Cells were sometimes fixed in 4% Formaldehyde (Sigma) for 20 min at 4°C for FACS analysis. For intra-cellular cytokine staining, cells were restimulated in cIMDM containing PMA (0.05 mg/mL phorbol 12-myristate 13-acetate (PMA) (Promega) and 0.1 mg/mL ionomycin (Sigma) in the presence of GolgiStop (BD) and GolgiPlug (BD) for 6 hours at 37°C. Cells were washed, centrifuged and fixed using the Fix/Perm kit (eBioscience) as per the manufacturer's instructions, or with PBS containing 2% Formaldehyde (Sigma) for 20min at 4°C. Cells were permeabilised in 1X permeabilisation buffer (eBioscience) for 30min at 4°C followed by FACS antibody staining in permeabilisation buffer for 25 min at 4°C. For all FACS sorting and analysis, viability of the cells was determined using the LIVE/DEAD Fixable Yellow or LIVE/DEAD Fixable Blue kit (Life Technologies).

2.7. Adoptive cell transfer model

Specified reporter mice were infected with 200 *H. polygyrus* larvae for 14 days. $CD4^+TCR\beta^+$*Foxp3*-RFP^+*Il4*-GFP^-CD25^{high} (HpTR) cells were FACS-purified (as described above) from CD4-enriched spleens and MLNs of *H. polygyrus* infected reporter mice day 14 post-infection. In some experiments, cells were FACS-purified as $CD4^+TCR\beta^+$*Foxp3*$^-RFP^+$*Il4*-GFP^-CD25^{low} (as specified in the figure legend). $CD4^+TCR\beta^+$*Foxp3*$^-RFP^-$*Il4*-$GFP^-CD25^-CD44^{low}$ naïve T cells (nT) were sorted naïve double-reporter mice. Sort purified T cells were counted (as described above) and diluted in sterile PBS for intra-venous (i.v) delivery. For each experiment, $0.5x10^6$ to $1.5x10^6$ HpTR or nT cells were injected into *Tcra*$^{-/-}$ mice infected with 200 *H. polygyrus* larvae on the day of transfer. Recipient mice were drug-cured and infected with a secondary *H. polygyrus* infection (as described above).

2.8. *In vitro* cell cultures and stimulation assays

2.8.1. T helper cell polarisation

Mice were infected with 200 *H. polygyrus* larvae for 14 days. $CD4^+TCR\beta^+$*Foxp3*-RFP^+*Il4*-GFP^-CD25^{high} (HpTR) cells were FACS-purified (as described above) from CD4-enriched spleens and MLNs of *H. polygyrus* infected reporter mice day 14 post-infection. $CD4^+TCR\beta^+$*Foxp3*$^-RFP^-$*Il4*-$GFP^-CD25^-CD44^{low}$ nT cells were FACS-purified from naïve double-reporter mice. Sort purified cells were resuspended in cIMDM at a concentration of $1x10^6$/mL. $1x10^5$ HpTR or nT cells were plated onto tissue-culture treated flat-bottom 96-well plates coated with CD3 (BioXCell; 1µg/mL) and CD28 (BioXCell; 10µg/mL) antibody at 37°C for 2-3 hours. A mix of recombinant IL-4 (Peprotech; 20ng/mL) and recombinant IL-2 (R&D; 20ng/mL) was added to the wells (final concentration of cells $1x10^5$, final concentration of cytokine 10ng/mL). At day 3, cells were removed from the plate and transferred to a round-bottom plate. Cells were harvested at day 7 for FACS analysis.

2.8.2. T cell-ILC2 co-cultures

$CD45^+Lin^-Thy1.2^+KLRG1^+Sca1^+$ (ILC2) cells were sort purified from the LP or MLN as described above. Cells were counted, centrifuged (1500rpm, 5min) and resuspended at a final concentration of $5x10^4$ cells per 50µl. ILC2s were cultured in cIMDM containing PMA (0.05 mg/mL phorbol 12-myristate 13-acetate (PMA) (Promega) and Ionomycin (0.1 mg/mL) (Sigma) for 24H. For some experiments (as specified in figure legends), the cell cultures were centrifuged after 3H and the supernatant containing PMA+I was replaced with fresh cIMDM for the remaining 21H. Supernatants were harvested 24H post-stimulation and stored at -20°C. $CD4^+TCR\beta^+$*Foxp3*$^-$$RFP^-$*Il4*-$GFP^-CD25^-CD44^{low}$ nT cells were FACS-purified from naïve double-reporter mice. Sort purified cells were resuspended in cIMDM at a concentration of $1x10^6$/mL. $1x10^5$ nT cells were plated onto tissue-culture treated flat-bottom 96-well plates coated with CD3 (1µg/mL) and CD28 (10µg/mL) antibody (BioXCell) at 37°C for 2-3 hours. Cells were resuspended in 50µl ILC2-derived supernatant with or without the addition of 10µg/ml of anti-IL-4 antibody (BioXCell).

2.8.3. Macrophage differentiation and culture

Bone marrow (BM) cells were flushed from the femur of male C57BL/6 mice, filtered through a 40µM filter and centrifuged (1500rpm, 5min). Cells were lysed in ACK lysis buffer (Life Technologies; 2mL per mouse for 1.5min), washed and centrifuged (1500rpm, 5min). Cells were counted and $5x10^6$ cells were plated in petri dishes in conditioned media (Dulbecco's Modified Eagle Medium with GlutaMAX (Life Technologies), 20% L-cell 929 (in-house media kitchen, Mill Hill Laboratory), 10% FBS, 10mM HEPES, 100 U/mL Penicillin and 100µg/mL Streptomycin (Gibco), 2.7mM L-glutamine (Gibco), 0.05mM 2-mercaptoethanol (Gibco), and 1mM Sodium Pyruvate (Lonza)). Additional conditioned media was added at day 4. Adherent cells, defined as bone-marrow derived macrophages (BMDMs), were harvested at day 7 and

resuspended in 1% DMEM with GlutaMAX (Life Technologies), 1% FBS, 10mM HEPES, 100 U/mL Penicillin and 100μg/mL Streptomycin (Gibco), 2.7mM L-glutamine (Gibco), 0.05 mM 2-mercaptoethanol (Gibco), and 1mM Sodium Pyruvate (Lonza). $1x10^6$ BMDMs were plated in 24-well flat bottom tissue-culture treated plates and left to rest for 24 hours. $1x10^5$ FACS-purified T cells were resuspended in 1% DMEM containing soluble CD3 (1μg/mL) and soluble CD28 (10μg/mL) antibody (BioXCell) and cultured with the BMDMs for 24H. As a control, BMDMs were co-cultured in the presence of recombinant IL-4 (Peprotech; 20ng/mL) and IL-13 (Peprotech; 20ng/mL) or media alone. Non-adherent cells were washed off after 24H and adherent activated BMDMs were harvested for downstream analysis.

2.8.4. Treg suppression assay

$CD4^+TCR\beta^+CD25^-CD44^{low}$ nT cells were FACS-purified from CD45.2 C57BL/6 mice. $CD4^+TCR\beta^+$ *Il4*-GFP^- *Foxp3*-RFP^+ (HpTR→ *Foxp3*-RFP^+), $CD4^+TCR\beta^+$ *Il4*-GFP^- *Foxp3*-RFP^- (HpTR→ DN) or $CD4^+TCR\beta^+$ *Il4*-GFP^+ *Foxp3*-RFP^- (HpTR→ *Il4*-GFP^+) cells were FACS-purified from the spleen and MLN of $Tcra^{-/-}$ mice day 42 following adoptive transfer. nT were counted, centrifuged (1500rpm, 5min) and resuspended in PBS containing Cell Tracker Violet (CTV) (5mM, Life Technologies). The cells were incubated for 20 min at 37°C (final cell density $1x10^6$/mL, final CTV concentration, 5μM). Following this, nT cells were quenched in pre-warmed PBS, centrifuged (1500rpm, 5min) and resuspended in cIMDM. nT cells were cultured alone or with an equal number of FACS-purified HpTR→ *Foxp3*-RFP^+, HpTR→ DN or HpTR→ *Il4*-GFP^+ cells at varying ratios (1:1, 1:5 and 1:10) in the presence of CD3/CD28 activator Dynabeads (Life Technologies) and harvested after 64h for FACS analysis.

2.8.5. Generation of bone marrow chimeric mice

BM cells were isolated by flushing adult femurs or by gently crushing bones isolated from juvenile $ROR\alpha^{sg/sg}$ and $Foxp3^{-/-}$ mice using a pestle and mortar. BM cells

were filtered through a 40μM filter and centrifuged (1500rpm, 5min). BM cells were lysed in ACK lysis buffer (Life Technologies; 2mL per mouse for 1.5min), washed, centrifuged (1500rpm, 5min) and stored in 90% FBS with 10% DMSO in liquid nitrogen until needed. To set up bone-marrow chimeras, cells were thawed and counted (as described above). The cells were mixed at the required ratios and diluted in sterile PBS for i.v delivery. *Tcra*$^{-/-}$ or TRE-IL-7R.BrtTA.C^{huCD2}*Il7r*$^{-/-}$ mice were irradiated with 900 RADs. 3-5x10^{6} BM cells (depending on the experiment) were transferred intravenously to mice. Mice were fed antibiotics for 3 weeks and the BM cells were left to reconstitute for 7-8 weeks before the start of the experiment.

2.9. RNA extraction and gene expression analysis

2.9.1. RNA extraction from tissue samples

Tissue samples were harvested and frozen in RNA*later*® at -20°C. Tissue samples were homogenised in 500μl Qiazol (Qiagen) using a Precellys Homogeniser (Precellys). 200μl chloroform was added and the samples were shaken and left at room temperature for 10min. The samples were spun in a table-top microcentrifuge at maximum speed for 15min. Supernatants were added to EtOH and samples were processed using the RNAeasy kit (Qiagen) according to the manufacturer's instructions and eluted in 30-50μl RNAse/DNAse-free water (5 Prime).

2.9.2. RNA extraction from cells

Sort purified cells were harvested and stored in RLT lysis buffer (Qiagen) at -20°C. The RNeasy kit (Qiagen) was used to perform RNA extractions according to the manufacturer's instructions and eluted in 30-50μl of RNAse/DNAse-free water (5 Prime). For quantitative real-time polymerase chain reaction (qRT-PCR), RNA concentrations were quantified using a Nanodrop 1000 (Thermo Scientific).

2.9.3. Real-Time PCR

RNA concentrations were quantified using a Nanodrop 1000 (Thermo Scientific). 0.1-1µg RNA was reverse transcribed using the Qiagen Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. For some experiments, the cDNA produced was diluted with RNAse/DNAse-free water before proceeding. cDNA was used for real-time PCR analysis using *Power* SYBR® Green Master Mix and analysed using an Applied Biosystems 7900HT Fast Real-Time PCR System (both Applied Biosystems). The PCR conditions were as follows: 95°C for 15min, 40 cycles of 94°C for 30s, 55°C for 30s, 70°C for 30s followed by a melting step of 95°C for 15s, 60°C for 15s, 95°C for 15s. Gene expression was normalised to the house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). In some experiments gene expression was expressed as fold-change relative to naïve or PBS treated mice (as specified in the figure legends). Sequences for the primers used are listed in Table 2. All primers were used at 1pmol/mL.

2.9.4. Microarray analysis and IPA analysis

$CD4^+TCR\beta^+$*Foxp3*-RFP^+*Il4*-GFP^-CD25^{high} (HpTR) cells were FACS-purified from CD4-enriched spleens and MLNs of *H. polygyrus* infected reporter mice day 14 post-infection. $CD4^+TCR\beta^+$*Foxp3*$^-$$RFP^-$*Il4*-$GFP^-CD25^-CD44^{low}$ nT cells were FACS-purified from naïve double-reporter mice. $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^+ (HpTR→ *Foxp3*-RFP^+), $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^- (HpTR→ DN) or $CD4^+TCR\beta^+$*Il4*-GFP^+*Foxp3*-RFP^- (HpTR→ *Il4*-GFP^+) were FACS-purified from HpTR $Tcra^{-/-}$ recipients and $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^+ (nT→ *Foxp3*-RFP^+), $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^- (nT→ DN) or $CD4^+TCR\beta^+$*Il4*-GFP^+*Foxp3*-RFP^- (nT→ *Il4*-GFP^+) were FACS-purified from nT $Tcra^{-/-}$ recipients day 42 following adoptive transfer. RNA was extracted from the FACS-purified populations and concentrated using a MiVac DNA concentrator (Barnstead, Genevac). The Systems Biology Unit at Mill Hill Laboratory, The Francis Crick Institute processed samples for microarray analysis. RNA quality

was determined using a 2100 Bioanalyzer (Agilent). RNA concentrations were determined using a Qubit 2.0 Fluorometer (Life technologies). cDNA was amplified from 20ng total RNA using the Ovation® Pico WTA system (version 2) (NuGEN) . Amplified cDNA was fragmented and labelled using the Encore Biotin Module (NuGEN). Labelled cDNA was hybridised to an Affymetric GeneChip® mouse Genome 430A 2.0 microarray using the GeneChip® Hybridization, Wash and Stain Kit (Affymetrix) and ran on the 450 Affymetrix GeneChip Fluidics Station (Affymetrix) followed by scanning on the 3000 7G Affymetrix GeneChip Scanner (Affymetrix). Microarray data was analysed using the GeneSpring software (Agilent). Samples were normalised using the Affymetrix MicroArray Suite 5 method (MAS5) and filtered by Flags and expression (20-100 percentile). Differentially expressed genes were determined using unpaired T-tests relative to nT control cells. Genes with false discovery rate-corrected P values <0.05 and fold change values ≥2.0 were considered significant. 3 biological replicates of each sample were used. Each biological replicate contains cells pooled from 3-4 mice. 3-4 way comparative analyses and hierarchical clustering were performed on significantly expressed genes using GeneSpring (Agilent). Predicted upstream regulators were determined using Ingenuity Pathways Analysis (Ingenuity systems).

2.10. Bisulphite modification of DNA and sequencing

2.10.1. Bisulphite modification of DNA

$CD4^+TCR\beta^+$*Foxp3*-RFP^+*Il4*-GFP^-CD25^{high} (HpTR) cells and $CD4^+TCR\beta^+$*Foxp3*-RFP^-*Il4*-GFP^+ (*Il4*-GFP^+) cells were FACS-purified from CD4-enriched spleens and MLNs of *H. polygyrus* infected reporter mice day 14 post-infection. $CD4^+TCR\beta^+$*Foxp3*-RFP^-*Il4*-$GFP^-CD25^-CD44^{low}$ nT cells were FACS-purified from naïve double-reporter mice. $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^+ (HpTR→ *Foxp3*-RFP^+), $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^- (HpTR→ DN) or $CD4^+TCR\beta^+$*Il4*-GFP^+*Foxp3*-RFP^- (HpTR→ *Il4*-GFP^+) were FACS-purified from HpTR $Tcra^{-/-}$ recipients day 42

following adoptive transfer. *Foxp3*-YFP-Cre^{+}*Il4*-GFP^{-} (*Foxp3*-YFP-Cre^{+}) *Foxp3*-YFP-Cre^{-}*Il4*-GFP^{+} (*Il4*-GFP^{+}) *Foxp3*-YFP-Cre^{-}*Il4*-GFP^{+}FP635^{+} (*Il4*-GFP^{+}FP635^{+}) cells were sorted form the spleen and MLN of fate-reporter mice day 7 following secondary infection. Sorted cells were centrifuged (2500 rpm, 5min) and cell pellets were digested in 1X TE buffer containing proteinase K (200μg/ml) and 0.5% sodium dodecyl sulphate (SDS, Life Technologies) at 55°C for 3-4 hours. DNA was extracted using Phenol/Chloroform (Sigma), centrifuged (13,000rpm, 10min) and washed in 100% chloroform (Sigma). The aqueous phase was removed and added to 100% ethanol for DNA precipitation. For large quantities of DNA, the DNA was fished using a glass pipette, washed in 70% ethanol and eluted in 30-40μl dH_2O. DNA concentrations were measured using a Nanodrop 1000 (Thermo Scientific). For smaller quantities, the DNA was precipitated in 100% ethanol at -20°C for 20min, then centrifuged (13,000rpm, 10min). Supernatant was gently removed and the pellet was eluted in 30-40μl dH_2O. DNA concentrations were measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life technologies). Bisulphite modification was performed as described previously (Clark et al., 1994). Briefly, 1μg of DNA was denatured in a final concentration of 0.3M NaOH at 37°C for 15min. 2M Meta Bisulphite (Sigma) and 100mM Hydroquinione (Sigma) were made up in distilled water and the pH was adjusted to 4.5-5. Denatured DNA was modified in Bisulphite (6.14 x $_{V}$DNA) containing 0.5mM Hydroquinone. The sample was gently mixed, overlayed with mineral oil and incubated at 55°C overnight (16-20hours). DNA was recovered from under the mineral oil and purified using ChIP DNA Clean & Concentrator kit (Zymo Research) according to the manufacturer's instructions and eluted in 50μl elution buffer. Freshly prepared NaOH was added to a final concentration of 0.3M at 37°C for 15min. The solution was neutralised with 3M Ammonium acetate. DNA was ethanol precipitated as described above and eluted in 20-50μl dH_2O. DNA concentrations were measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life technologies). Efficiency of DNA modification was assessed by

qRT-PCR with methylation specific primers (see Table 2) designed on UroGene (Li and Dahiya, 2002).

2.10.2. Cloning and sequencing

PCR reactions were performed on MasterCycler Gradient S PCR cycler (Eppendorf) in a final volume containing 1X PCR buffer, 0.5U Taq Polymerase (Thermo Scientific), 0.4mM dNTPs, 0.2-1pmol each of forward and reverse primers and 0.1μg DNA. The amplification conditions were 94°C for 3min, 10 cycles of 94°C for 30s, 63°C down to 53°C for 40s (touchdown), 72°C for 45s, 40 cycles of 94°C for 30s, 53°C for 30s and 72°C for 45s, and a final extension step of 72°C for 5min. Primers used for amplification of bisulphite modified DNA for sequencing (see Table 2) were designed based on previous studies (Floess et al., 2007) using MethPrimer primer design software (Li and Dahiya, 2002). PCR products were gel purified using QiaQuick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was concentrated using a speed vacuum concentrator. DNA was cloned into OneShot TOP10 Chemically competent E. coli according to the manufacturer's instructions and cells were plated on ampicillin plates (100mg/L) with X-gal (20mg/ml). Clones were cultured overnight in LB Broth (in-house media kitchen, Mill Hill Laboratory, The Francis Crick Institute) containing 0.1mg/mL ampicillin (Sigma). 10-16 clones were sent for sequencing (Source Bioscience). Sequencing results were analysed using Seqbuilder and online Quantification Tool for Methylation Analysis (QUMA) (Kumaki et al., 2008).

2.11. Cytokine measurements

2.11.1. ELISA

IL-5 and IL-13 were measured in cell culture supernatants using DuoSet ELISA kits, according to the manufacturer's instructions (R&D systems – Biotechne).

2.11.2. Flow Cytomix

IL-4, IL-2, IL-5 and IL-13 were measured in cell culture supernatants using the FlowCytomix™ flow cytometry multi analyte detection system according to the manufacturer's instructions (eBioscience).

2.12. Western Blot

For immunoblotting, cells were lysed in 1X RIPA buffer (500mM Tris HCl Ph2.5, 150mM NaCl, 2mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet-P40) containing protein inhibitors as per the manufacturer's instructions (diluted 1:50; Roche), 5mM NaF, 1mM Na3VO4, 100nM Okadoic acid, 2mM Na4P2O7 and MilliQ water. Cell lysates were normalised to equal total protein content using the Pierce BCA Protein Assay Kit (Life Technologies) and resolved on 10% Criterion^{TM} TGX^{TM} Gels (Biorad). Separated proteins were transferred onto Trans-Blot® Turbo^{TM} PVDF transfer (Biorad) membranes. Membranes were blocked in 0.1% PBST (Sigma) containing 20% milk (Sigma) and then incubated with primary (pSTAT6, STAT6; Cell Signalling, αTubulin; in-house) and secondary (Rabbit IgG; GE Healthcare) antibodies in 0.1% PBST (Sigma) containing 10% milk (Sigma). Membranes were washed in PBST and specific bound antibodies were visualised by chemiluminescence (Immobilon; Merck Millipore).

2.13. Statistical analysis

Data sets were compared by unpaired t-tests (two-tailed Mann Whitney test) using GraphPad Prism (V.5.0). Some graphs were analysed using one-tailed Mann Whitney test (as specified in the figure legends). Differences were considered significant at *$P \leq 0.05$.

2.14. FACS antibodies and FACS-sorting staining panel

Marker	Clone	Conjugate	[C] μg/mL
CD4	RM4-5	APC, BioL; eFluor 450, eBio	0.5-1.0
CD4	MCD0430	Pacific Orange, Invitrogen	0.5
CD8	53-6.7	APC, BioL	0.5
CD3	145-2C11	APC, BioL	1.0
TCRαβ	H57-597	PeCy7, PerCPCy5.5, BioL; APC, eBio	0.5-1.0
TCRγδ	GL3	APC, BioL	1.0
CD44	IM7	PeCy7, Pacific Blue, BioL; PerCPCy5.5 eBio	1.0-2.5
CD25	PC61	APCCy7, BioL; PerCPCy5.5 eBio	1.0
CD103	2E7	APC, eBio	1.0
CD49b	DX5	APC, BioL; PB, eBio	1.0-2.0
CD11c	N418	APC, BioL; PeCy7, eBio	1.0
CD11b	M1/70	APC, BioL	0.1
CD19	6D5	APC, BioL	0.5
CD45.1	A20	APC, eBio	1.0
CD45.2	104	PeCy7, BioL	1.0
CD45	30-F11	APCCy7, BioL; FITC eBio	0.5-1.0
CD69	H1-2F3	PB, eBio	1.0
CTLA-4	UC10-4B9	APC, BioL	1.0
GITR	ebioAITR	eFluor 450, eBio	1.0
Foxp3	FJK-16S	PE, eBio	1.0
IL-13	eBio13A	Alexa Fluor 288, eBio	2.5
IL-4	11B11	PE, eBio	1.0
IL-5	554396	APC, BD Biosciences	1.0
Thy1.2	53-2.1	PeCy7, BioL	0.5
KLRG1	2F1	PErCPe710, eBio	1.0
IL7Ra	A7R34	PE, BioL	1.0

FCER1	MAR-1	APC, BioL	1.0
NK1.1	PK136	APC, BioL	1.0
Sca1	E13-161.7	PB, eBio	2.5
Siglec-F	E50-2240	PE, BioL	
c-kit	2B8	PerCPCy5.5, BioL	1.0
Gr-1	RB68C5	APC, BioL	0.5
Ter119	TER-119	APC, BioL	1.0
CXCR5	2G8	Biotin, BD Biosciences	2.5
PD-1	29F.1.A12	PeCy7, BioL	1.0

ILC2 sorting panel: Live, lymphocytes, CD45$^+$, Lineage$^-$(CD3, CD4, CD8, CD19, CD11c, CD11b, NK1.1, TCRβ, TCRγδ, Gr-1, CD49b, Ter119), Thy1.2$^+$, KLRG1$^+$, Sca1$^+$

HpTR sorting panel: Live, lymphocytes, CD4$^+$, TCRβ$^+$, *Il4*-GFP$^-$, *Foxp3*-RFP$^+$, CD25^{high}

2.15. PCR primers

RT-PCR Primers

	Forward	**Reverse**
Hprt	GCCCTTGACTATAATGAGTACTTCAGG	TTCAACTTGCGCTCATCTTAGG
Il4	ACGAGGTCACAGGAGAAGGGA	AGCCCTACAGACGAGCTCACTC
Il5	TGACAAGCAATGAGACGATGAGG	ACCCCCACGGACAGTTTGATTC
Il13	CCTCTGACCCTTAAGGAGCTTAT	CGTTGCACAGGGGAGTCTT
Arg1	GGAAAGCCAATGAAGAGCTG	GCTTCCAACTGCCAGACTGT
Ym1	CATGAGCAAGACTTGCGTGAC	GGTCCAAACTTCCATCCTCCA
Retnla	CCCTCCACTGTAACGAAGACTC	CACACCCAGTAGCAGTCATCC
Retnlb	ATGGGTGTCACTGGATGTGCTT	AGCACTGGCAGTGGCAAGTA
Muc5ac	CAGGACTCTCTGAAATCGTACCA	AAGGCTCGTACCACAGGGA
Mcl3a3	CATCGCCATAGACCACGACG	TTCCAGCTCTCGGGAATCAAA

Il9	ATGTTGGTGACATACATCCTTGC	TGACGGTGGATCATCCTTCAG
Il22	GTGAGAAGCTAACGTCCATC	GTCTACCTCTGGTCTCATGG
Il17A	ACCCTGGACTCTCCACCGCAA	GGCTGCCTGGCGGACAATCG
Rorct	GGAGCTCTGCCAGAATGACC	CAAGGCTCGAAACAGCTCCAC
Gata3	CGGGTCGGCCAGGCAAGATG	AGGGGACCTCCCAGCAGGC
Il10	ATGCTGCCTGCTCTTACTGACTG	CCCAAGTAACCCTTAAAGTCCTGC
Il4ra	TCCCATTTTGTCCACCGAAT	GTTTCTAGGCCCAGCTTCCA
Il2	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC
Il33	CACATTGAGCATCCAAGGAA	AACAGATTGGTCATTGTATGTACTCAG
Foxp3	GTGGGCACGAAGGCAAAG	CCTTGTTTTGCGCTGAGAGTCT

Bisulphite sequencing primers		
2a	TGGGTTTTTTTGGTATTTAAGAAAG	AAAAAACAAATAATCTACCCCACAA
2b2	GAAATTTGTGGGGTAGATTATTTGT	AACTAACCAACCAACTTCCTACACTAT
3	TTTTAAGTTTAAAATTAGTTTGGTTAA	CTCAAATCCTTTTTCTATCAAAAATAT

Methylation specific primers		
Mod	TGATTTTTTTAAAATATAAAGAAATACGG	TCCCAAATACTAAAATCAAAAACA TACG
Unmod	TGATTTTTTTAAAATATAAAGAAATATGG	TCCCAAATACTAAAATCAAAAACATACAC
Gen	TGACTCTTCTAAAACACAAAGAAACACGG	TCCCAAGTGCTGGGATCAAAGGCATGCG

Chapter 3: IL-4-secreting ILC2s are essential for the differentiation of Th2 cells following helminth infection

3.1. Introduction

ILC2s have emerged as key players in the development of type 2 immune responses. Gain and loss of function experiments have demonstrated that ILC2s were both sufficient and necessary for the functional expulsion of acute infection with the intestinal helminth, *N. brasiliensis* (Oliphant et al., 2014). However the function of ILC2s in a model of chronic intestinal infection such as an infection with the natural murine helminth *Heligmosomoides polygyrus* remains unclear. ILC2 numbers were shown to expand early following *H. polygyrus* infection (Mackley et al., 2015) and correlated with genetic resistance to *H. polygyrus* (Filbey et al., 2014). In addition, ILC2s co-localise with $CD4^+$ T cells in the mesenteric lymph nodes (Mackley et al., 2015). In light of recent work demonstrating antigen-dependent co-operation between ILC2 and T cells for the development of type-2 responses (Oliphant et al., 2014), we wanted to test whether ILC2s contributed to the differentiation of Th2 cells following infection with *H. polygyrus,* and whether this was through their ability to secrete Th2-associated cytokines.

ILC2s secrete type-2 cytokines such as IL-13, IL-5 and IL-9 in response to epithelial cell-derived alarmins (Moro et al., 2010), with ILC2-derived IL-13 being particularly important for immunity to *N. brasiliensis*. Transfer of WT, but not IL-13-deficient ILC2s resulted in the expulsion of *N. brasiliensis* in lymphodeficient mice (Neill et al., 2010). Furthermore, IL-13 production by ILC2s was shown to be required for the recruitment of $CD40^+$ DCs to the local draining lymph nodes and differentiation of Th2 cells resulting in *N. brasiliensis* clearance (Halim et al., 2014). However in addition to IL-13, IL-5 and IL-9, both murine and human ILC2s have been shown to secrete IL-4 (Mjosberg et al., 2012; Doherty et al., 2013) and IL-2 (Crellin et al., 2010; Molofsky et al., 2013), however the role of these cytokines from ILC2s has not been reported. Both IL-4 and IL-2 are essential for the differentiation of Th2 cells *in vitro* (Liao et al., 2008),

however the contribution of ILC2-derived IL-4 or IL-2 to Th2 differentiation remains unknown.

A variety of innate cells secrete IL-4 however their relative contribution to Th2 differentiation *in vivo* is still debated. Following the observation that ILC2s secrete IL-4*,* we hypothesised that ILC2-derived IL-4 may be an important early source of IL-4 for Th2 differentiation. IL-4 blockade *in vitro* (Oliphant et al., 2014) and *in vivo* (Halim et al., 2014) did not impair ILC2 effector function and IL-13 production in the context of *N. brasiliensis* or papain lung challenge respectively, however immunity to *N. brasiliensis* does not require IL-4 (Lawrence et al., 1996; Halim et al., 2012) suggesting that this may not be the most appropriate model for studying ILC2-derived IL-4. Conversely, IL-4 is both necessary (Urban et al., 1991) and sufficient (Urban et al., 1995; Herbert et al., 2009) for immunity to *H. polygyrus.* For these reasons we decided to test the role for ILC2-derived IL-4 in the context of *H. polygyrus* infection.

In this chapter, we tested the requirement of ILC2-derived IL-4 for the differentiation of Th2 cells during primary infection with *H. polygyrus.* Although ILC2s were increased following infection and following treatment with IL-2 complex (IL-2c), they were insufficient to mediate expulsion of *H. polygyrus* in the absence of T cells. Instead, we hypothesised that ILC2s cooperated with T cells and that ILC2-derived IL-4 was required for the differentiation of Th2 cells *in vivo*. We found that ILC2s isolated from the small intestine of *H. polygyrus* infected mice expressed *Il4*-GFP and secreted IL-4 protein *in vitro*. Furthermore, ILC2-derived IL-4 was sufficient to drive Th2 differentiation *in vitro* and ILC2s were required for the development of Th2 responses *in vivo.* In addition, we identified that the ILC2s did not require IL-2 for their development or effector function suggesting that in the context of early Th2 differentiation, ILC2s provide help to adaptive immune cells and not vice-versa.

3.2. Results

3.2.1. ILC2 expansion correlated with the development of type-2 responses during primary infection with *H. polygyrus*

3.2.1.1. Development of type-2 immune responses early following primary infection with *H. polygyrus*

We infected C57BL/6 mice with *H. polygyrus* and measured the gene expression of key type-2 response genes at days 5, 7, and 10 post-infection in the small intestine by qRT-PCR. We observed a significant elevation of the type-2 response as early as day 5 (d5) following infection in comparison to naïve mice (d0) (**Fig. 3.1**) including elevated levels of *Il13, Il5* and *Il4* throughout the timecourse (**Fig. 3.1**). Furthermore, the expression of epithelial-cell derived IL-33 (*Il33*), known to promote type-2 responses and activate ILC2s, was significantly elevated from day 3 post-infection. In addition, goblet cell-derived Relmβ (*Retnlb*), which is secreted downstream of IL-13 signalling, was significantly upregulated from d5 in comparison to uninfected mice (d0). Finally, the expression of genes associated with AAMΦ following IL-4 and IL-13 exposure such as Relmα (*Retnla*) and Arginase (*Arg1*), were significantly increased at day 5 post-infection, and followed the same pattern thereafter. Increased expression of type-2 genes also correlated with heightened mucus production by intestinal goblet cells as assessed by period acid-Schiff (PAS) staining, particularly at d7 and d10 (**Fig 3.1.B**), correlating with the highest expression of type-2 cytokines.

3.2.1.2. Expansion of ILC2s day 5 following primary infection with *H. polygyrus.*

Killer lectin-like receptor 1 (KLRG1) is an e-cadherin receptor (Henson and Akbar, 2009) expressed on many cells of the immune system including NK cells (Blaser et al., 1998) and activated $CD4^+$ T cells (Voehringer et al., 2002). Within the ILC population, GATA-3^+ ILC2s were shown to uniquely express KLRG1 (Hoyler et al.,

2012), permitting its use as a marker for ILC2s for FACS-based analysis. FACS-based gating strategies for identifying ILC2s had previously defined them as $Sca1^+$ (Moro et al., 2015) however because all $KLRG1^+$ cells also co-expressed Sca-1, throughout this study we chose to identify the ILC2 subset as either $KLRG1^+$ or $KLRG1^+Sca1^+$. In this study we defined $KLRG1^-$ cells containing a mixture of group-1 ILCs (ILC1) and group-3 ILCs (ILC3) as ILCs (Hoyler et al., 2012).

ILC2s have been shown to expand and produce cytokines such as IL-13 and IL-5 in response to IL-33 signalling (Artis and Spits, 2015). Following the observation that *Il33, Il13* and *Il5* expression were all significantly elevated at day 5 post-infection (**Fig. 3.1**), we hypothesised that ILC2 numbers would be increased at day 5 both in the tissue and local draining lymph node. We infected wild-type mice with *H. polygyrus* and observed a significant increase in the proportion and total number (**Fig 3.1.D**) of $CD45^+Lin^-Thy1.2^+KLRG1^+$ ILC2s in the lamina propria (LP) of mice infected with *H. polygyrus,* as shown in representative FACS plots of the lamina propria (**Fig.3.1 C**) and graphically (**Fig 3.1.D**). We also saw a significant expansion of the ILC2s in the MLN at day 5 following infection (**Fig.3.1.E**). In addition, we saw a significant increase in the number of $KLRG1^-$ ILCs in the LP at d5 (**Fig. 3.1 F**), as previously reported (Mackley et al., 2015).

3.2.1.3. Expansion of ILC2s correlated with the differentiation of Th2 cells

$CD4^+$ Th2 cells play an essential role in promoting early type-2 effector responses to intestinal helminths (Allen and Maizels, 2011). We therefore measured type-2 cytokine production by intracellular cytokine staining in $CD4^+$ T cells in both local (MLN) and peripheral (spleen) lymphoid organs at days 5, 7 and 10 post-infection. In the spleen, we saw a significant increase in the proportion and total number of $CD4^+CD44^+IL\text{-}4^+$ (**Fig.3.2 A** and **B**) and $CD4^+CD44^+IL\text{-}13^+$ Th2 cells (**Fig.3.2 C** and **D**) as early as day 5 post-infection and a further significant increase at day 7 (**Fig 3.2 A and C**). In the MLN, the proportion of IL-4- (**Fig.3.2 E**) and IL-13-producing Th2 cells

(**Fig.3.2 G**) was higher than in the spleen, with up to 10% of $CD4^+$ T cells producing IL-4 (**Fig.3.2 E**) and 6% of $CD4^+$ T cells producing IL-13 at d10 (**Fig 3.2. G**). The proportion and total number of $CD4^+CD44^+IL\text{-}4^+$ (**Fig.3.2 E** and **F**) and $CD4^+CD44^+IL\text{-}13^+$ Th2 cells in the MLN (**Fig.3.2 G** and **H**) were significantly increased from day 5 following infection.

In summary, a characteristic type-2 response developed early following *H. polygyrus* infection, as exemplified by the expression of genes and cytokines associated with the activation of AAMΦ, Th2 cells and ILC2s. The proportion and absolute numbers of ILC2s increased at day 5 post-infection in the tissue and peripheral lymphoid organs, correlating with the differentiation of Th2 cells and the initiation of a polarised type-2 response in the intestinal tissue.

3.2.2. ILC2s are a major source of IL-4 in the tissue following *H. polygyrus* infection

3.2.2.1. Phenotyping intestinal $KLRG1^+$ ILC2s

To further characterise *H. polygyrus*-elicited ILC2s we FACS-purified $CD45^+Lin^-Thy1.2^+KLRG1^+Sca1^+$ ILC2s from the LP of $Rag2^{-/-}$ mice and measured the expression of several genes known to be related to ILC2 development and function. As a control, we purified $CD45^+Lin^-Thy1.2^+KLRG1^-Sca1^{+/-}$ ILCs. Purified $KLRG1^+$ ILC2s expressed high levels of *Gata3* and type-2 cytokines *Il13* and *Il5* in comparison to $KLRG1^-$ ILCs (**Fig 3.3 A**). Conversely, purified $KLRG1^-$ ILCs expressed high levels of *Rorct, Il17* and *Il22,* characteristic of the ILC3 phenotype (**Fig 3.3 A**). Purified ILC2s were morphologically similar to previously published images (Moro et al., 2010; Robinette et al., 2015) and similar to purified *Il4*-GFP^+ $CD4^+$ T cells (Th2), indicative of their lymphoid origin (**Fig 3.3 B**). Concordant with the mRNA expression, *in vitro* re-stimulated $KLRG1^+$ ILC2s but not $KLRG1^-$ ILCs secreted high concentrations of IL-13 and IL-5 protein following re-stimulation with PMA and Ionomycin or with IL-2/IL-25/IL-33, as measured by FlowCytomix™ (**Fig. 3.3 C**).

3.2.2.2. ILC2s express *Il4*-GFP, *Il4* and *Il2* mRNA and secrete IL-4 and IL-2 protein

In addition to secreting IL-13, IL-5 and IL-9 ILC2s have been shown to secrete other type-2 cytokines including IL-4 and IL-2 (Artis and Spits, 2015). Indeed, following *H. polygyrus* infection, purified ILC2s expressed *Il4* and *Il2* (**Fig. 3.4 A**) and secreted high concentrations of both IL-4 and IL-2 after re-stimulation with PMA and Ionomycin *ex vivo* (**Fig. 3.4 B**). To identify factors required for IL-4 secretion, we stimulated ILC2s with IL-2/IL-25/IL-33 or leukotriene D4 (LTD_4), a member of the arachidonic pathway previously shown to induce IL-4 production by ILC2s (Doherty et al., 2013). IL-4 secretion, in contrast to IL-13 and IL-5, was not induced in response to IL-2/IL-25/IL-33 (**Fig. 3.4 C**), however we found that LTD_4 could induce IL-4 secretion by ILC2s in a dose-dependent manner (**Fig. 3.4 C**), as previously described (Doherty et al., 2013). We also infected *Il4*-*GFP*Foxp3-*RFP* mice and found that the majority of $KLRG1^+$ ILC2s were *Il4*-GFP^+*Foxp3*-RFP^-. Indeed, ILC2s constitutively expressed *Il4*-GFP both in naïve and *H. polygyrus* infected mice (**Fig. 3.4 D**). Consequently, the absolute number of *Il4*-GFP-expressing ILC2s significantly increased in the LP at d5 post-infection (**Fig. 3.4 E**).

3.2.2.3. ILC2s are a dominant cellular source of *Il4*-GFP in the LP of *H. polygyrus* infected mice

A diversity of immune cells have been reported to express *Il4*-GFP early in ontogeny (Gessner et al., 2005) however their relative contribution to Th2 differentiation *in vivo* is still not clearly understood. In particular, basophils are thought to play an essential role for the differentiation of Th2 cells *in vivo* (Voehringer, 2013). However basophil-deficiency (Schwartz et al., 2014) or basophil depletion (Smith et al., 2012) had no impact on the development of Th2 cells in a primary infection with *H. polygyrus*. Instead, basophils were required for functional expulsion of *H. polygyrus* in a secondary infection (Herbst et al., 2012). To characterise the dominant cellular sources of IL-4 during a primary *H. polygyrus* infection, we determined the composition

of *Il4*-GFP$^+$ cells at day 5 post-infection in the lamina propria and local gut draining MLN. We identified CD4$^+$ T cells as *Il4*-GFP$^+$CD4$^+$CD49b$^-$, ILC2s as *Il4*-GFP$^+$CD4$^-$ CD49b$^-$lineage$^-$Thy1.2$^+$KLRG1$^+$ and basophils as *Il4*-GFP$^+$CD4$^-$CD49b$^+$ (**Fig 3.5 A**). Furthermore, we identified a large proportion of *Il4*-GFP$^+$ cells as non-basophil, non-CD4, non-ILC, FSC-H^{low}SSC-A^{high} cells, suggesting they could be eosinophils (**Fig. 3.5 A**). We saw significantly higher numbers of *Il4*-GFP$^+$ ILC2s than basophils in the lamina propria at day 5 post-infection (**Fig. 3.5 B**), and CD4$^+$ T cells formed the major part of the *Il4*-GFP$^+$ population in the MLN (**Fig. 3.5 B**).

3.2.3. ILC2s develop and function in the absence of IL-2

As described above, ILC2s isolated from the lamina propria of *H. polygyrus* infected mice expressed *Il2* mRNA and secreted IL-2, confirming previous findings from mouse and human studies (Crellin et al., 2010; Moro et al., 2010; Monticelli et al., 2011; Molofsky et al., 2013). Furthermore, ILC2s expressed the high affinity IL-2 receptor CD25 (IL-2Rα) (Moro et al., 2010). Many studies report that IL-2 can increase cytokine production by ILC2s *in vitro,* when compared with IL-25 and IL-33 alone (Moro et al., 2010). Furthermore, IL-2 has been shown to be sufficient to drive IL-9 secretion by ILC2s (Wilhelm et al., 2011), and IL-9 further promotes IL-5 and IL-13 secretion (Turner et al., 2013; Mohapatra et al., 2015). These studies suggest IL-2 is important for the effector function of ILC2s, however the absolute requirement of exogenous or autocrine IL-2 for ILC2 development and function have not been studied.

To address this question, we made use of *Il2*$^{-/-}$*Rag2*$^{-/-}$ mice that lack T and B cells and are deficient in any source of IL-2, and compared them to *Rag2*$^{-/-}$ mice (Moro et al., 2010). We saw no significant differences in the proportion (**Fig. 3.6 A**) or absolute number (**Fig. 3.6 B**) of KLGR1$^+$ ILC2 (black bars) or KLRG1$^-$ ILCs (white bars) in the lamina propria or MLN between the two groups. In addition, we saw no significant impact of IL-2 deficiency on the expansion of ILC2s at day 5 post-infection with *H. polygyrus* (black bars) in the LP or MLN (**Fig. 3.7 A**). Furthermore, a high

proportion of *Il2*$^{-/-}$ ILC2s secreted IL-5 and IL-13 (**Fig. 3.7 B**) and were actively proliferating at day 5 post-infection, as measured by Ki-67 staining (**Fig. 3.7 C**), similar to IL-2-sufficient ILC2s. Overall, these experiments demonstrate that ILC2s are not dependent on IL-2 for their development, proliferative capacity or cytokine secretion.

3.2.4. ILC2s are insufficient to drive sterile immunity to *H. polygyrus*

3.2.4.1. IL-2 complex treatment expands ILC2s in the tissue and mesenteric lymph nodes

As discussed, ILC2s express high levels of CD25 and secrete significant amounts of type-2 cytokines in response to IL-2 stimulation *in vitro.* To determine whether ILC2s were sufficient to control *H. polygyrus*, we made use IL-2c, which has been shown to expand and activate ILC2s *in vivo* (Roediger et al., 2013; Oliphant et al., 2014; Van Gool et al., 2014). Treatment of *Rag2*$^{-/-}$ mice with a dose of IL-2c previously used in lymphocomplete mice (Boyman et al., 2006) caused severe haemorrhaging resembling vascular leakage syndrome (Klatzmann and Abbas, 2015) and ultimately resulting in their death. Consequently, we treated *Rag2*$^{-/-}$ mice with lower doses of IL-2c (IL-2c^{low} [0.5μg:5μg] and IL-2c^{med} [2.5μg:25μg]) on days 0, 2 and 4 post-infection, when L4 larvae are establishing in the intestinal wall. We characterised lineage^{-}Thy1.2^{+} cells at day 5 post-infection, as shown in the representative FACS plots of the MLN (**Fig 3.8 A**). Treatment with both IL-2c^{low} and IL-2c^{med} doses led to a significant expansion in the proportion and absolute number of ILC2s in the LP (**Fig. 3.8 B**). Because we had previously seen that the majority of lineage^{-}Thy1.2^{+} cells in the MLN of *Rag2*$^{-/-}$ mice were ILC2s (**Fig. 3.6 A**), IL-2c treatment did not significantly impact the proportion of ILC2s (**Fig. 3.8 C**), however the absolute number of ILC2s in the MLN was significantly increased following both IL-2c^{low} and IL-2c^{med} treatments (**Fig. 3.8 B**). In accordance with these results, the proportion of Ki-67^{+} proliferating ILC2s was increased following IL-2c treatment (**Fig. 3.9 C**). Furthermore, IL-2c

treatment further increased CD25 expression on ILC2s (**Fig. 3.9 A**), but not on KLRG1- ILCs (**Fig. 3.8 B**).

3.2.4.2. IL-2 complex treatment impairs *H. polygyrus* larval development resulting in reduced adult worm establishment

We hypothesised that IL-2c-mediated expansion of ILC2s would lead to an increased type-2 response in the tissue and impair the development of invading larvae. To test this, we infected *Rag2*$^{-/-}$ mice, treated them with IL-2c^{med} or PBS at days 0, 2 and 4 and harvested the mice at day 9 post-infection when adult worms have normally emerged into the lumen (Reynolds et al., 2012). At day 9 post-infection, the majority of adult worms had emerged into the lumen of PBS treated mice (black bars) and only a small number were still trapped in the wall (white bars) (**Fig. 3.10 A**). Strikingly, IL-2c^{med} treatment resulted in a large number of larvae trapped in the intestinal wall (white bars) and a significant reduction in the emergence of adult worms in the lumen (**Fig. 3.10 A** and **B**). Following an identical treatment regimen, we looked at a later time-point following infection to assess whether IL-2c treatment early during infection merely reflected a delay in the emergence of worms, or whether the tissue-embedded larvae were killed. We saw a significant reduction in the total number of adult worms in the lumen at day 22, with little evidence of any worms still trapped in the wall (**Fig. 3.10 A**), suggestive of IL-2c-mediated larval killing.

To identify potential mechanisms of larval killing, we measured the expression of type-2 effector genes in the small intestine of IL-2c treated mice 5 days post-infection. IL-2c^{med} treatment increased the expression of *Il5, Il13* and *Il4* in the whole tissue (**Fig. 3.10 C**), correlating with the expansion of ILC2s (**Fig. 3.8 B**). IL-2c^{med} treatment also resulted in the increased expression of *Retnlb, Retnla, Arg1* and increased expression of the goblet-cell proliferation marker *Mclca3* (Gob5) (**Fig. 3.10 C**).

3.2.4.3. Depletion of ILC2s partly impairs IL-2c-mediated larval killing

To test the role of ILC2s in promoting larval killing following IL-2c treatment, we depleted ILC2s from *Rag2*$^{-/-}$ mice using an anti-Thy1.2 antibody (as previously published (Turner et al., 2013)) and determined whether larval killing occurred in the absence of ILC2s (see model, **Fig. 3.11 A**). As we had previously shown (**Fig. 3.8**), IL-2c treatment led to a significant increase in the absolute number of ILC2s in the MLN of IL-2c-treated mice co-treated with isotype control, but not anti-Thy1.2 antibody (**Fig. 3.11 B**), suggesting the anti-Thy1.2 treatment efficiently depleted ILC2s. As expected, a significant number of larvae were trapped in the intestinal wall of IL-2c treated mice at day 9 post-infection (**Fig. 3.11 C**). Surprisingly, the majority of worms also remained trapped in ILC2-depleted mice, (**Fig. 3.11 C**) suggesting that IL-2c treatment promoted larval killing in an ILC2-independent manner. In conclusion, IL-2c mediated expansion of ILC2s correlated with increased type-2 responses in the tissue but only partial immunity to *H. polygyrus,* suggesting they have limited anti-helminth potential against *H. polygyrus*. We therefore explored whether ILC2s were influencing adaptive immune mechanisms.

3.2.5. ILC2s promote the differentiation of nT cells to *Il4*-GFP^{+} cells *in vitro* in an IL-4 and IL-2-dependent manner

To test whether ILC2-derived IL-4 was required for Th2 differentiation, we designed an *in vitro* assay to measure Th2 polarisation (normally dependent on IOL-4 and IL-2 *in vitro*) by ILC2-derived supernatant (see model, **Fig. 3.12 A**). To obtain sufficient numbers of ILC2s, we expanded ILC2s *in vivo* with IL-2c. *Rag2*$^{-/-}$ or *Il2*$^{-/-}$ *Rag2*$^{-/-}$ mice were infected with *H. polygyrus* and treated with IL-2c on days 0, 2 and 4. At day 5 post-infection, IL-2c-expanded ILC2s were FACS-purified and re-stimulated with P+I for 3 hours. Following this 3-hour stimulation, ILC2s were washed to remove the P+I and resuspended in fresh cIMDM for a remaining 21 hours before harvesting the supernatant. Similarly to ILC2s obtained from the lamina propria of WT mice (**Fig.**

3.3 and 3.4), ILC2s isolated from the MLN of *Rag2*$^{-/-}$ mice secreted large concentrations of IL-13, IL-5, IL-4 and IL-2 (white bars, **Fig. 3.12 B**). Furthermore, *Il2*$^{-/-}$ ILC2s secreted similar concentrations of IL-13 and IL-5 as WT ILC2s, and as expected, secreted undetectable levels of IL-2 (black bars, **Fig. 3.12 B**). Surprisingly however, *Il2*$^{-/-}$ ILC2s secreted lower levels of IL-4 than WT ILC2s (**Fig. 3.12 C**) which was not due to increased cell death (**Fig. 3.12 D**). To test whether IL-2 was required for ILC2-derived IL-4, we purified ILC2s from the LP of *Rag2*$^{-/-}$ and *Il2*$^{-/-}$*Rag2*$^{-/-}$ mice and re-stimulated them with P+I *in vitro.* Compared to MLN-derived ILC2s, we saw no differences in IL-4 production by LP-derived ILC2s (**Fig. 3.12 C**) suggesting that ILC2s form the MLN may have a greater dependence on IL-2 for IL-4 production.

To test whether ILC2-derived supernatant could support Th2 differentiation, we purified naïve CD4^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{-}CD25^{-}CD44^{-} T cells (nT) from *Il4*-GFP*Foxp3*-RFP mice and cultured them with the supernatant of re-stimulated WT ILC2 or *Il2*$^{-/-}$ ILC2 in the presence of anti-CD3 and anti-CD28 for 4 days. In addition, we tested whether ILC2-derived IL-4 contributed to Th2 differentiation by adding anti-IL-4 blocking antibody to some of the cultures. As a positive and negative control, we cultured nT cells with recombinant IL-4 or media alone, respectively. After 4 days in culture we measured *Il4*-GFP expression as an indication of Th2 commitment. Strikingly, up to 90% of the cells cultured with ILC2 supernatant were *Il4*-GFP^{+}, in comparison to 50% of cells cultured with recombinant IL-4 alone (**Fig. 3.12 E**). Furthermore, the proportion of *Il4*-GFP^{+} cells was significantly reduced in samples cultured with supernatant from *Il2*$^{-/-}$ ILC2 cultures (**Fig. 3.12 E**). The addition of anti-IL-4 to the supernatants of either WT ILC2 or *Il2*$^{-/-}$ ILC2 cultures entirely inhibited the differentiation of *Il4*-GFP+ cells (**Fig. 3.12 E**). Furthermore, differentiation of nT cells into *Il4*-GFP^{+} cells correlated with increased concentrations of ILC2 supernatant (**Fig. 3.12 F**). Neither the addition of IL-2 to the cultures or varying concentration of IL-4 could rescue the difference in the proportion of *Il4*-GFP^{+} cells seen between WT and *Il2*$^{-/-}$ cultures.

3.2.6. IL-4 production by ILC2s is required for early development of Th2 cells

Following the observation that ILC2s could promote Th2 differentiation *in vitro*, we tested whether ILC2-derived IL-4 supported Th2 differentiation *in vivo* during the development of a Th2 response to *H. polygyrus*. To test this, we used a mixed bone marrow chimera approach to restrict IL-4 deficiency to ILC2s. To do this, we used lethally irradiated *Il7r*-deficient mice that are deficient in ILC1, ILC2 and ILC3 (Hoyler et al., 2012) and *Rora*-deficient mice (RORα$^{sg/sg}$ mice*),* which are specifically deficient in ILC2s (Halim et al., 2012; Wong et al., 2012). We reconstituted *Il7r*-deficient mice with 100% *Rora*-deficient bone marrow creating a mouse that is specifically deficient in ILC2 cells. In addition, we reconstituted a cohort of mice with 80% *Rora*$^{-/-}$ bone marrow and 20% WT or *Il4*$^{-/-}$ bone marrow. In these chimeric mice, all ILC2s were either WT (80% *Rora*$^{-/-}$:20% WT) or *Il4*-deficient (80% *Rora*$^{-/-}$:20% *Il4*$^{-/-}$), with the majority (80%) of other haematopoietic cells being IL-4-sufficient (see model, **Fig. 3.13 A**).

3.2.6.1. *Il4*$^{-/-}$ ILC2s fail to develop in bone-marrow chimera

Following 8 weeks of reconstitution, we infected the chimeric mice with *H. polygyrus* and assessed the development of ILC2s and Th2 cells at day 7 post-infection. We checked for reconstitution by FACS-staining for congenically marked WT bone-marrow (data not shown) however we were unable to distinguish the CD45.2$^+$ IL-4Rα-deficient bone marrow from the CD45.2$^+$ RORα group. There were no defects in the proportion of lineage$^-$Thy1.2$^+$ cells between the different groups (**Fig. 3.13 B**) however as expected, there were significantly fewer KLRG1$^+$ ILC2s in mice reconstituted with 100% *Rora*$^{-/-}$ bone marrow alone (–) in comparison to mice reconstituted with 80% *Rora*$^{-/-}$ and 20% WT bone marrow (WT) (**Fig. 3.13 C**). Surprisingly, we observed significantly fewer ILC2s in the lamina propria in chimeric mice given 80% *Rora*$^{-/-}$ and 20% *Il4*$^{-/-}$ bone marrow (*Il4*$^{-/-}$) (**Fig. 3.13 C**).

3.2.6.2. ILC2-deficiency correlates with the impaired development of Th2 responses

To measure the impact of ILC2-deficiency on Th2 differentiation we measured cytokine production from splenic T cells by intra-cellular cytokine staining. We saw no significant differences in the proportion and number of $CD4^+TCR\beta^+$ cells suggesting *Rora*-deficiency did not grossly impact the development of $CD4^+$ T cells (**Fig. 3.13 D**). However we saw a reduced proportion of IL-4^+, IL-13^+ $CD4^+$ T cells and a significantly reduced proportion of IL-5^+ $CD4^+$ T cells in mice reconstituted with *Rora*$^{-/-}$ bone marrow alone (–) compared to mice with ILC2s (WT) (**Fig. 3.13 E**). Strikingly, this resulted in significantly fewer absolute numbers of IL-4^+, IL-13^+ and IL-5^+ $CD4^+$ T cells (**Fig. 3.13 F**). Similarly, there was a reduced proportion of IL-4^+, IL-13^+ and IL-5^+ $CD4^+$ T cells in mice reconstituted with *Il4*$^{-/-}$ ILC2s (*Il4*$^{-/-}$) compared to mice with ILC2s (WT) (**Fig. 3.13 E**). Consequently, this resulted in fewer absolute numbers of IL-4^+ $CD4^+$ T cells and significantly fewer IL-13^+ and IL-5^+ $CD4^+$ T cells (**Fig. 3.13 F**), even though absolute numbers of $CD4^+$ T cells were unchanged (**Fig. 3.13 D**). Overall, these results demonstrate that ILC2s are required for Th2 differentiation *in vivo* during primary infection with *H. polygyrus* and that ILC2-derived IL-4 can drive Th2 differentiation *in vitro.* However it remains unclear whether ILC2-derived IL-4 is required for Th2 differentiation *in vivo*.

3.3. Discussion

IL-4 is critical for the differentiation of Th2 cells following primary infection with the intestinal helminth *H. polygyrus* (Urban et al., 1991; Urban et al., 1991) and is required for the functional expulsion of *H. polygyrus* following a secondary challenge infection (Urban et al., 1991; Urban et al., 1995). Conventional $CD11c^+$ DCs were shown to be critically required for the initiation of Th2 responses following *H. polygyrus* infection (Gause et al., 1996; Smith et al., 2012) but are not thought to be a source of IL-4 themselves (MacDonald and Pearce, 2002). Although stimulation of naïve T cells

by dendritic cells was sufficient to promote IL-4 secretion by T cells *in vitro* in the presence of antigen and in the absence of any supplemented IL-4 (Jankovic et al., 2000; Noben-Trauth et al., 2000), the differentiation of Th2 cells *in vivo* was almost completely abolished in mice lacking IL-4Rα (Noben-Trauth et al., 1997) indicating that IL-4 plays a central role in Th2 differentiation *in vivo*. Taken together, these studies indicate that DCs and IL-4 are critical for Th2 differentiation *in vivo,* however the source of IL-4 required for optimal differentiation of Th2 cells in the context of *H. polygyrus* infection is still unclear. NKT cells, basophils and eosinophils have all been described to produce IL-4 but none of these cells appear to be required for Th2 differentiation following *H. polygyrus* infection (Voehringer et al., 2004; Mohrs et al., 2005).

ILC2s are characterised by their secretion of IL-5 and IL-13 however they have also been shown to transcribe *Il4* and *Il2 in vivo* using cytokine reporters (Fallon et al., 2006; Price et al., 2010; Molofsky et al., 2013; Roediger et al., 2015) and to secrete IL-4 and IL-2 following re-stimulation *ex vivo* (Molofsky et al., 2013). The function of ILC2-derived IL-4 and IL-2 has not been tested, and in this chapter, we aimed to determine whether ILC2-derived IL-4 and IL-2 could promote the differentiation of Th2 cells *in vitro* and *in vivo.* Using *Il2*-deficient mice, we found that neither paracrine or autocrine IL-2 was required for the optimal expansion of and function of ILC2s *in vivo.* Furthermore, we found that ILC2s were not sufficient to mediate expulsion of primary infection with *H. polygyrus* in the absence of an adaptive immune response, as has been suggested in the context of other helminth infections. Instead, we found that ILC2-derived IL-4 could promote the differentiation of naïve T cells to Th2 cells *in vitro* and *in vivo,* identifying a novel role for ILC2s in anti-helminth immunity.

H. polygyrus has a strictly enteric lifecycle and induces a strong polarised type-2 response early in infection (Reynolds et al., 2012). It has previously been observed that the absolute number of ILC2s correlated with the strength of the type-2 response in mice with varying genetic susceptibilities to *H. polygyrus* (Filbey et al., 2014; Mackley et al., 2015). Furthermore, the absolute number of ILC2s have been reported to increase in the spleen and MLN of C57BL/6 mice at days 6, 7 and 10 post-infection

(Zaiss et al., 2013; Filbey et al., 2014; Mackley et al., 2015). These studies suggest that ILC2s are dynamically regulated and may play a role in immune responses to primary infection with *H. polygyrus.* We were particularly interested in determining the role for ILC2s at early time-points following infection when *H. polygyrus* larvae were embedded in the intestinal sub-mucosa **(Fig. 3.1 B)** and are known to secrete highly immunogenic products (Hewitson et al., 2013). Furthermore, we had observed increased gene expression of cytokines known to promote ILC2s or to be secreted by ILC2s in the intestinal tissue of mice as early as day 5 post-infection **(Fig. 3.1 A)**. We also found that ILC2s expanded early following infection in the tissue as well as the local draining LN, correlating with the differentiation of Th2 cells **(Fig. 3.3 A-H)**. This is the first description of increased ILC2 numbers in the intestinal tissue rather than MLN following *H. polygyrus* infection, perhaps due to the difficulty in obtaining live preparations of cells from inflamed tissue (Moro et al., 2015). For technical reasons, we were limited to studying immune responses between days 0 and 7 following primary *H. polygyrus* infection, as cells isolated after day 7 were not reliably recoverable. ILC2 expansion was previously shown to precede the expansion of Th2 cells in the context of *N. brasiliensis* infection (Fallon et al., 2006), however both ILC2 and Th2 cells were concomitantly increased at day 5 post-infection. A more detailed kinetic analysis in local tissue and draining lymph nodes following *H. polygyrus* infection would be required to determine whether ILC2s precede Th2 cell differentiation in this setting, which would support the hypothesis that ILC2s promote Th2 differentiation.

3.3.1. Identifying a role for neglected ILC2-derived cytokines

A recently published transcriptional array identified that ILC2s purified from the small intestine of naïve mice express *Il4* and *Il2* (Robinette et al., 2015). We confirmed this in ILC2s isolated from *H. polygyrus* mice and found that LP-derived ILC2s expressed *Il4* and *Il2* as well as the canonical cytokines *Il5* and *Il13*. Importantly, the authors of this study purified ILC2s from the small intestine of mice using a similar

staining strategy as ours (containing anti-KLRG1 antibody, as described in chapter 2). *Il4* expression was not reported in transcriptional arrays of lung-derived ILC2s following allergic inflammation (Motomura et al., 2014) or viral infection (Monticelli et al., 2011), however there have been many reports of *Il4*-GFP expression by lung ILC2s following *N. brasiliensis* infection (Fallon et al., 2006; Price et al., 2010; Molofsky et al., 2013), suggesting there are either disparities between the methods used to analyse *Il4* expression, or that expression of *Il4* is highly context dependent. Alternatively, this suggests that *Il4* expression in lung ILC2s is induced following infection with helminths rather than constitutively expressed. We characterised *Il4*-GFP expression in LP-derived ILC2s and found that the majority of ILC2s in both naïve and *H. polygyrus* infected mice expressed *Il4*-GFP, suggesting they were genetically primed to secrete IL-4 **(Fig. 3.4 C and D)**. Previously published work using *Il4*-GFP mice crossed with KN2 mice (expressing huCD2 under the control of the IL-4 promoter to identify cells that secrete IL-4) indicated that the highest correlation of *Il4*-GFP expression with IL-4 protein production occurred in gut-associated tissues such as the MLN and LP (Mohrs et al., 2005). Therefore we hypothesise that a high proportion of *Il4*-GFP-expressing ILC2s could be secreting IL-4 protein *in vivo*.

ILC2s secrete IL-5 and IL-13 in response to stimulation with combinations of IL-2, IL-25, IL-33 and TSLP (Artis and Spits, 2015). We confirmed these findings and showed that ILC2s stimulated with IL-25, IL-33 and IL-2 secreted IL-5 and IL-13, but not IL-4. Indeed, IL-4 was only seen following PMA and Ionomycin or LTD4, as previously reported (Doherty et al., 2013). We found that ILC2s but not KLRG1$^-$ ILCs expressed and secreted IL-2 in response to P+I, in contrast to a recently published study suggesting that ILC3s formed the dominant IL-2-expressing cells in the lung (Roediger et al., 2015). Again, the differences may be explained by the apparent heterogeneity between ILC populations isolated from the lung and the LP, or to sorting strategies. To definitively test whether *Il4*-GFP$^+$ ILC2s produce IL-4 protein *in vivo* and increase upon infection, we could infect *Il4*-GFP/KN2 mice and measure huCD2

expression on the surface of *Il4*-GFP$^+$ ILC2s in naïve mice and following *H. polygyrus* infection.

Many cells including NKT cells, eosinophils and mast cells have been described to express *Il4*-GFP (Gessner et al., 2005; Mohrs et al., 2005), however their role in the differentiation of Th2 cells is not clear. Basophils (Perrigoue et al., 2009) and CD4$^+$ T cells themselves (Noben-Trauth et al., 2000) have been proposed to be potential sources of IL-4 for Th2 differentiation in various settings. Following our observation that purified ILC2s expressed *Il4* and secreted IL-4 *in vitro,* we wanted to characterise *Il4* expression *in vivo* and determine the relative contribution of ILC2s to *Il4*-expressing cells (Mohrs et al., 2001). We found higher numbers of *Il4*-GFP$^+$ ILC2s than CD4 cells and basophils in the LP but not the MLN, suggesting ILC2s may play a role in priming T cells in the tissue but not the MLN. Alternatively, very low numbers of ILC2s could be sufficient to drive the differentiation of Th2 cells in the MLN. Recent imaging of the MLN following *H. polygyrus* infection showed 30% of lamina propria ILC2s migrate from the small intestine to the MLN following infection (Mackley et al., 2015), suggesting that ILC2s may be recruited to the MLN for the activation of T cells.

Similar to previously published data (Voehringer et al., 2004), we found that the majority of *Il4*-GFP$^+$ cells were phenotypically characteristic of eosinophils. However, eosinophils are not thought to contribute to the differentiation of Th2 cells following *N. brasiliensis* (Voehringer et al., 2006) or *S. mansoni* (Swartz et al., 2006) infection. Furthermore, depletion of eosinophils had no impact on the production of IgE following primary infection with *H. polygyrus* (Urban et al., 1991) suggesting that eosinophils are not required for Th2 development during *H. polygyrus* infection. Instead, eosinophils may be an important source of IL-4 for the amplification of type-2 immune responses.

IL-2 has been shown to enhance cytokine production by ILC2s *in vitro* (Moro et al., 2010) and expand ILC2s *in vivo* (Roediger et al., 2013; Oliphant et al., 2014), however it remained unclear whether ILC2s require IL-2 for their development or function. The best way to test this would be to characterise ILC2s isolated from IL-2-deficient mice, however these mice succumb to T-cell mediated inflammation due to a

deficiency in the development of Treg cells (Sadlack et al., 1993). We therefore chose to compare the development of ILC2s in T-cell deficient mice to limit the development of disease. We compared *Rag2*-deficient and IL-2-deficient mice on a *Rag2*-deficient background and found IL-2 was not fundamentally required for the development, expansion or cytokine-producing function of ILC2s. Furthermore, given that ILC2s are a source of IL-2 themselves, these results suggested that ILC2-derived IL-2 was not required in an autocrine manner. However these studies were restricted to T and B cell deficient *Rag2*$^{-/-}$ mice, and it is conceivable that in a wild-type setting ILC2-derived IL-2 is required to stimulate IL-2-responsive cells such as T cells. For example, Treg cells express CD25 and are critically dependent on IL-2 for their development and maintenance, however they do not produce IL-2 (Setoguchi et al., 2005; Marson et al., 2007). ILC2-derived IL-2 may exert an indirect regulatory role by promoting Treg maintenance in the tissue. In addition, ILC2-derived IL-2 could be promoting NK cell proliferation for example in the context of early immune responses to LCMV (Biron et al., 1990), although the role of NK cells in protective anti-helminth immune responses is currently unclear. To characterise the kinetics of IL-2 secretion by ILC2s in the small intestine we could use *Il2*^{Cre} fate-reporter mice (Yamamoto et al., 2013) that were recently used to determine *Il2* expression by ILCs in the inflamed lungs (Roediger et al., 2015). Furthermore, to test whether ILC2-derived IL-2 influenced T cell responses, we could restrict IL-2 deficiency to ILC2s by generating mixed bone marrow chimeras of 80% *Rora*$^{sg/sg}$ and 20% *Il2*$^{-/-}$*Rag*$^{-/-}$ bone marrow and studying the generation and maintenance of T cells, in particular the generation of Treg cells.

The sensitivity of ILC2s to IL-2 has been manipulated *in vivo* by treating *Rag*-deficient mice with IL-2:anti-IL-2 complexes (IL-2c) (Boyman et al., 2006) to activate and expand ILC2 cells. IL-2c treatment resulted in systemic eosinophilia (Van Gool et al., 2014), exacerbated atopic dermatitis (Roediger et al., 2013), enhanced lung inflammation during eosinophilic crystalline pneumonia (ECP) (Roediger et al., 2015) and expulsion of *N. brasiliensis* from susceptible *Rag2*$^{-/-}$ mice (Oliphant et al., 2014),

correlating with the expansion of ILC2s. We also observed a dose-dependent expansion in the number of ILC2s in the LP and MLN of *Rag2*-deficient mice following IL-2c treatment. Furthermore, high doses of IL-2c led to the increased expression of surface CD25, indicative of an IL-2 positive feedback loop described following IL-2c stimulation of Treg cells (Spangler et al., 2015). Taken together these results suggest that although IL-2 was not fundamentally required for ILC2 function it can promote the expansion and activation of ILC2s, which could impact the amplification of immune responses. Indeed, T cells secrete high levels of IL-2 upon activation (Seder et al., 1994), and have been previously suggested to provide a source of IL-2 for ILC2 expansion *in vivo* (Wilhelm and Stockinger, 2011). Alternatively, NK cells were recently shown to deplete IL-2 from the microenvironment thereby modulating Treg numbers (Gasteiger et al., 2013), which could also be the case for ILC2s.

Another effect of IL-2 on ILC2s could be the induction of cytokine production. For example, IL-2 was shown to be required for IL-9 secretion by ILC2s (Wilhelm et al., 2011) and consequently for efficient lung repair following *N. brasiliensis* infection (Turner et al., 2013). Furthermore, *Il4* expression was detectable in inflamed lungs of ECP-diseased *Rag2*$^{-/-}$ mice following IL-2c treatment, suggesting that IL-2 could promote IL-4 production by non-T cells in the lung, perhaps from ILC2s (Roediger et al., 2015). Indeed, IL-2 is required for optimal IL-4 production by T cells (Ben-Sasson et al., 1990) by promoting accessibility of the *Il4* locus (Cote-Sierra et al., 2004) suggesting it may be required for ILC2-derived IL-4 production. However, we found that *in vitro* stimulation of ILC2s with IL-2 alone or in the presence of IL-25 and IL-33 did not promote IL-4 secretion. Furthermore, we only observed reduced concentrations of IL-4 from ILC2s isolated from the MLN of IL-2 deficient mice but not LP-derived ILC2s. Whether there are differential requirements for IL-2 signalling between ILC2 populations in the tissue compared to ILC2s in lymphoid organs is currently unclear. Taken together, these results suggest that IL-2 is not fundamentally required for IL-4 production by ILC2s, in contrast to T cells, highlighting an interesting divergence in the transcriptional and post-transcriptional regulation of IL-4 mRNA. As mentioned, NKT

cells (Stetson et al., 2003) constitutively express *Il4* and were found to contain pre-formed cytokine transcripts correlating with permissive chromatin modifications at the *Il4* locus (Stetson et al., 2003). Similarly, pre-formed IFN-γ transcripts are retained in the nucleus of NK cells following transcription, and released upon secondary stimulation (Hodge et al., 2002). Following the observation that ILC2s constitutively express *Il4*-GFP*,* it is conceivable that they are transcriptionally regulated in a similar way to NK T cells and contain pre-formed *Il4* mRNA transcripts. ILC2s could be primed for the rapid production of IL-4 and represent a biologically important source of IL-4 for the differentiation of Th2 cells. Experiments using the transcriptional inhibitor actinomycin D could be done to determine whether pre-formed *Il4* transcripts are stored in ILC2s for rapid translation and secretion.

Although IL-2 was not fundamentally required for ILC2 function, Th2 differentiation was impaired in cells cultured with *Il2*$^{-/-}$ ILC2s. This may not be due to reduced IL-4 secretion by *Il2*$^{-/-}$ ILC2s, as culturing nT cells with a range of IL-4 concentrations (1-15ng/μl) to account for the differences measured in the *Il2*$^{-/-}$ ILC2 supernatants made little impact on the proportion of CD4^{+}*Il4*-GFP^{+} cells. Furthermore, the differences did not occur as a direct consequence of an IL-2 deficiency, as exogenous IL-2 did not rescue Th2 differentiation. Instead, ILC2-derived IL-2 may play an indirect role on promoting Th2 differentiation, via an autocrine feedback loop supporting the secretion of an unidentified cytokine or growth factor from ILC2s. For example, ILC2s have been shown to express *Il6* (Robinette et al., 2015), which can promote early IL-4 secretion by Th2 cells (Rincon et al., 1997). Whether ILC2-derived IL-6 could be promoting Th2 differentiation by ILC2s in addition to IL-4 is unclear. Further characterisation of ILC2-derived cytokines using multi-analyte detection systems or mass spectrometry may uncover a role for novel molecules in promoting Th2 differentiation.

In summary, both the function of ILC2-derived IL-2 as well as the requirement for IL-2 signalling on ILC2s for optimal immune responses is still unclear and requires further investigation.

3.3.2. Regulation of cytokine production by ILC2s

The signals required for IL-4 secretion by murine ILC2s are not well defined. We have shown that ILC2s produce IL-4 in response to P+I *in vitro* but not in response to IL-2, IL-33 and IL-25. In T cell cultures, PMA stimulates enhanced PKC activation, mimicking Nf-κb activation downstream of the TCR (Visekruna et al., 2012) however ILCs do not express antigen receptors such as the TCR, suggesting PMA mimics PKC activation downstream of another receptor such as a cytokine receptor.

ILC2s have been shown to express genes associated with lipid metabolism including *Alox5,* a lipoxygenase that catalyses the synthesis of leukotriene A4 (Robinette et al., 2015). Two separate studies identified the expression of the surface receptor CysTLR1 (*Cystlr1*) on ILC2s (Doherty et al., 2013; Robinette et al., 2015), which recognises cysteinyl leukotrienes including LTC4, LTD4 and LTE4 that are derived from arachidonic acid through *Alox5* catalysis (Ford-Hutchinson et al., 1994). Leukotrienes are known to play a pathophysiological role in asthma and *Alox5* polymorphisms are associated with increased leukotriene production and reduced lung function (Mougey et al., 2013). As such, leukotriene antagonists are therefore used to control chronic asthma in patients (Montuschi, 2010). Similar to a previous report (Doherty et al., 2013), we found that LTD_4 could promote IL-4 production by LP-derived ILC2s following *H. polygyrus* infection to similar levels as P+I stimulation. Mast cells are known to secrete proteins including leukotrienes and prostaglandins in the context of helminth infections (Rogerio and Anibal, 2012). Furthermore, mast cells are activated early following *H. polygyrus* infection (Hepworth et al., 2012), and are required for early Th2 differentiation following *H. polygyrus* infection (Hepworth et al., 2012). Indeed, the supernatant from IgE-activated mast cells was sufficient to stimulate IL-4-secretion from Th2 cells (Xue et al., 2012). Finally, mast cells and ILC2s form close associations *in vivo* (Roediger et al., 2013), supporting a model of mast cell-derived LTD_4 stimulating IL-4 secretion by ILC2s. To test the role for mast cells in

promoting ILC2-derived IL-4, we could measure IL-4 production by ILC2s isolated from mast-cell deficient mice *Kit*^{W}/*Kit*$^{W\text{-}v}$ (Hepworth et al., 2012). Prostaglandin D2 (PGD2), a lipid mediator derived from arachidonic acid pathway and known to promote Th2 responses (Kalinski et al., 1997), could also stimulate IL-4 production by lung-derived ILC2s *in vitro* (Xue et al., 2014), however its role in promoting IL-4 production by LP-derived ILC2s is still unclear. Interestingly, helminth-derived excretory/secretory products contain proteins that resemble members of the arachidonic pathway (Maizels and Yazdanbakhsh, 2003). It is therefore conceivable that *H. polygyrus*-secreted proteins act directly on ILC2s to promote IL-4 production. To test this, we could culture ILC2s with *H. polygyrus* excretory/secretory proteins (HES) isolated from larval stages to test whether helminth secretions can simulate IL-4 production.

Basophil-derived IL-4 was proposed to stimulate the function of ILC2s in the skin and the lung (Kim et al., 2014; Motomura et al., 2014), suggesting that IL-4 from other cellular sources may drive the production of IL-4 by ILC2s. Indeed, IL-4Rα was highly expressed on ILC2s in the lung (Motomura et al., 2014), however *Il4ra* expression on ILC2s isolated from the small intestine was not significantly different than on other ILC subsets (Robinette et al., 2015) suggesting that intestinal ILC2s may not require IL-4 for their activation. To conclusively test this, we could isolate ILC2s from the small intestine of basophil-deficient (*Mcpt8*$^{-/-}$) or *Il4ra*-deficient mice and measure IL-4 production. Furthermore, NKT cells (Stetson et al., 2003), basophils and eosinophils (Voehringer et al., 2004) have all been shown to constitutively express *Il4* mRNA in a STAT6-independent manner, which suggests that IL-4 may not be required for ILC2-derived *Il4* expression. To test this we could characterise *Il4*-GFP expression by ILC2s in *Il4*-GFP/*Stat6*$^{-/-}$ mice (Voehringer et al., 2004).

3.3.3. Determining a wider impact of ILC2s for anti-helminth immunity

H. polygyrus establishes chronic infections in susceptible hosts, despite the development of strong Th2 responses (Reynolds et al., 2012). Treatment with

supraphysiological levels of IL-4 using IL-4c was sufficient to mediate the expulsion of a primary infection (Urban et al., 1995; Herbert et al., 2009), suggesting that the amplification of type-2 responses is sufficient to promote expulsion of the parasite. Similarly, IL-2c treatment was shown to significantly expand IL-5 and IL-13-producing ILC2s resulting in the expulsion of *N. brasiliensis* in susceptible lymphodeficient mice (Oliphant et al., 2014). We investigated the impact of IL-2c treatment on the establishment of *H. polygyrus,* and found that IL-2c treatment resulted in the trapping of larvae in the intestinal wall*,* correlating with the expansion of ILC2s and enhanced type-2 responses. This phenotype was similar to that observed during immune responses to secondary infection with *H. polygyrus* (Reynolds et al., 2012), however we saw no significant cellular infiltrate around the embedded larvae suggesting that IL-2c may not be promoting a similar mechanism of expulsion. Although the depletion of ILCs significantly reduced IL-2c-mediated larval killing, a large number of worms remained trapped in the wall. Collectively, these results suggest IL-2c treatment promotes partial immunity to *H. polygyrus* but is insufficient to mediate the complete expulsion seen following IL-4c treatment (Urban et al., 1995; Herbert et al., 2009). These results also suggest that IL-2c mediates larval killing in an ILC-independent manner, however it remains unclear how. To conclusively test the requirement for ILCs in this system, we could infect ILC-deficient mice such as *Il7*$^{-/-}$*Rag2*$^{-/-}$ mice (Moro et al., 2010) and measure worm burden following IL-2c treatment. This would allow us to directly test the impact of ILCs on the larval killing and exclude the role of other Thy1.2-expressing cells that may have been depleted following anti-Thy1.2 antibody treatment, such as NK cells and basophils.

To date, the role of ILC2-derived cytokines in mediating expulsion following IL-2c treatment has not been tested. Adoptively transferred IL-13 competent but not *Il13*$^{-/-}$ ILC2s could mediate *N. brasiliensis* expulsion from *Rag2*$^{-/-}$ mice (Neill et al., 2010), which suggests IL-13 may play an important role for ILC2-mediated expulsion of *N. brasiliensis.* To test whether ILC2-derived cytokines mediate larval killing following IL-2c treatment, both in the context of *H. polygyrus* or *N. brasiliensis* infection, it would be

interesting to cross existing cytokine-deficient mice such as *Il13*$^{-/-}$ and *Il4*$^{-/-}$ mice onto a lymphodeficient background and assess *H. polygyrus* expulsion following IL-2c treatment. In this way we could address whether ILC2-derived cytokines are required to promote downstream immune responses in the absence of T cells, by stimulating the alternative activation of macrophages or the recruitment of eosinophils as has been previously shown in wild-type mice (Molofsky et al., 2013; Nussbaum et al., 2013).

3.3.4. Teaming-up: ILC2s as an early trigger of Th2 differentiation

Although there are situations where ILC2s may play a role in the absence of the adaptive immune system, it's increasingly thought that ILCs provide crucial signals to T cells either through their presentation of antigen (Hepworth et al., 2013; Oliphant et al., 2014) or cytokine production (Halim et al., 2014). In particular, ILC2s have been shown to be important for optimal Th2 differentiation and development of type-2 responses *in vivo* (Halim et al., 2012; Wong et al., 2012; Gold et al., 2014; Halim et al., 2014; Mirchandani et al., 2014; Oliphant et al., 2014). Furthermore, ILC2s promoted T cell proliferation and increased type-2 cytokine production *in vitro* (Mirchandani et al., 2014) in a contact- and antigen-dependent manner (Oliphant et al., 2014). Mechanistically, ILC2-derived IL-13 was shown to promote the recruitment of DCs to the tissue-draining lymph nodes, thus indirectly promoting Th2 differentiation (Oliphant et al., 2014), however whether ILC2-derived cytokines have a direct role on Th2 cells remains unclear. We have shown that ILC2s secrete IL-4 and IL-2 *in vitro*, which are two fundamental requirements for Th2 differentiation (Paul, 2010). In contrast to previous experiments that describe an essential role for ILC expression of MHC Class II for ILC2-mediated T cell help, (Mirchandani et al., 2014; Oliphant et al., 2014) our experiments suggest ILC2-derived cytokines were sufficient to mediate Th2 differentiation in the presence of TCR stimulation. Given the importance of CD11c^{+} cells for the differentiation of Th2 cells in the context of *H. polygyrus* infection (Smith et al., 2012), it is conceivable that CD11c^{+} dendritic cells provide TCR engagement and

co-stimulation, while ILC2s provide IL-4 for the differentiation of Th2 cells *in vivo.* Indeed, both $CD11c^+$ DCs (Leon et al., 2012) and ILC2s (Mackley et al., 2015) were shown to migrate from the lamina propria into the MLN early following *H. polygyrus* infection. Interestingly, both ILC2s and $CD11c^+CXCR5^+$ DCs localised in the interfollicular spaces of the lymph node rather than the T cell zones (Leon et al., 2012; Mackley et al., 2015). This unusual localisation of DCs was required for the differentiation of Th2 and TfH cells in response to *H. polygyrus* (Leon et al., 2012). Given the similar localisation of ILC2s, it is conceivable that ILC2s are an important source of IL-4 for the priming of Th2 cells in the MLN. Furthermore, ILC3s, which possess antigen-presentation capacity (Hepworth et al., 2013) and migrate to the MLN following *H. polygyrus* infection (Mackley et al., 2015), were shown to express significant levels of CXCR5 (Robinette et al., 2015) suggesting they could directly provide antigen and co-stimulation to T cells in the interfollicular spaces. Thus, it is also conceivable that expanded ILC3s migrate to the MLN and provide antigen and TCR engagement whilst ILC2s provide the IL-4.

We found that the differentiation of Th2 cells was significantly impaired in ILC2-deficient mice, indicating that ILC2s are required for the differentiation of Th2 cells. This confirms previously published data showing impaired Th2 cells in response to *N. brasiliensis* (Oliphant et al., 2014) and constitutes the first report of a functional role for ILC2s in the context of *H. polygyrus* infection. Although IL-4 was not required for the development of ILC2s in the BM or skin (Kim et al., 2014), we found that $Il4^{-/-}$ ILC2s were compromised in their ability to migrate or repopulate the lamina propria, suggesting that ILC2-derived IL-4 may play a role in the survival or maintenance of ILC2s. As a consequence of ILC2 deficiency seen in $Il4^{-/-}$ mice, Th2 responses were severely compromised, similarly to that seen in ILC2-deficient mice. We can therefore conclude that the number of ILC2s in the lamina propria correlated with the development of Th2 responses to *H. polygyrus*, similarly to that described in mice of differing genetic backgrounds (Filbey et al., 2014). To further address the role of ILC2-derived IL-4 *in vivo,* we could generate mice with an ILC2-specific deletion in IL-4 using

Rora-IRES-Cre knock-in mice (Wu et al., 2010) crossed with *Il4/Il13*^{flox} mice (Voehringer et al., 2009), however ILC2-derived IL-13 was also shown to be required for the differentiation of Th2 cells (Oliphant et al., 2014). A better system would be to cross *Rora*-IRES-Cre mice to *Il4*^{flox} mice instead, however these mice are not currently available. In summary, as a consequence of the impaired reconstitution of ILC2s from *Il4*$^{-/-}$ BM, we weren't able to specifically test a role for ILC2-derived IL-4.

Taken together, these results highlight an important role for ILC2s early in the development of Th2 responses to primary *H. polygyrus* infection however whether ILC2s also influence memory Th2 cells is currently unclear. Indeed, re-activation of memory Th2 cells is required for functional expulsion (Urban et al., 1991; Finkelman et al., 2000) and does not depend on DCs (Gause et al., 1996). It is conceivable that ILC2s may be involved in the reactivation of memory responses through antigen-presentation or by directly promoting type-2 responses through their cytokine production. However given that IL-4 is not required for the activation of memory Th2 cells, as discussed in the next chapter, it's unlikely that ILC2-derived IL-4 is contributing at that stage. To test whether ILC2 cells are required for the reactivation of memory Th2 responses we would need to temporally and selectively delete ILC2 cells following the diphtheria toxin treatment of *Rora*-Cre/DTR mice, however these mice are not currently available.

3.3.5. Requirement for improved mouse models for the study of ILC2s

Studying ILC2 function *in vivo* is currently hindered by the lack of available tools to specifically delete ILC2s, highlighting gaps in our knowledge of unique molecules and TFs required for ILC2 development. An explanation for this is that these TFs may have evolved in innate cells before being co-opted by T cells (Roediger and Weninger, 2015). Indeed, TFs such as *Gata3, Tcf7, Gfi1* and *Bcl11b* are uniquely expressed in the group-2 ILCs and not other ILC subsets, however they are also important in the development of CD4^{+} T cells or the differentiation of T helper cells. Although *Rora*-

deficient Th2 cells were not impaired in their ability to differentiate into Th2 cells *in vitro* (Halim et al., 2012), it is conceivable that RORα plays a role in Th2 cell function *in vivo.* Furthermore, Th17 cells (Yang et al., 2008) and some subsets of Tregs (Feuerer et al., 2010) were also shown to express RORα, suggesting the deletion of *Rora* may have non-specific effects on other immune cells in addition to ILC2s. Furthermore, RORα plays a critical role during neurological development and RORα$^{sg/sg}$ mice develop severe neurological disease within a few weeks of birth (Sidman et al., 1962). The role of RORα has therefore been characterised using BM reconstituted mice (in our study and others), or through conditional deletion of *Rora* under the control of the IL-7 receptor promoter (*Il7r*-Cre), which limited *Rora* deletion to T cells and ILCs (Oliphant et al., 2014). Furthermore, Oliphant et al also generated a second ILC2-deficient mouse model by deleting *Icos* (expressed on T helper cells and ILC2s and required for T helper cell function) specifically in ILC2 cells. To do this, a floxed Dtx receptor (DTR) sequence was introduced within the *Icos* locus under the control of the CD4 promoter (Oliphant et al., 2014), which meant that upon diphtheria toxin treatment, the target sequence was deleted in CD4 cells and not ILC2s leading to disrupted *Icos* function specifically in non-CD4 expressing ILC2s (Oliphant et al., 2014). Additionally, *Il5*-Cre or *Il13*-Cre mice were crossed to ROSA-DTA mice and subsequently treated with diphtheria toxin to eliminate IL-5 and IL-13-expressing cells (Molofsky et al., 2013; Van Dyken et al., 2014; Molofsky et al., 2015). Although ILC2s are important producers of these cytokines, DT treatment would result in the deletion of other innate and adaptive immune cells secreting IL-5 and IL-13 such as CD4$^+$ T cells, and therefore these mice are an imperfect model for studying the relationship between ILC2 and Th2 cells.

In summary, although currently available tools have demonstrated there are important relationships between ILC2s and the immune system, the identification of TFs unique to the development or function of ILC2s will be necessary to definitively test the requirement and involvement of ILC2s for the development of immunity or disease.

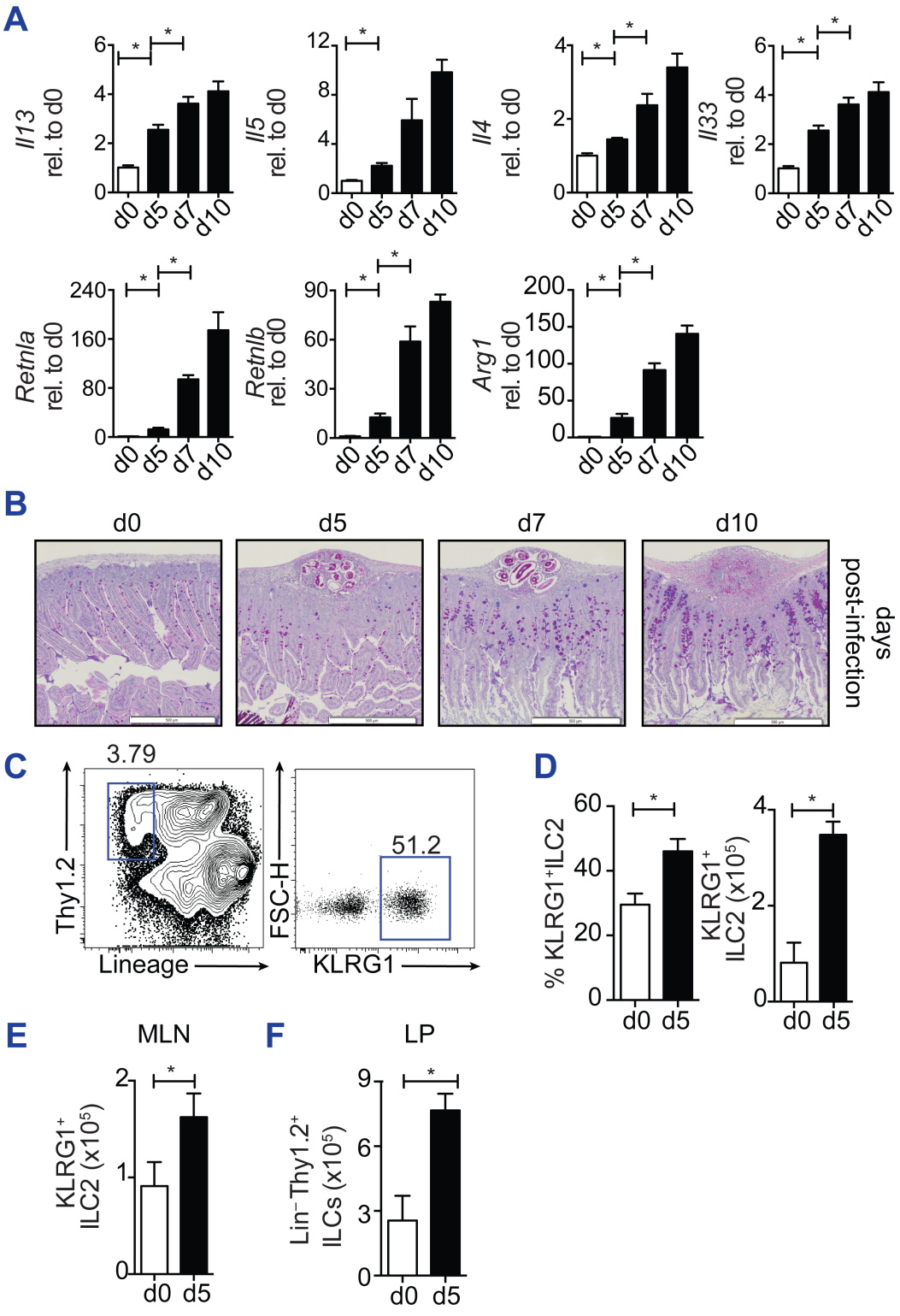

A
Il13 rel. to d0
Il5 rel. to d0
Il4 rel. to d0
Il33 rel. to d0
Retnla rel. to d0
Retnlb rel. to d0
Arg1 rel. to d0
d0 d5 d7 d10
B
d0
d5
d7
d10
days post-infection
C
3.79
Thy1.2
Lineage
FSC-H
KLRG1
51.2
D
% KLRG1+ILC2
KLRG1+ ILC2 (x10^5)
E
MLN
KLRG1+ ILC2 (x10^5)
F
LP
Lin-Thy1.2+ ILCs (x10^5)

Figure 3.1. ILC2 expansion correlates with the development of type-2 responses following primary infection with *H. polygyrus*. C57BL/6 mice were infected with 200 *H. polygyrus* larvae and harvested at days 0, 5, 7 and 10 post-infection. A) *Il33, Retnla, Retnlb, Arg1, Il13, Il5 and Il4* gene expression in the small intestine, expressed as fold change relative to day 0. B) Mucus staining (AB-PAS) of small intestine sections taken at days 0, 5, 7 and 10 post-infection. C) Representative FACS plots of small intestine lamina propria (LP) cells showing the frequency of $KLRG1^+$ cells within the $Lineage^-Thy1.2^+$ gate. D) Frequency and absolute number of $KLRG1^+$ cells in the LP at days 0 (white bars) and 5 (black bars) post-infection. E) Absolute number of $KLRG1^+$ cells in the mesenteric lymph nodes (MLN) at days 0 (white bars) and 5 (black bars) post-infection. F) Absolute number of $KLRG1^-$ cells in the LP at days 0 (white bars) and 5 (black bars) post-infection. Data are representative of 3 independent experiments with 4-5 mice per group. Statistical test applied Mann-Whitney, two-tailed, * denotes $p<0.05$.

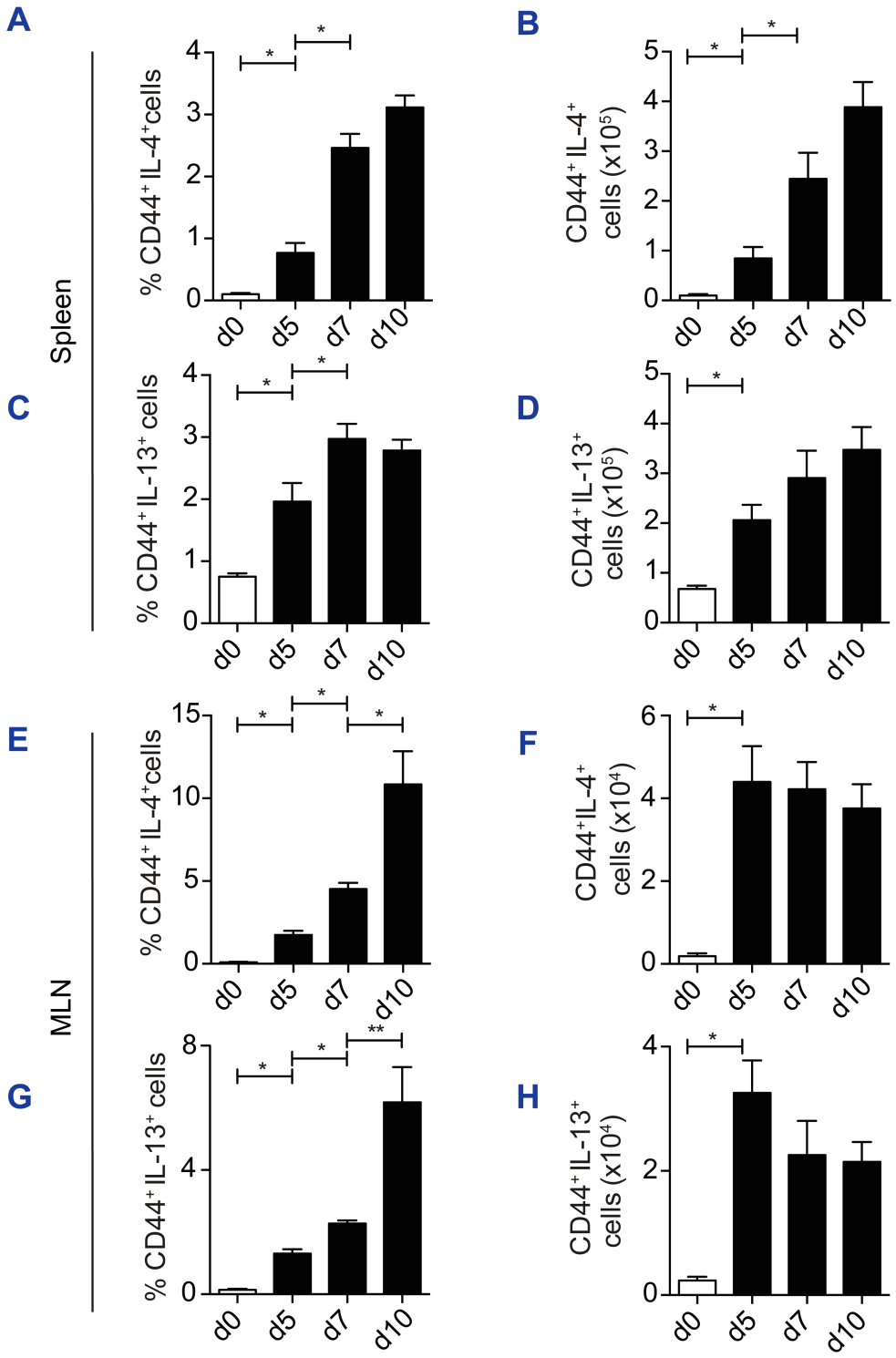


Figure 3.2. Timecourse of Th2 differentiation following primary infection with *H. polygyrus*. C57BL/6 mice were infected with 200 *H. polygyrus* larvae and harvested at days 0, 5, 7 and 10 post-infection. Frequency (A and C) and total number (B and D) of splenic $CD44^+IL\text{-}4^+$ cells and $CD44^+IL\text{-}13^+$ cells as measured by intra-cellular cytokine staining. Frequency (E and G) and total number (F and H) of $CD44^+IL\text{-}4^+$ cells and $CD44^+IL\text{-}13^+$ cells in the MLN as measured by intra-cellular cytokine staining. Statistical test applied Mann-Whitney, two-tailed, * denotes $p<0.05$.

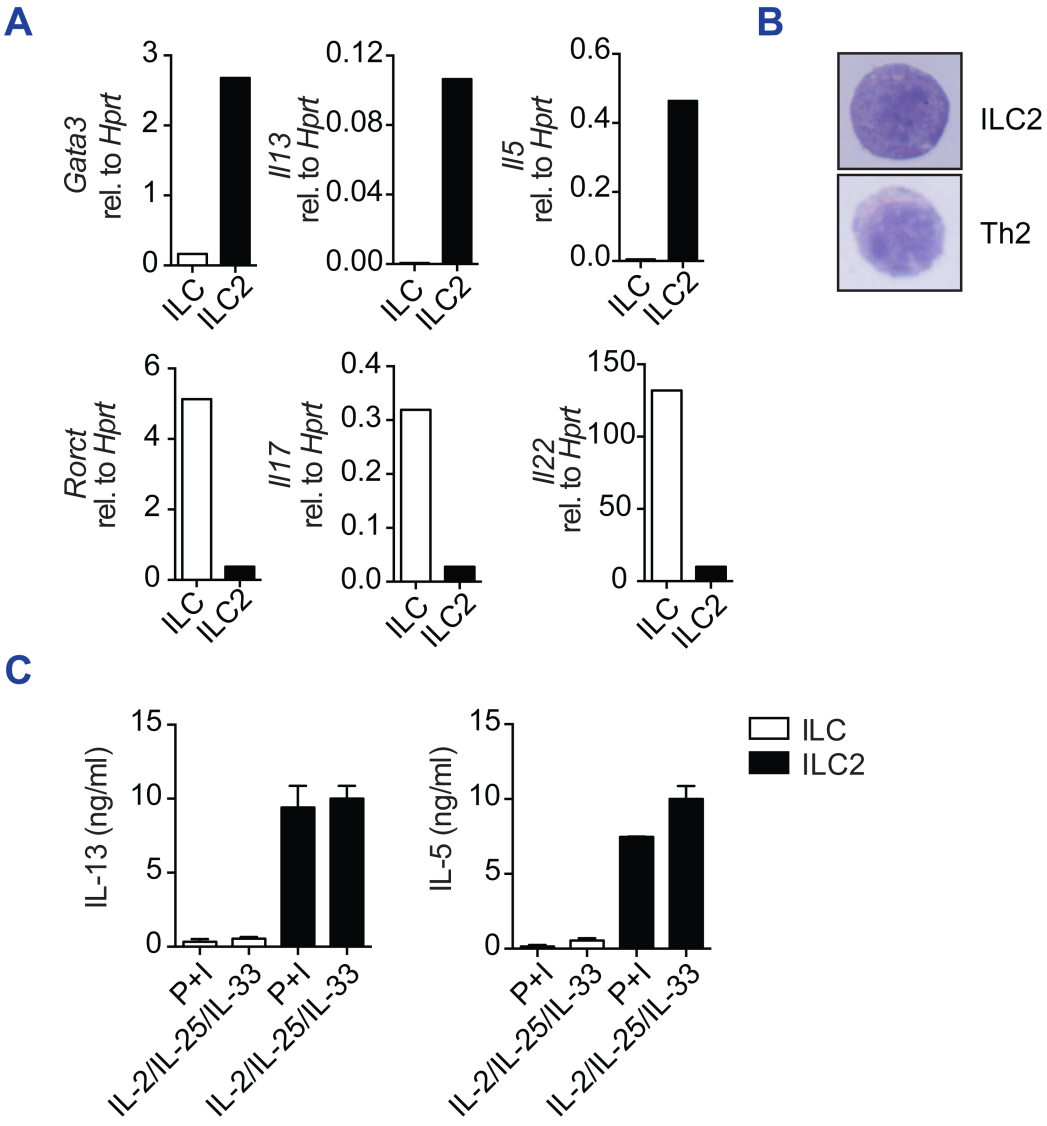


Figure 3.3. Intestinal KLRG1$^+$ ILC2s express characteristic type-2 cytokines. *Rag2*$^{-/-}$ mice were infected with 200 *H. polygyrus* larvae and harvested at day 5 post-infection. A) Expression of key innate lymphoid cell associated genes (*Gata3, Il13, Il5, Rorct, Il17, and Il22*) in FACS-purified LP ILCs (Lin$^-$Thy1.2$^+$KLRG1$^-$) and ILC2s (Lin$^-$Thy1.2$^+$KLRG1$^+$), expressed relative to *Hprt*. Data is representative of 3 independent experiments and cells were pooled from 4-6 mice. B) Cytospins of FACS-purified ILC2 cells isolated from *Rag2*$^{-/-}$ mice day 5 post-infection and CD4$^+$TCRβ$^+$*Il4*-GFP$^+$ (Th2) cells isolated from *Il4*-GFP mice infected for 14 days with *H. polygyrus*. C) Quantification of IL-5 and IL-13 production by LP FACS-purified ILCs (white bars) and ILC2s (black bars) re-stimulated with PMA and ionomycin or IL-2/IL-25/IL-33 for 24h, as measured by Flow Cytomix. Data is representative of 2 independent experiments and cells were pooled from 4-6 mice.

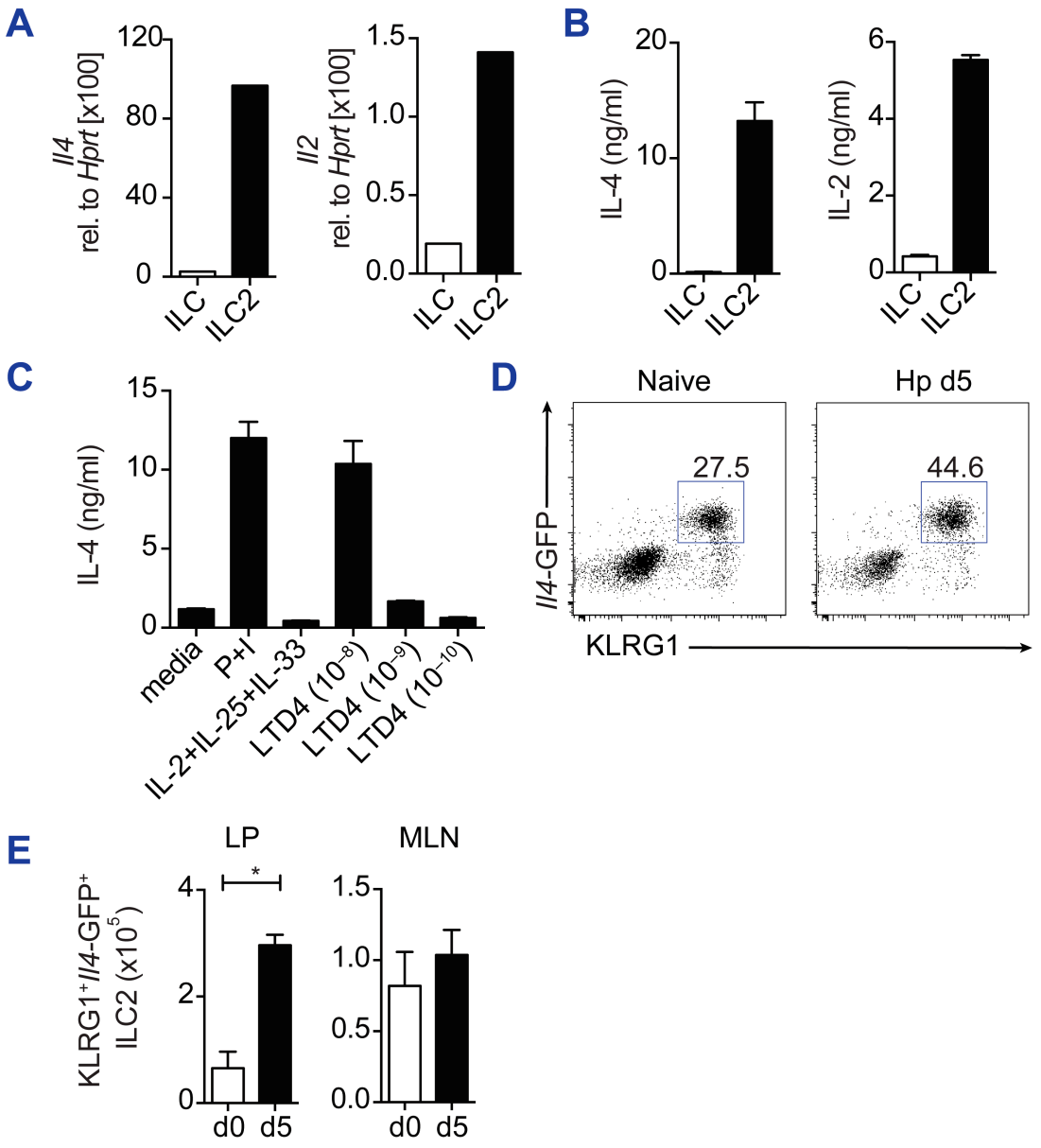


Figure 3.4. ILC2s express and secrete IL-4 and IL-2, and constitutively express *Il4*-GFP. *Rag2*$^{-/-}$ mice were infected with 200 *H. polygyrus* larvae and harvested at day 5 post-infection. A) *Il4* and *Il2* gene expression in FACS-purified LP ILCs and ILC2s, expressed relative to *Hprt*. Cells were pooled from 4-6 mice. B) Quantification of IL-4 and IL-2 production by FACS-purified LP ILCs and ILC2s re-stimulated with PMA and ionomycin for 24h, as measured by Flow Cytomix. C) Quantification of IL-4 production by FACS-purified MLN ILC2s re-stimulated with PMA and ionomycin, IL-2/IL-25/IL-33 or LTD_4 for 24h. *Il4*-GFP*Foxp3*-RFP mice were infected with 200 *H. polygyrus* larvae and harvested at day 5 post-infection. D) Representative FACS plots of small intestine LP cells showing the frequency of KLRG1$^+$*Il4*-GFP$^+$ cells within the Lineage$^-$Thy1.2$^+$ gate. E) Total number of KLRG1$^+$*Il4*-GFP$^+$ cells in the LP and MLN at days 0 (white bars) and 5 (black bars) post-infection. Data are representative of 3 independent experiments with 4 mice per group. * denotes $p<0.05$.

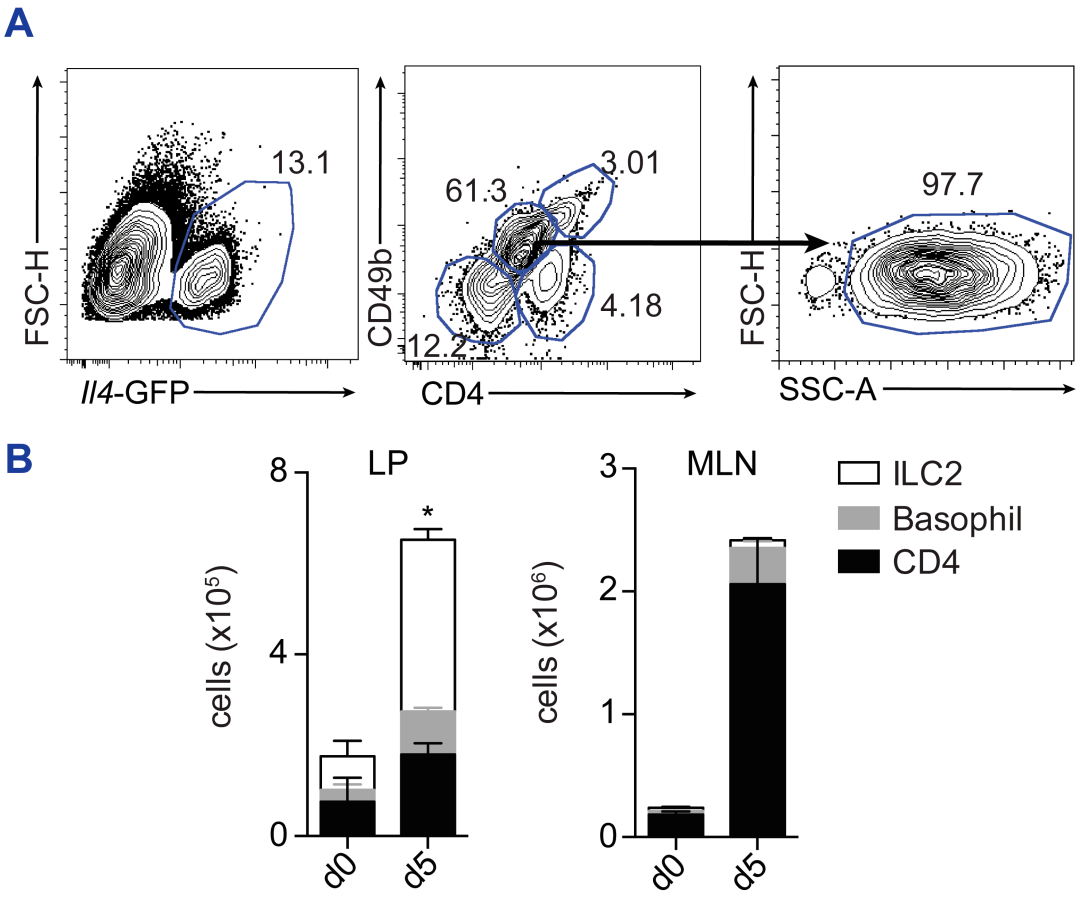


Figure 3.5. ILC2s form a significant part of the *Il4*-GFP$^+$ population in the LP at day 5 post-infection. *Il4*-GFP*Foxp3*-RFP mice were infected with 200 *H. polygyrus* larvae and harvested at day 5 post-infection. A) Representative FACS plots of small intestine LP cells showing the frequency of CD4$^+$, CD49b$^+$ CD4$^-$CD49b$^-$ cells within the *Il4*-GFP$^+$ gate, and all non-CD4, non-CD49b, non-CD4$^-$CD49b$^-$ cells. Representative FACS showing the frequency of FSC-H^{low}SSC-A^{high} cells within the non-CD4/non-CD49b/non-CD4$^-$CD49b$^-$ gate. B) Total number of CD4$^+$ Th2 cells (black bar; CD45$^+$*Il4*-GFP$^+$CD4$^+$), basophils (grey bar; CD45$^+$*Il4*-GFP$^+$CD49b$^+$) and ILC2s (white bar; CD45$^+$*Il4*-GFP$^+$CD4$^-$CD49b$^-$Thy1.2$^+$KLRG1$^+$) in the LP and MLN at days 0 and 5 post-infection. C) Total number of ILC2s (CD45$^+$*Il4*-GFP$^+$CD4$^-$CD49b$^-$ Thy1.2$^+$KLRG1$^+$) and CD4$^+$ Th2 cells (CD45$^+$*Il4*-GFP$^+$CD4$^+$) in the LP and MLN at day 5 post-infection. Data are representative of 3 independent experiments with 4 mice per group. * denotes $p<0.05$.

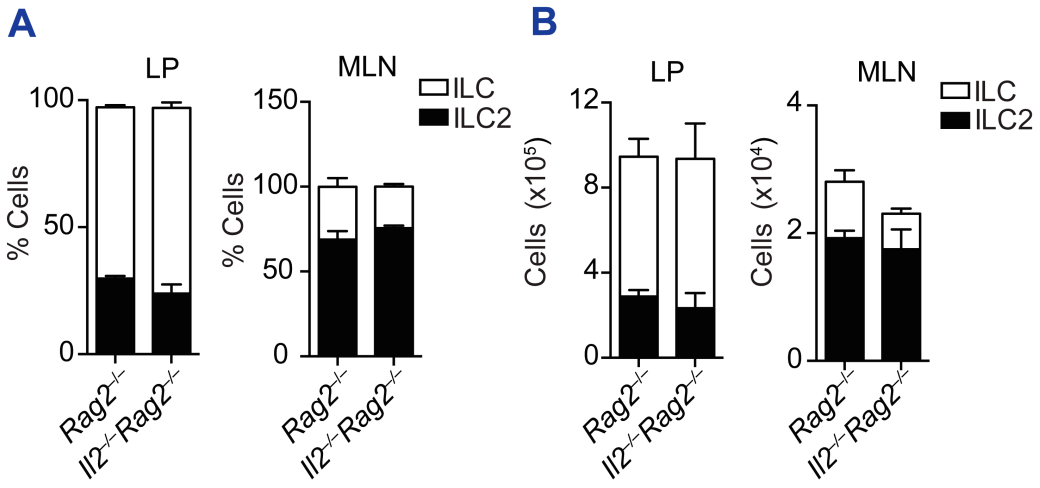


Figure 3.6. ILC2s develop normally in the absence of IL-2. A) Frequency and total number (B) of ILCs (white bars; Lin$^-$Thy1.2$^+$KLRG1$^-$) and ILC2s (black bars; Lin$^-$Thy1.2$^+$KLRG1$^+$) in the LP and MLN of naïve *Rag2*$^{-/-}$ and *Il2*$^{-/-}$*Rag2*$^{-/-}$ mice. Data are representative of 3 independent experiments with 3-5 mice per group.

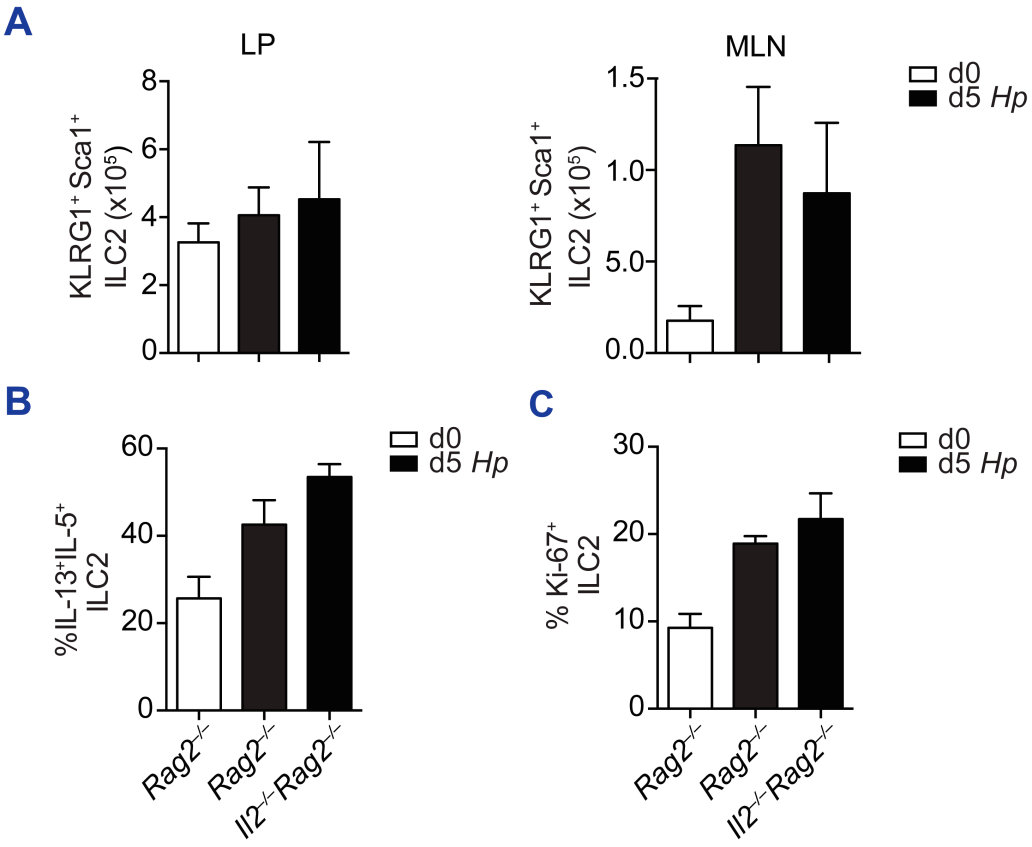


Figure 3.7. IL-2 is not required for the expansion, cytokine production or proliferation of ILC2s. *Rag2*$^{-/-}$ or *Il2*$^{-/-}$*Rag2*$^{-/-}$mice were infected with 200 *H. polygyrus* larvae and harvested at day 5 post-infection. A) Absolute number of ILC2s (Lin^{-}Thy1.2^{+}KLRG1^{+}) in the LP and MLN of naïve *Rag2*$^{-/-}$ (white bars) and infected *Rag2*$^{-/-}$ and *Il2*$^{-/-}$*Rag2*$^{-/-}$ mice (black bars) day 5 post-infection. Data are representative of 3 independent experiments with 3-5 mice per group. B) Frequency of IL-5^{+}IL-13^{+} ILC2s (Lin^{-}Thy1.2^{+}KLRG1^{+}) in the MLN of naïve *Rag2*$^{-/-}$ (white bars) and infected *Rag2*$^{-/-}$ and *Il2*$^{-/-}$*Rag2*$^{-/-}$ mice (black bars) day 5 post-infection. C) Frequency of Ki-67^{+} ILC2s (Lin^{-}Thy1.2^{+}KLRG1^{+}) in the MLN of naïve *Rag2*$^{-/-}$ (white bars) and infected *Rag2*$^{-/-}$ and *Il2*$^{-/-}$*Rag2*$^{-/-}$ mice (black bars) day 5 post-infection. Data are representative of 3 independent experiments with 3-5 mice per group.

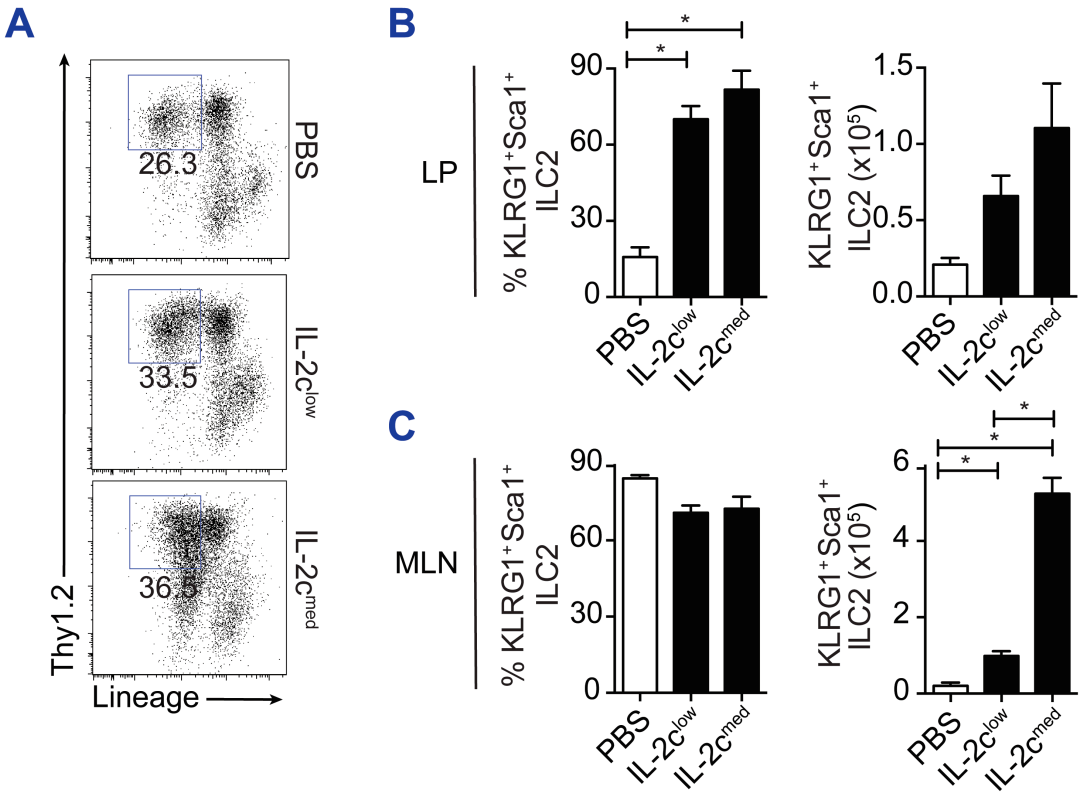


Figure 3.8. IL-2 complex (IL-2c) treatment expands ILC2s in the tissue and mesenteric lymph nodes. *Rag2*$^{-/-}$ were infected with 200 *H. polygyrus* larvae and treated with low-dose IL-2c (IL-2c^{low}) and medium-dose (IL-2c^{med}) or with PBS as a control. Mice were harvested 5 days post-infection. A) Representative FACS plots of MLN cells showing the frequency of lineage^{-} Thy1.2^{+} cells following treatment. Frequency and total number of ILC2s (Lin^{-}Thy1.2^{+}KLRG1^{+}) in the LP (B) and MLN (C) of treated mice. Data are representative of 3 independent experiments with 3-4 mice per group. * denotes $p<0.05$.

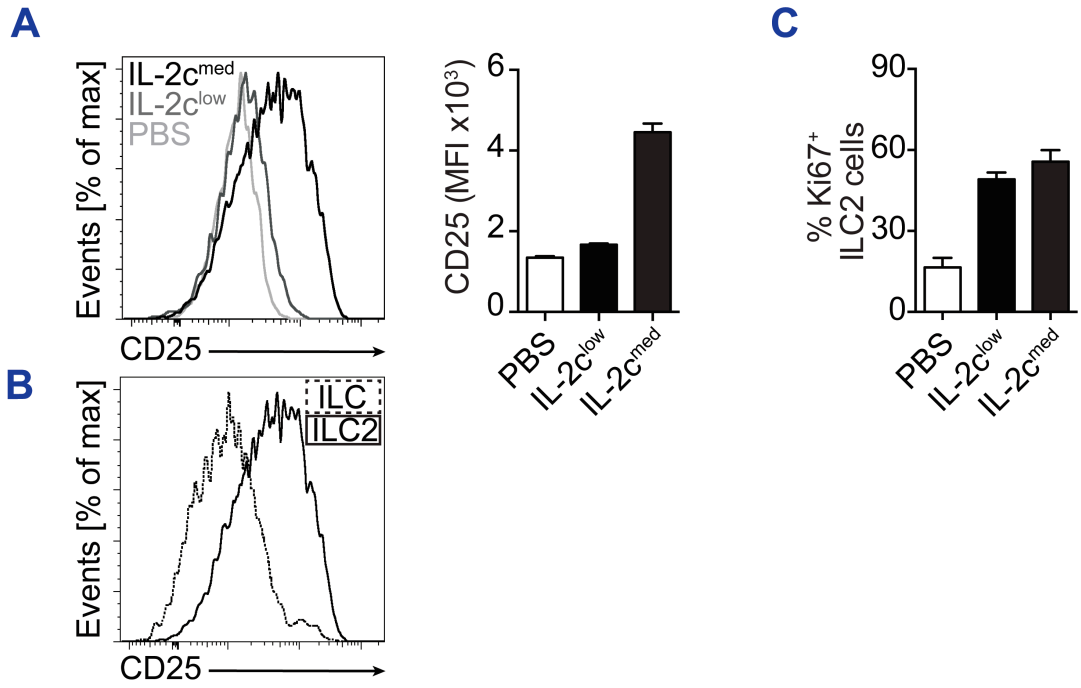


Figure 3.9. IL-2c treatment increases the surface expression of CD25 on ILC2s and their proliferation. *Rag2*$^{-/-}$ were infected with 200 *H. polygyrus* larvae and treated with low-dose IL-2c (IL-2c^{low}) and medium-dose (IL-2c^{med}) or with PBS as a control. Mice were harvested day 5 post-infection. A) Representative FACS plots of MLN cells showing the frequency of Lineage^{-} Thy1.2^{+} cells following treatment. Frequency and total number of ILC2s (Lin^{-}Thy1.2^{+}KLRG1^{+}) in the LP (B) and MLN (C) of IL-2c-treated mice. Data are representative of 3 independent experiments with 3-4 mice per group. * denotes $p<0.05$.

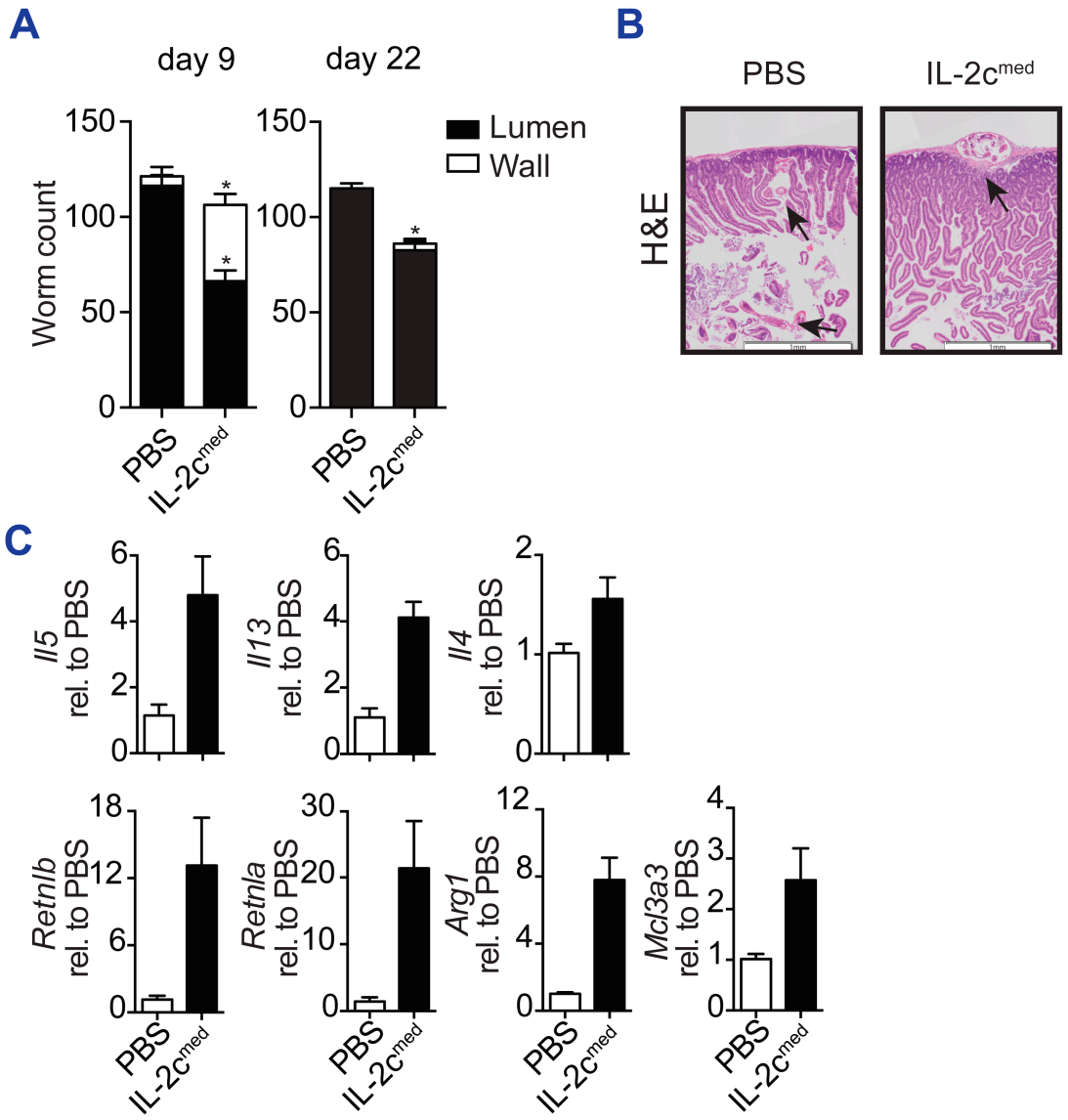


Figure 3.10. IL-2c treatment impairs larval development resulting in reduced adult worm development. *Rag2*$^{-/-}$ were infected with 200 *H. polygyrus* larvae and treated with IL-2c^{med} or PBS as a control. A) Worm count of *H. polygyrus* larvae in the wall (black bars) and adult worms in the lumen (white bars) of mice at day 9 and day 22 post-infection. B) Immune cell infiltration (H&E) in small intestine histological sections. Arrows point to wall-embedded and luminal worms. C) *Il5, Il13, Il4, Retnla, Retnlb, Arg1* and *Mcl3a3* (Gob5) gene expression in the small intestine of IL-2c treated mice, day 5 post-infection, expressed as fold change relative to PBS treated mice. Data are representative of 3 independent experiments with 3-4 mice per group. * denotes $p<0.05$

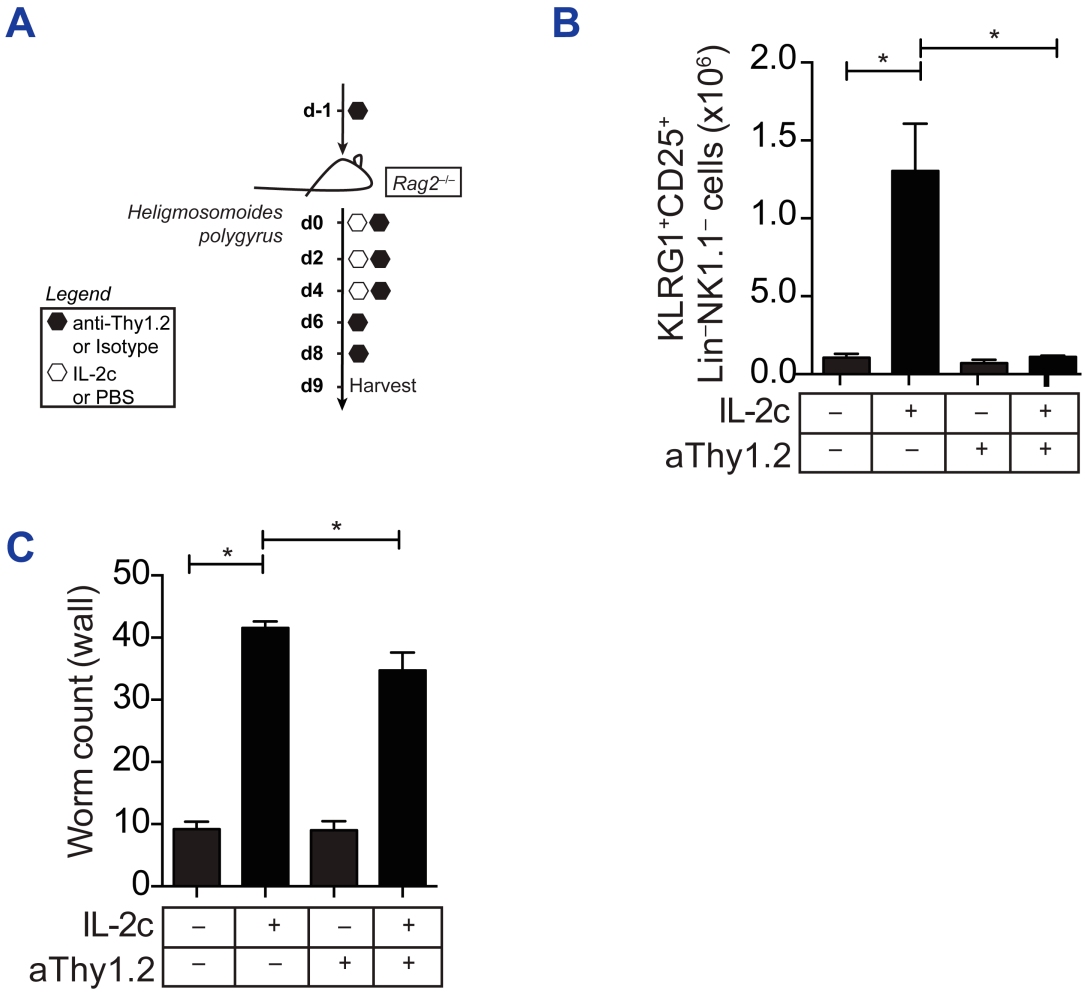


Figure 3.11. Depletion of ILC2s partly impairs IL-2c-mediated larval killing.

A) Experimental system: *Rag2*$^{-/-}$ were pre-treated with anti-Thy1.2 or an isotype control. Following this, the mice were infected with *H. polygyrus* and treated with anti-Thy1.2 or isotype control as well as IL-2c as illustrated. B) Absolute number of ILC2s (Lineage^{-}NK1.1^{-} KLRG1^{+}CD25^{+}) in the LP of IL-2c and anti-Thy1.2 antibody treated mice day 9 post-infection. C) Worm count in the intestinal wall of IL-2c and anti-Thy1.2 antibody treated mice day 9 post-infection. Data are representative of 2 independent experiments with 4-6 mice per group. * denotes $p<0.05$.

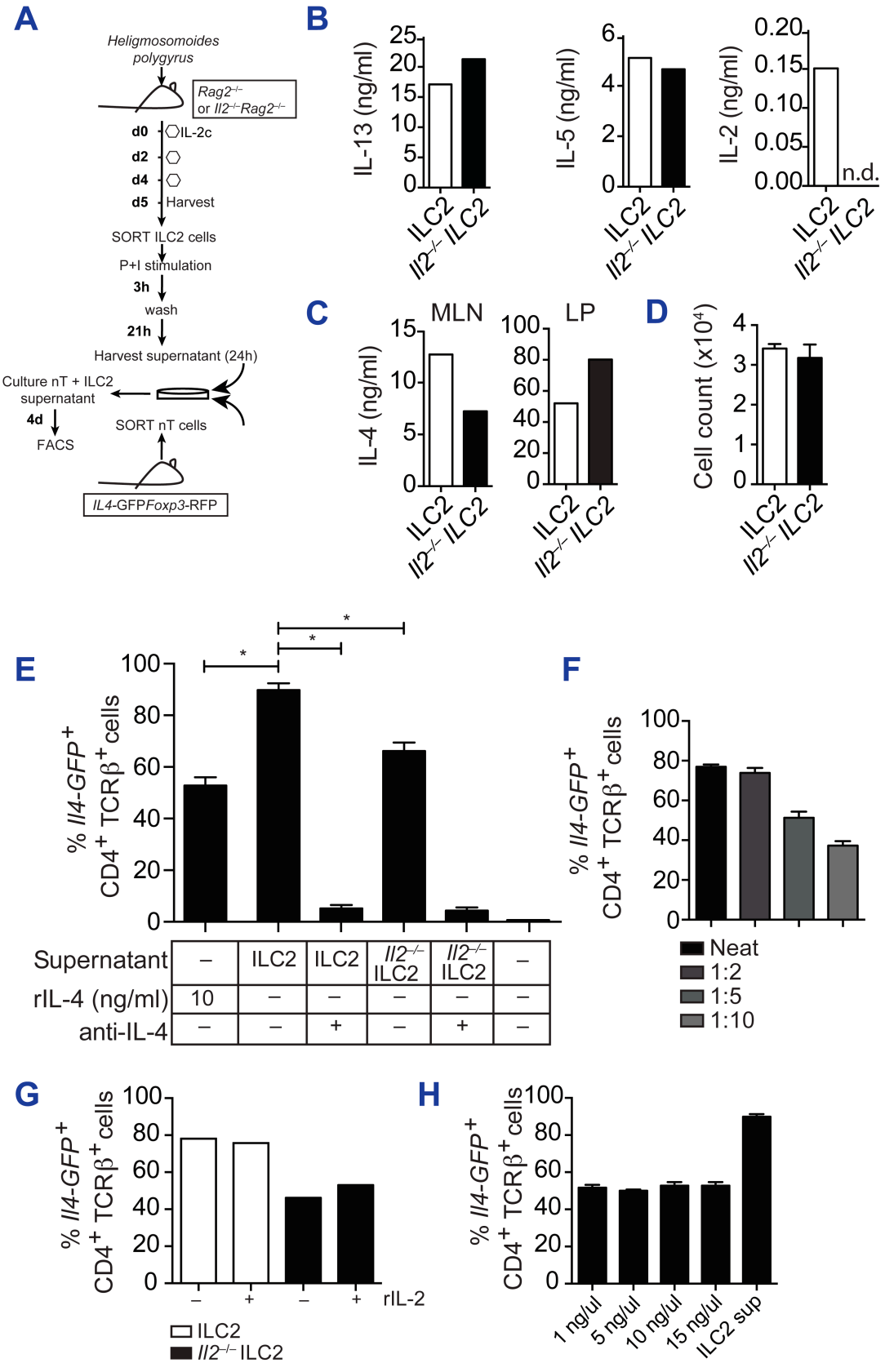

A
Heligmosomoides polygyrus
Rag2−/− or Il2−/−Rag2−/−
d0 IL-2c
d2
d4
d5 Harvest
SORT ILC2 cells
P+I stimulation
3h
wash
21h
Harvest supernatant (24h)
Culture nT + ILC2 supernatant
4d
FACS
SORT nT cells
IL4-GFPFoxp3-RFP
B
IL-13 (ng/ml)
IL-5 (ng/ml)
IL-2 (ng/ml)
n.d.
ILC2
Il2−/− ILC2
C
MLN
LP
IL-4 (ng/ml)
D
Cell count (x10⁴)
E
% Il4-GFP+ CD4+ TCRβ+ cells
Supernatant
rIL-4 (ng/ml)
anti-IL-4
F
Neat
1:2
1:5
1:10
G
rIL-2
H
1 ng/ul
5 ng/ul
10 ng/ul
15 ng/ul
ILC2 sup

Figure 3.12. ILC2s drive the differentiation of naïve T cells *in vitro* in an IL-4 and IL-2-dependent manner. A) Experimental model: ILC2 cells were expanded using IL-2 complex and FACS-purified from the MLN of 3-4 *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il2*$^{-/-}$ mice. Following this the cells were stimulated with PMA + Ionomycin for 3H, washed and resuspended in complete media and harvested at 24H. B) Quantification of IL-13, IL-5 and IL-2 as well as C) IL-4 production in the supernatant of re-stimulated ILC2s or *Il2*$^{-/-}$ ILC2s (3-24H) measured by Flow Cytomix. D) Live cell count after 24H in culture. Error bars represent individual wells. Naïve T cells (nT; CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{-}CD44^{-}CD25^{-}) were FACS-purified from *Il4*-GFP*Foxp3*-RFP mice and cultured with the supernatant from either ILC2 or *Il2*$^{-/-}$ ILC2 cultures, in the presence or absence of anti-IL-4 blocking antibody. Cells were cultured with recombinant IL-4 (10ng/ml) and IL-2 (10ng/ml) or cIMDM as controls. Cells were pooled from 2-3 mice and error bars represent individual wells. E) Frequency of *Il4*-GFP^{+} cells in the CD4^{+}TCRβ^{+} gate as assessed by FACS. Data are representative of 3 independent experiments. nT cells were sorted from *Il4*-GFP*Foxp3*-RFP mice and cultured with supernatant ILC2 cultures at indicated dilutions (F) or with the addition of recombinant IL-2 (G). nT cells were sorted from *Il4*-GFP*Foxp3*-RFP mice and cultured with recombinant IL-4 at the indicated concentrations. Data is representative of 1 experiment. * denotes $p<0.05$.

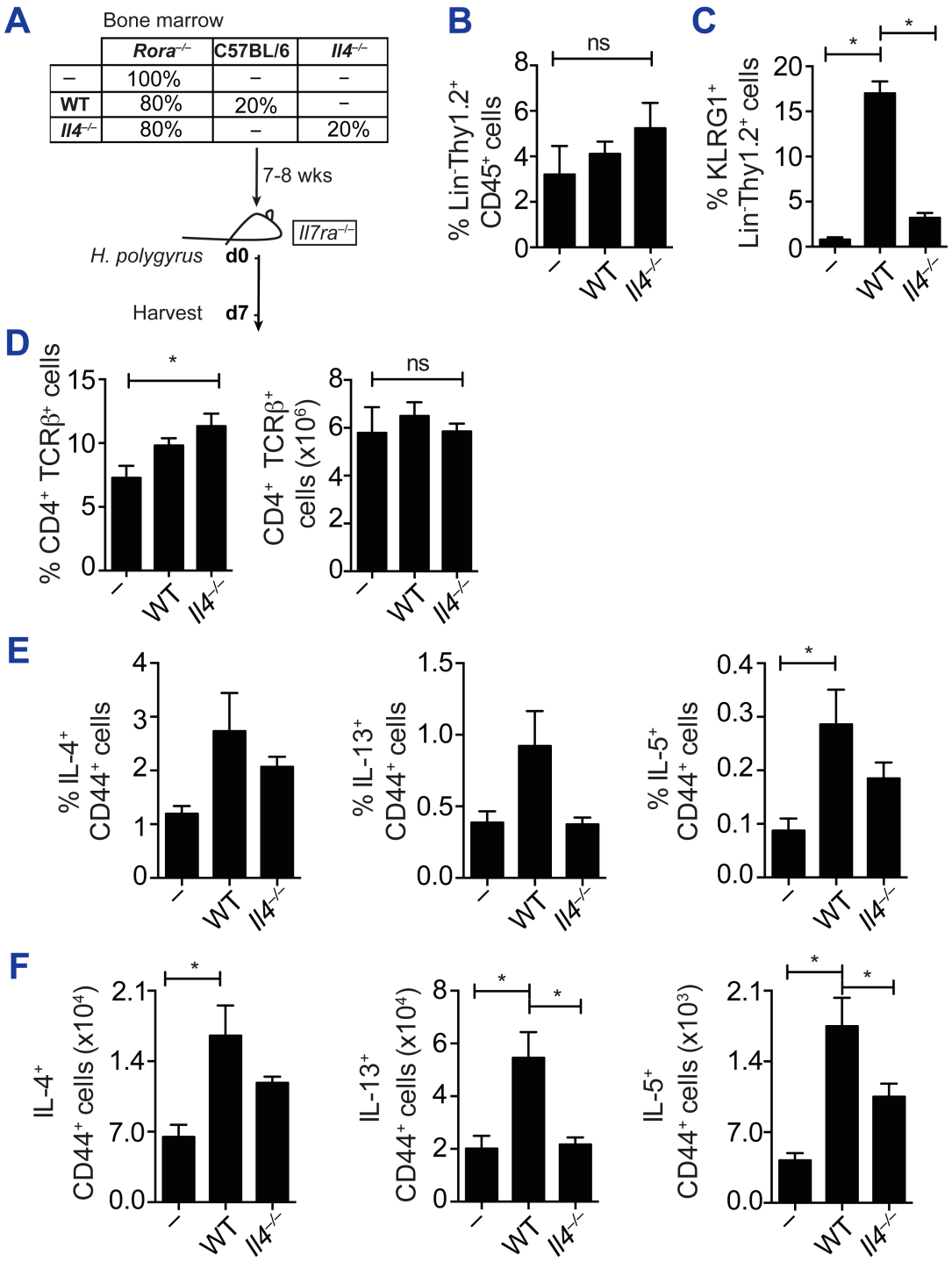

A
Bone marrow
Rora−/− C57BL/6 Il4−/−
– 100% – –
WT 80% 20% –
Il4−/− 80% – 20%
7-8 wks
Il7ra−/−
H. polygyrus d0
Harvest d7
B
% Lin-Thy1.2+ CD45+ cells
ns
C
% KLRG1+ Lin-Thy1.2+ cells
D
% CD4+ TCRβ+ cells
CD4+ TCRβ+ cells (x10^6)
E
% IL-4+ CD44+ cells
% IL-13+ CD44+ cells
% IL-5+ CD44+ cells
F
IL-4+ CD44+ cells (x10^4)
IL-13+ CD44+ cells (x10^4)
IL-5+ CD44+ cells (x10^3)
–
WT
Il4−/−

Figure 3.13. ILC2s are required for the development of Th2 cells during primary infection with *H. polygyrus*. A) Experimental system: *Il7ra*$^{-/-}$ mice were sub-lethally irradiated and reconstituted with *Rora*$^{-/-}$ (RORα$^{sg/sg}$) ILC2-deficient bone marrow or a mix of 80% *Rora*$^{-/-}$ bone marrow and 20% C57BL/6 (WT) or 20% *Il4*$^{-/-}$ (IL-4KO) bone marrow. 7-weeks post-reconstitution, chimeric mice were infected with 200 *H. polygyrus* larvae and harvested at day 7 post-infection. Frequency of ILCs (Lineage^{-}Thy1.2^{+}) (B) and ILC2s (Lineage-Thy1.2+KLRG1+) (C) in the LP of RORα$^{sg/sg}$ (–), RORα$^{sg/sg}$:WT (WT) or RORα$^{sg/sg}$:IL-4KO (*Il4*$^{-/-}$) mice d7 post-infection as assessed by FACS. D) Frequency and total number of CD4^{+}TCRβ^{+} cells in the spleen of chimeric mice as assessed by FACS. E) Frequency of IL-4^{+}, IL-13^{+} and IL-5^{+} of the CD4^{+}CD44^{+} cells in the spleen of chimeric mice as assessed by intra-cellular cytokine staining and FACS. F) Absolute number of IL-4^{+}, IL-13^{+} and IL-5^{+} of the CD4^{+}CD44^{+} cells in the spleen of chimeric mice as assessed by intra-cellular cytokine staining and FACS. Data are representative of 2 experiments with 6-7 mice per group. * denotes $p<0.05$.

Chapter 4: Foxp3$^+$ cells adopt a Th2 phenotype, contributing to protective type-2 immunity against *Heligmosomoides polygyrus*.

4.1. Introduction

Foxp3$^+$ CD4$^+$ Treg cells are suppressive cells essential for the maintenance of immune homeostasis (Bennett et al., 2001; Brunkow et al., 2001) and prevention of excessive immune responses to infection (reviewed in (Belkaid, 2007)). The stability of the Treg population is maintained through the co-ordination of diverse mechanisms including cytokine signalling, transcription factor expression, post-transcriptional and post-translational modifications (Barbi et al., 2014). Despite this, many studies have identified that CD4$^+$ Treg cells can lose the expression of *Foxp3 in vitro* or after transfer into lymphopenic hosts (Coomes et al., 2013). Additionally, the use of fate-mapping tools has identified a population of ex-Foxp3 cells that previously expressed, but have subsequently down-regulated *Foxp3* expression *in vivo* in the context of autoimmune disease (Bailey-Bucktrout et al., 2013; Komatsu et al., 2014) and allergic inflammation (Noval Rivas et al., 2015). Furthermore, a significant fraction of ex-Foxp3 cells acquired T helper cell function and contributed to disease pathogenesis. To date, it is unclear whether ex-Foxp3 cells contribute to protective rather than pathogenic immune responses, in particular in the context of infection. In addition, due to a limited understanding of the mechanisms driving Treg to T effector cell conversion, the contribution of Treg conversion to functional immune responses remains unclear.

The immune response to the intestinal helminth *H. polygyrus* is characterised by the simultaneous development of a polarised type-2 response, orchestrated by CD4$^+$ Th2 cells, and immune-regulatory mechanisms, including the expansion of CD4$^+$ Foxp3$^+$ T regulatory cells. The balance established between these responses is thought to limit host damage, but as a consequence permits the survival, establishment and chronicity of infection and subsequent disease morbidity (Gause et al., 2013). CD4$^+$ Th2 cells secrete cytokines that activate a cascade of downstream type-2 effector responses and promote functional immunity to intestinal helminths (Allen and

Maizels, 2011). Emerging evidence suggests Th2 cells are required for sterile immunity to re-infection following anti-helminth vaccination (Hewitson et al., 2015). Interestingly, Th2 cells have contrasting pathogenic and regulatory roles within the immune response, possibly depending upon the time and requirements of the immune response. For example Th2 cells contribute to the development of allergic inflammation and can also promote wound healing and tissue repair during the resolution of inflammation (Wynn, 2015). Despite their important role as orchestrators of type-2 responses, the factors driving Th2 differentiation *in vivo* remain unclear. A better understanding of the cellular and molecular pathways leading to Th2 differentiation *in vivo* is therefore required for the improved design of effective vaccines and new therapeutics and to identify new molecular targets to prevent Th2-mediated allergic reactions.

In the previous chapter we identified a novel role for IL-4-secreting ILC2s in the differentiation of Th2 cells during acute *H. polygyrus* infection. In this chapter, we asked whether $CD4^+$ $Foxp3^+$ T regulatory cells convert to Th2 cells during *H. polygyrus* infection and contribute to the protective type-2 response. Many studies have shown a close relationship between Treg and Th2 cells and loss of *Foxp3* expression has often been associated with the spontaneous acquisition of a Th2 phenotype (Sawant et al., 2012; Muto et al., 2013). In addition, IL-4, which is required for Th2 differentiation *in vivo* (Kopf et al., 1993), can suppress *Foxp3* expression *in vivo* (Noval Rivas et al., 2015) and directly lead to the re-methylation of the *Foxp3* locus *in vitro*, thereby compromising Treg stability (Feng et al., 2014).

We hypothesised that canonical IL-4 signalling drives Treg conversion to Th2 cells and that ex-Treg cells contribute to the memory Th2 response during a secondary, challenge infection with *H. polygyrus.* To test this hypothesis, we generated *Il4*-GFP*Foxp3*-RFP double-reporter mice to simultaneously monitor Th2 and Treg responses. Using these mice, we demonstrated that *Foxp3*-RFP^+CD25^{high} cells could lose *Foxp3* expression and acquire *Il4*-GFP expression following adoptive transfer into lymphodeficient mice infected with *H. polygyrus.* To determine the frequency of Th2

cells that had derived from a Treg-past *in vivo*, we used a *Foxp3* fate-reporter system crossed with *Il4*-GFP reporter mice (*Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635}) and identified a population of *Il4*-GFP^{+}*R26R*$^{FP635+}$ cells *in vivo* which were currently transcribing *Il4* and were fate-marked (FP635^{+}) indicating that they had derived from a Foxp3-expressing past. The proportion and number of these *Il4*-GFP^{+} ex-Foxp3 cells expanded during a secondary infection with *H. polygyrus*. Finally, we have deleted IL-4R signalling specifically on *Foxp3*-expressing Treg cells, using recently generated *Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635}*Il4ra*$^{fl/fl}$ mice, to test the Treg cell-intrinsic requirement of IL-4 signalling for Treg conversion. These mice will also be used to test the functional importance of IL-4Rα signalling on Treg cells during *H. polygyrus* expulsion.

4.2. Results

4.2.1. Secondary infection with *H. polygyrus* correlates with increased type-2 responses and larval killing

To study the conversion of CD4^{+} Foxp3^{+} T cells to CD4^{+} Th2 cells following infection with *H. polygyrus*, we generated double-reporter mice by crossing existing fluorescent cytokine reporter strains *Foxp3*-RFP mice (Wan and Flavell, 2005) and *Il4*-GFP mice (Mohrs et al., 2001). *Il4*-GFP*Foxp3*-RFP mice enabled us to simultaneously monitor Th2-commited and Treg cells based on their expression of lineage-defining factors *Foxp3* and *Il4* and to FACS-purify live Th2 or Treg cells for adoptive transfer studies.

H. polygyrus establishes a chronic infection in C57BL/6 mice lasting between 8-20 weeks (Reynolds et al., 2012) however drug-cured mice develop a highly protective memory response resulting in killing of subsequent invading larvae (Reynolds et al., 2012). Using this system and with the same genetic background of mice, we can compare the immune response during a primary infection (Hp 1°), which leads to a chronic infection, with the immune response during a secondary challenge infection (Hp 2°), which leads to rapid killing of invading larvae. To do this, we infected *Il4*-

GFP*Foxp3*-RFP mice with *H. polygyrus,* drug-treated them on day 14 and 15 post-infection and subsequently gave them a secondary, challenge infection on day 28 (Hp 2°) and analysed the immune response at d35 or d42 (model; **Fig. 4.1 A**). We infected a second group of *Il4*-GFP*Foxp3*-RFP mice and analysed the immune response at day 7 or 14 following a primary infection (Hp 1°) (model; **Fig. 4.1 A**), which could otherwise lead to a chronic infection. Validating this model system in my hands, we observed significantly fewer adult worms in the lumen of immune Hp 2° mice compared to susceptible Hp 1° mice 14 days post-infection (**Fig. 4.1 B**). Immunity to secondary infection correlated with increased mucus staining on intestinal sections (as visualised by AB-PAS stain) and increased accumulation of immune cells around the *H. polygyrus* larvae in the intestinal wall (as assessed by H&E staining) at day 7 post-infection in secondary infected mice (**Fig. 4.1 C**). In addition, immunity to secondary infection correlated with a significant increase in the expression of several genes associated with type-2 effector responses such as *Retnlb*, *Retnla, Arg1* and *Gob5* in the small intestine (**Fig. 4.1 D**).

4.2.2. Characterising Treg and Th2 cells during *H. polygyrus* infection

4.2.2.1. Shift from a regulatory to an effector T cell response during secondary infection with *Heligmosomoides polygyrus*

We characterised the frequency of *Il4*-GFP-expressing and *Foxp3*-RFP-expressing $CD4^+$ T cells in mice with a primary or secondary infection by FACS analysis. We observed an increased proportion of $CD4^+$*Foxp3*-RFP^-*Il4*-GFP^+ Th2 cells in Hp 1° mice compared to naïve mice, which was further increased during a secondary infection (Hp 2°), as shown in the representative FACS plots of the MLN (**Fig. 4.2 A**). $CD4^+$*Foxp3*-RFP^-*Il4*-GFP^+ Th2 cells were significantly increased in the PP and the MLN (**Fig. 4.2 C and D**) of Hp 1° mice compared to naïve mice, and further significantly increased in Hp *2*° mice compared to Hp 1° mice in the spleen, MLN and PP (**Fig. 4.2 A, B and C**). There were no significant changes in the proportion of

CD4$^+$*Foxp3*-RFP$^+$*Il4*-GFP$^-$ Treg cells in the spleen or MLN (**Fig. 4.2 A and B**) however Treg cells were increased in the PP of Hp 1° mice in comparison to naïve and Hp 2° mice (**Fig. 4.2 D**). Using this data we calculated the ratio of *Il4*-GFP expression over *Foxp3*-RFP expression and observed a significant increase in the ratio of Th2 to Treg cells between Hp 1° and Hp 2° mice in the spleen, MLN and PP (**Fig. 4.2 E**). Taken together this data indicated that immunity to *H. polygyrus* following secondary infection, correlated with a dominant CD4$^+$ Th2 effector population in systemic, secondary and tissue-associated lymphoid organs.

4.2.2.2. IL-2c treatment during *H. polygyrus* infection leads to the expansion of Treg and Th2 cells and cannot be used to preferentially expand Treg cells

As previously discussed in chapter 3, the complex of IL-2 and IL-2 antibody (JES6-1A12 clone), or IL-2 complex (IL-2c), has been shown to preferentially expand CD4$^+$Foxp3$^+$ cells (Boyman et al., 2006) and has been extensively used to manipulate the balance between Treg and T effector populations in the context of inflammation. For example, IL-2c was shown to restore Treg numbers and limit Th1-mediated islet cell damage during the development of type-1 diabetes in NOD mice (Tang et al., 2008) and to expand Treg numbers and limit Th2-mediated airway allergy (Wilson et al., 2008). Furthermore, IL-2c treatment was shown to restore Treg numbers following *Toxoplasma gondii* infection and limit a pathogenic Th1 response (Oldenhove et al., 2009). In light of our data showing IL-2c expansion of CD25-expressing ILC2s (chapter 3), we wanted to investigate the impact of IL-2c treatment on CD4$^+$ T cell responses following *H. polygyrus* infection, with the aim of promoting Th2 cells and *H. polygyrus* expulsion. We treated naïve and *H. polygyrus* infected mice (at days 12, 14 and 16 post-infection) with a previously published dose of IL-2c (IL-2c^{high}; 5:50μg) and observed a significant expansion in the proportion of *Foxp3*-RFP$^+$ Treg cells in the spleen of both naïve and *H. polygyrus* infected mice (**Fig. 4.3 A**). Furthermore, we saw a two-fold expansion in the proportion of *Il4*-GFP$^+$ cells in naïve and *H. polygyrus*

infected mice (**Fig. 4.3 A**). Due to the high levels of CD25 expression on Treg cells (Sakaguchi et al., 1995), lower doses of IL-2c have been shown to preferentially expand Treg cells over T effector cells (Tang et al., 2008; Klatzmann and Abbas, 2015). Therefore, we wanted to test whether we could use variable doses of IL-2c to modulate the balance between Treg and Th2 cells. We treated *H. polygyrus* infected mice with IL-2c^{high} (5:50μg) and IL-2c^{low} (0.5:5μg) doses. We found that treatment with IL-2c^{low} did not induce a significant expansion of either Treg or Th2 cells compared to PBS treated mice (**Fig. 4.3 C**). From these experiments we concluded that IL-2c can stimulate the expansion of diverse CD25-expressing cells including CD4$^+$ Th2 and Treg cells, however we were unable to test whether differing doses of IL-2c led to the preferential expansion of Treg or Th2 cells and would need to test an intermediate dose in any future experiments.

4.2.2.3. The *Il4*-GFP*Foxp3*-RFP reporter faithfully reports *Il4* and *Foxp3* mRNA expression

Throughout these experiments, we identified a population of CD4$^+$*Il4*-GFP$^+$*Foxp3*-RFP$^+$ double-positive cells (DP) in both naïve and *H. polygyrus* infected mice (**Fig. 4.2 A**). We FACS-purified DP cells, single-positive *Il4*-GFP$^+$ (Th2) and *Foxp3*-RFP$^+$ (Treg) cells from the spleen and MLN of Hp 1° mice and analysed their expression of characteristic Th2 and Treg associated genes (*Il4, Foxp3, Il4ra, Il2ra, Gata3* and *Il10*). DP cells expressed *Il4,* albeit at lower levels than Th2 cells (**Fig. 4.4**), correlating with a lower *Il4*-GFP mean fluorescence intensity (MFI) observed by FACS analysis (**Fig. 4.2**). DP cells expressed similar levels of the Th2 master TF GATA-3 as Th2 cells. GATA-3 was also slightly increased in Treg cells compared to naïve T cells, in line with its essential role in the development of all CD4$^+$ T cell subsets (Wang et al., 2011; Wohlfert et al., 2011). DP cells expressed similar levels of *Foxp3* as Treg cells (**Fig. 4.4**), correlating with their similar *Foxp3*-RFP MFI levels (**Fig. 4.4**). Although Treg cells expressed similar levels of *Il4ra* to naïve T cells, *Il4*-GFP$^+$ cells expressed much

lower levels (**Fig. 4.4**), in accordance with the previously reported downregulation of IL-4Rα on differentiated Th2 cells *in vivo* (Perona-Wright et al., 2010). DP cells expressed intermediate level of *Il4ra* (**Fig. 4.4**) and *Il2ra* in comparison to *Foxp3*-RFP$^+$ cells and *Il4*-GFP$^+$ cells (**Fig. 4.4**). Interestingly, DP cells expressed significantly higher levels of *Il10* than both *Foxp3*-RFP$^+$ cells and *Il4*-GFP$^+$ cells. Taken together, these results show that *Il4*-GFP$^+$*Foxp3*-RFP$^+$ DP cells display a distinct gene expression profile. In addition, this data highlights the utility of *Il4*-GFP*Foxp3*-RFP mice to faithfully identify characteristic CD4+ T cell lineages and identify sub-populations of cells co-expressing lineage-specific TFs and cytokines.

4.2.3. *Foxp3*-RFP$^+$CD25^{high} Treg cells convert to *Il4*-GFP$^+$ cells and are transcriptionally re-wired following adoptive transfer into *H. polygyrus* infected TCRα-deficient mice

4.2.3.1. *Foxp3*-RFP$^+$CD25^{high} Treg cells convert to *Il4*-GFP$^+$ cells during secondary *H. polygyrus* infection

IL-4 can directly inhibit *Foxp3* expression *in vitro* (Feng et al., 2014) and *in vivo* (Noval Rivas et al., 2015). Furthermore, a proportion of Foxp3$^+$ cells can lose *Foxp3* expression and their suppressive potential in the context of inflammation (Barbi et al., 2014). Foxp3$^+$ cells expressing high levels of CD25 were shown to have a demethylated *Foxp3* locus, correlating with their functional stability *in vitro* (Komatsu et al., 2009) and *in vivo* following adoptive transfer (Miyao et al., 2012), however the stability of these cells was not tested during inflammation or in the context of infection. Following our observations that the Treg and Th2 ratio shifts towards a Th2 response during a memory response to *H. polygyrus,* we hypothesised that Treg cells contributed to the pool of memory Th2 cells, losing their regulatory phenotype and converting to Th2 cells. To test whether *Foxp3*-RFP$^+$ cells that develop following *H. polygyrus* infection could convert to *Il4*-GFP$^+$ cells, we FACS-purified CD4$^+$TCRβ$^+$*Il4*-GFP$^+$*Foxp3*-RFP$^+$ cells expressing high levels of CD25 (CD25^{high}) (HpTR) from the

spleen and MLN of mice 14 days following infection with *H. polygyrus,* a time when the parasite has established a chronic infection correlating with the peak of the differentiation of *Il4*-GFP-expressing $CD4^+$ T cells (**Fig. 4.5 A**). We hypothesised that a proportion of Treg cells developing in the face of a strong Th2 response would convert to Th2 cells following adoptive transfer (**Fig. 4.5 A**). We transferred these cells to T cell deficient (*Tcra*$^{-/-}$) mice, transferring FACS-purified naïve T cells (nT) as a control population into separate *Tcra*$^{-/-}$ mice. We infected the recipient *Tcra*$^{-/-}$ mice with *H. polygyrus* on the day of transfer*,* drug-cured them on days 14 and 15 post-infection and re-infected them at day 28 (model; **Fig. 4.5 B**). After 14 days of secondary infection, a high proportion of transferred HpTR cells lost *Foxp3*-RFP expression and CD25 expression. Furthermore, a proportion of transferred HpTR cells expressed *Il4*-GFP, as shown in representative FACS plots of transferred cells in the MLN (**Fig 4.5 D**). Approximately 10% of adoptively transferred HpTR cells (black bars) were *Foxp3*-RFP^- *Il4*-GFP^+ in the spleen and MLN, in comparison to 20% of transferred nT cells (white bars) (**Fig. 4.5 E**). More strikingly in the PP, 25% of transferred HpTR were *Foxp3*-RFP^-*Il4*-GFP^+, similarly to transferred nT cells (**Fig. 4.5 E**). In addition to expressing *Il4*-GFP, a proportion of transferred HpTR cells (black bars) secreted IL-4 protein in the MLN as measured by intra-cellular cytokine staining, to a similar extent as transferred nT cells (**Fig. 4.6 A**). Transferred HpTR cells failed to produce significant levels of IL-5 or IL-13 in comparison to nT cells (**Fig. 4.6 A**) suggesting that converted cells had acquired some, but not all, characteristics of Th2 cells. Together, these data identified that $CD25^{high}$*Foxp3*-RFP^+ cells have the potential to convert to IL-4-expressing and -secreting cells, to a similar extent as transferred naïve T cells, and suggest that differences in the tissue microenvironment may contribute to different levels of conversion.

4.2.3.2. MLN-derived *Foxp3*-RFP^{+}CD25^{high} Treg cells convert to the highest extent in the Peyer's patches

To test the impact of the tissue microenvironment on the ability of *Foxp3*-RFP^{+} cells to convert to *Il4*-GFP^{+} cells, we isolated HpTR cells from either the spleen or MLN of CD45.1 or CD45.2 congenically marked double-reporter mice and co-transferred CD45.1^{+}*Foxp3*-RFP^{+} (spleen) or CD45.2^{+}*Foxp3*-RFP^{+} (MLN) cells in equal proportions into the same *Tcra*$^{-/-}$ host. We infected the recipient *Tcra*$^{-/-}$ mice with *H. polygyrus,* drug-cured the mice and measured the proportion of *Il4*-GFP+ cells (model; **Fig. 4.6 B**). We found that 10-12% of transferred HpTR cells were *Foxp3*-RFP^{-}*Il4*-GFP^{+} in the spleen and MLN and 25-30% were *Foxp3*-RFP^{-}*Il4*-GFP^{+} in the PP (**Fig. 4.6 C**), similarly to previous experiments (**Fig. 4.5 D**). Gating on the *Il4*-GFP^{+} cells in the PP, we identified that the majority (around 80%) of *Il4*-GFP^{+} cells originated from transferred MLN HpTR cells (white bars, **Fig. 4.6 D**), as did the majority of *Il4*-GFP^{-} cells (data not shown), suggesting MLN cells proliferated more following adoptive transfer. In a separate analysis, gating on MLN (CD45.2) or spleen (CD45.1)-derived HpTR cells, we found a higher proportion of MLN-derived cells expressed *Il4*-GFP compared to spleen-derived cells (**Fig. 4.6 E**). These results suggest that MLN-derived Treg cells proliferate and convert to a larger extent than spleen-derived Treg cells, in particular in the Peyer's patches.

4.2.3.3. *Foxp3*-RFP^{+} cells convert to *Il4*-GFP^{+} cells in the absence of antigen and of high levels of CD25 expression

Following the observation that a population of HpTR cells had the capacity to either co-express *Il4*-GFP or down-regulate *Foxp3* and express *Il4*-GFP, we wanted to test whether the infection status of either the donor or recipient mouse influenced the ability of the cells to convert. To test this, we isolated CD25^{high}*Foxp3*-RFP^{+} cells from naïve (nTR cells) or *H. polygyrus* infected double-reporter donor mice (HpTR cells) and adoptively transferred the purified Treg cells into either naïve (white bars) or *H.*

polygyrus infected (black bars) *Tcra*$^{-/-}$ recipient mice (**Fig. 4.7 A**). We observed a similar proportion of *Il4*-GFP^{+} cells in naïve or infected recipients following the transfer of CD25^{high}*Foxp3*-RFP^{+} cells from naïve or infected donor mice, as shown in representative graphs of the Peyer's patches (**Fig. 4.7 A**) suggesting that the antigen-specificity of the donor or host may not influence Treg conversion in this adoptive transfer model. Furthermore, we wanted to test whether the levels of CD25 expression previously shown to correlate with Treg stability (Miyao et al., 2012) influenced their conversion to *Il4*-GFP expressing cells. To test this, we isolated either CD25^{high}*Foxp3*-RFP^{+} or CD25^{low}*Foxp3*-RFP^{+} cells from *H. polygyrus* infected double-reporter mice and adoptively transferred these cells to *Tcra*$^{-/-}$ mice. Adoptively transferred CD25^{high} or CD25^{low} HpTR cells expressed similar levels of *Il4*-GFP as shown in representative graphs of the Peyer's patches (**Fig. 4.7 B**), suggesting that CD25 expression may not determine the degree of Treg conversion to *Il4*-GFP–expressing cells following adoptive transfer.

4.2.3.4. *Il4*-GFP^{+} ex–*Foxp3*-RFP^{+} cells are transcriptionally re-wired

To characterise the transcriptional profile of *Il4*-GFP^{+} ex-Foxp3 cells (HpTR→ *Il4*-GFP^{+}), we FACS-purified donor HpTR cells from double-reporter mice and compared them to *Il4*-GFP+ cells isolated following the adoptive transfer of HpTR (HpTR→ *Il4*-GFP^{+}) or nT cells (nT→ *Il4*-GFP^{+}). We performed a gene expression microarray on 3 biological replicates for each group and filtered significantly expressed genes that were 2-fold up-regulated compared to naïve T cell controls. We performed hierarchical clustering between HpTR, HpTR→ *Il4*-GFP^{+} and nT→ *Il4*-GFP^{+} populations and found that HpTR→ *Il4*-GFP^{+} and nT→ *Il4*-GFP^{+} were more closely clustered together than with HpTR cells (**Fig. 4.8 A**). Following this, we compared differentially expressed genes between the samples, presented as a Venn diagram (**Fig. 4.8 B**). A significant number of genes (913) were similarly expressed between all cell types (**Fig. 4.8 B**). Strikingly, very few genes (172 genes) were similarly expressed

between HpTR and HpTR→ *Il4*-GFP$^+$ cells, suggesting converted cells had significantly re-wired their transcriptional profile following adoptive transfer. Indeed, 907 genes were commonly expressed between HpTR→ *Il4*-GFP$^+$ and nT→ *Il4*-GFP$^+$ cells, which constitutes around 50% of the significantly expressed genes for both cell types. Few genes (297) were commonly expressed between HpTR and nT→ *Il4*-GFP$^+$ cells (**Fig. 4.8 B**). Furthermore we selected a few candidate genes representative of Treg and Th2 cells and looked at their expression in the three subsets. HpTR→ *Il4*-GFP$^+$ and nT→ *Il4*-GFP$^+$ but not HpTR significantly expressed the Th2 hallmark cytokines *Il4, Il13* and *Il2* and had downregulated *Il4ra* expression (**Fig. 4.8 C**). HpTR→ *Il4*-GFP$^+$ and nT→ *Il4*-GFP$^+$ had also downregulated the Treg-associated genes *Foxp3, Il2ra, Ctla4* and *Il10* (**Fig. 4.8a C**), suggesting that HpTR→ *Il4*-GFP$^+$ had lost their Treg phenotype and acquired a Th2 effector phenotype. We confirmed loss of *Foxp3* and gain of *Il4* expression by qRT-PCR (**Fig. 4.8a D**). In addition to the classical CD4$^+$ T cell lineage genes listed above, we also saw high levels of *Cxcr5* and *Il21,* typically associated with Tfh cells, expressed in HpTR→ *Il4*-GFP$^+$. In addition, the IL-33R (*Il1rl1*) was expressed on HpTR and nT→ *Il4*-GFP$^+$ but not on HpTR→ *Il4*-GFP$^+$ (**Fig. 4.8a C**). Computational analysis using Ingenuity Pathways Analysis software (IPA) (Kramer et al., 2014) was used to identify putative upstream signalling pathways that may contribute to the observed gene expression profile in HpTR, HpTR→ *Il4*-GFP$^+$ and nT→ *Il4*-GFP$^+$ cells. *Il4* was predicted to be upstream of the transcriptional profile of the three populations (**Fig. 4.8 E**). Additionally, *Csf2, Il1b,* and *Tnf* were the most highly predicted cytokines responsible for regulating the transcriptional profile of HpTR→ *Il4*-GFP$^+$ cells (**Fig. 4.8 F**). *Il4* also featured as a highly significant upstream regulator when compared to all molecules upstream and featured in the 5 highest predicted regulators (bold, **Fig. 4.8 F**). The transcriptional profiling of HpTR and HpTR→ *Il4*-GFP$^+$ suggested that loss of *Foxp3*-RFP expression correlated with broad transcriptional changes and the acquisition of T effector cell properties. To test whether the similarities in the transcriptional profile of HpTR→ *Il4*-GFP$^+$ and nT→ *Il4*-GFP$^+$ were a consequence of the lymphodeficient environment in *Tcra*$^{-/-}$ mice, we also compared the expression

profile of HpTR cells that had maintained *Foxp3*-RFP following adoptive transfer (HpTR→ *Foxp3*-RFP$^+$), and HpTR cells that had lost *Foxp3*-RFP expression but not upregulated *Il4*-GFP expression (HpTR→ DN). We performed hierarchical clustering between HpTR, HpTR→ *Foxp3*-RFP$^+$, HpTR→ DN and HpTR→ *Il4*-GFP$^+$ cells. From the dendogram, HpTR cells were the most distant, whereas the three groups isolated following adoptive transfer clustered together (**Fig. 4.8b C**). Of these, HpTR→ HpTR and HpTR→ DN clustered closest together. Interestingly, the HpTR→ *Il4*-GFP$^+$ samples clustered apart from HpTR→ DN (**Fig. 4.8b C**), suggesting that neither the loss of *Foxp3* or signals from the lymphodeficient environment were entirely responsible for driving the transcriptional profile of HpTR→ *Il4*-GFP$^+$. Instead, these results suggest that sorting ex-HpTR cells based on *Il4*-GFP expression cells led to the identification of a unique subset of cells.

4.2.3.5. *Il4*-GFP$^+$ ex–*Foxp3*-RFP$^+$ cells retain a demethylated *Foxp3* locus

Foxp3 expression is essential for the development of CD4+ T regulatory cells (Fontenot et al., 2003), however recent studies identified an important group of proteins and TFs that cooperate with *Foxp3* to maintain Treg function (Fu et al., 2012; Rudra et al., 2012) termed the Foxp3 interactome (Hori, 2012). Furthermore, commitment towards a Treg lineage correlates with the establishment of a unique demethylation pattern on the *Foxp3* gene and on other genes associated with Treg function such as *Il2ra* and *Ctla4* (Okada et al., 2014). As mentioned previously, CD25 expression on *Foxp3*-expressing cells correlated with the proportion of demethylated CpG islands both at the CD25 coding gene, *Il2ra,* as well as on the *Foxp3* locus (Ohkura et al., 2012). From our previous experiments, a proportion of *Foxp3*-RFP$^+$CD25^{high} cells were unstable *in vivo* and lost *Foxp3* expression, which correlated with the re-wiring of their transcriptional profile. In light of this, we wanted to determine the methylation status of the *Foxp3* locus in our donor *Foxp3*-RFP$^+$CD25^{high} cells and compare them to cells that had lost *Foxp3*-RFP and *Foxp3* mRNA expression following adoptive transfer. We

hypothesised that converted cells would have re-methylated their *Foxp3* locus. To quantify the methylation of the *Foxp3* locus we performed bisulphite modification of DNA (Clark et al., 1994), which converts all unmethylated cytosines to uracils, and leaves methylated cytosines untouched. We designed primers to amplify the CNS2 region of the *Foxp3* locus containing 12 CpG islands previously shown to be specifically demethylated in Foxp3^{+}CD25^{+} cells (Floess et al., 2007). In addition, we designed primers for a control region of the *Foxp3* locus containing 5 CpG islands previously shown to remain methylated in Treg cells (Floess et al., 2007) (Fig. 4.8 A). We PCR-amplified bisulphite modified DNA from our different populations and cloned the PCR products. We sent multiple clones from each sample for sequencing, and analysed the data using the online methylation analysis programme QUMA (Kumaki et al., 2008). We FACS-purified *Foxp3*-RFP^{+}CD25^{high} cells (HpTR), naïve T cells (nT) and HpTR cells that had lost *Foxp3*-RFP expression and unregulated *Il4*-GFP (HpTR→ *Il4*-GFP^{+}) and assessed their methylation status. As expected, the majority of the CpG islands within the three populations were methylated at the control region of the *Foxp3* locus (as illustrated in **Fig. 4.9 A and B**). Furthermore, a high proportion of the CpG islands in the *Foxp3* CNS2 of naïve T cells (nT) (95%) were methylated (white bars, **Fig. 4.9 A and B**) as previously described (Miyao et al., 2012). Conversely, only a small proportion (15-20%) of the CpG islands were methylated in HpTR cells (grey bars; **Fig. 4.9 A and B**), as expected. Surprisingly, this was also true in HpTR→ *Il4*-GFP^{+} cells that had lost *Foxp3* expression, where a very low proportion (5-10%) of CpG islands were methylated (black bars, **Fig. 4.9 A and B**). We also FACS-purified *Foxp3*-RFP^{+}CD25^{high} from naïve double-reporter mice (nTR) and *Foxp3*-RFP^{-}*Il4*-GFP^{+} cells from double reporter mice infected with *H. polygyrus* for 14 days. As expected, a low proportion of CpG islands were methylated in the *Foxp3* CNS2 (15-20%), but not the control region of nTR cells similarly to HpTR cells (**Fig. 4.9 D**). Conversely, a high proportion of CpG islands were methylated in both the *Foxp3* CNS2 and control region of *Foxp3*-RFP^{-}*Il4*-GFP^{+} cells, similarly to nT cells (**Fig. 4.9 D**). In light of the surprising finding that HpTR→ *Il4*-GFP^{+} cells had retained a demethylated *Foxp3* CNS2, we

isolated HpTR→ DN cells that had lost *Foxp3* expression but not acquired *Il4*-GFP expression. In contrast to HpTR→ *Il4*-GFP$^+$, a high proportion of HpTR→ DN cells had re-methylated their *Foxp3* locus (**Fig. 4.9 E**). Lastly, we FACS-purified cells which had maintained *Foxp3* expression following adoptive transfer (HpTR→ *Foxp3*-RFP$^+$) to account for the effects of the lymphodeficient environment on changes in methylation status. We found the majority of HpTR→ *Foxp3*-RFP$^+$ cells had maintained a demethylated *Foxp3* locus, which correlated with the maintenance of *Foxp3* expression (**Fig. 4.9 E**). Taken together, these experiments revealed that although HpTR→ *Il4*-GFP$^+$ cells lost *Foxp3* expression and were transcriptionally more similar to Th2 cells, they retained a demethylated *Foxp3* locus, similar to Treg cells.

Methylation of the *Foxp3* TSDR has been shown to correlate with Treg suppressor function. We therefore wanted to test whether HpTR→ *Il4*-GFP$^+$ cells that maintained a demethylated *Foxp3* locus (**Fig. 4.9 A and B**) had retained their suppressor function. To address this, we performed an *in vitro* Treg suppression assay by co-culturing CD4$^+$TCRβ$^+$CD25$^-$CD44$^-$ nT cells alone or with an equal number of FACS-purified HpTR→ *Foxp3*-RFP$^+$, HpTR→ DN or HpTR→ *Il4*-GFP$^+$ cells in the presence of TCR stimulation. A high proportion (~70%) of nT cells cultured alone or with HpTR→ DN proliferated *in vitro* as assessed by CTV staining (**Fig. 4.10 A**). In comparison, we saw fewer nT cells proliferating when cultured with HpTR→ *Il4*-GFP$^+$ or HpTR→ *Foxp3*-RFP$^+$ cells, where proliferation was almost completely blocked (**Fig. 4.10 A**). Together, these results suggest that the methylation status of the *Foxp3* locus correlated with the suppressive capacity of the cells.

4.2.3.6. Adoptive transferred Treg and nT cells are insufficient to mediate functional worm expulsion

Although the population of HpTR→ *Il4*-GFP$^+$ cells maintained elements of their Treg past, including a demethylated *Foxp3* locus, the above studies also highlighted fundamental changes in their transcriptional profile, including evidence of Th2 effector

function. To test whether the cells could function as Th2 effector cells in the adoptive transfer model, we measured their ability to stimulate downstream type-2 responses in *Tcra*$^{-/-}$ recipients following secondary infection with *H. polygyrus*. One of our first observations was that although the frequency of *Il4*-GFP^{+} cells originating from HpTR cells was similar to the frequency of *Il4*-GFP^{+} cells originating from naïve T cells (nT→ *Il4*-GFP^{+}), the absolute number of both populations was significantly different, with much fewer numbers of HpTR→ *Il4*-GFP^{+} cells, suggesting they had a lower proliferative capacity (**Fig. 4.10 B**), although this needs confirming with labelling experiments. In addition, although we had previously detected IL-4 protein secretion by CD4^{+} T cells by intra-cellular cytokine staining, levels of IL-4 secreted by re-stimulated HpTR→ *Il4*-GFP^{+} cells *in vitro* were undetectable by ELISA (**Fig. 4.10 C**). Furthermore, we could not detect serum IgE antibody levels in mice that received HpTR cells, in comparison to mice that received nT cells (**Fig. 4.10 D**). Similarly, mRNA expression of alternative macrophage activation-related genes, *Arg1* and *Chil3l3*, was only marginally elevated in the small intestine of mice that received HpTR in contrast to mice that received nT cells (**Fig. 4.10 E**). Finally, we assessed the ability of transferred cells to promote the expulsion of *H. polygyrus* following secondary infection (as previously shown **Fig. 4.1**). Using this adoptive transfer system, neither nT or HpTR recipients eliminated a secondary infection with *H. polygyrus* (**Fig. 4.10 F**). Collectively, these results suggested that the adoptive transfer model was a useful system to identify the ability of T cells to convert their phenotype, but was insufficient to test the functional consequences of this change *in vivo*, including their their ability to promote antibody class-switching as well as alternative activation of macrophages, possibly due to their limited ability to expand. In addition, although nT→ *Il4*-GFP^{+} expanded and induced a type-2 effector response, they were also unable to promote functional expulsion of *H. polygyrus.* As a consequence of these observations, we concluded that this model was insufficient to test the ability of HpTR→ *Il4*-GFP^{+} cells to functionally expel *H. polygyrus*. Nevertheless, we observed that a proportion of FACS-purified *Foxp3*-RFP^{+}CD25^{high} HpTR cells had downregulated *Foxp3*-RFP reporter expression and

mRNA expression following adoptive transfer into *H. polygyrus* infected *Tcra*$^{-/-}$ mice. Of these ex-Foxp3 cells, a fraction upregulated *Il4*-GFP and *Il4* mRNA expression (HpTR→ *Il4*-GFP^{+} cells) and exhibited a unique transcriptional profile.

4.2.4. ex-Foxp3 *Il4*-GFP^{+}FP635^{+} cells develop *in vivo* and contribute to the memory Th2 response to *H. polygyrus*

4.2.4.1. ex-Foxp3 *Il4*-GFP^{+}FP635^{+} cells develop *in vivo* and expand in secondary responses to *H. polygyrus*

We hypothesised that *Il4*-GFP+ ex-Foxp3 cells also developed in a wild-type setting *in vivo.* Following the observation that there was a strong shift from a regulatory to an effector response in secondary responses to *H. polygyrus* (**Fig. 4.2**), we further hypothesised that *Il4*-GFP+ ex-Foxp3 cells contributed to the Th2 memory population during *H. polygyrus* infection. To test whether *Il4*-GFP+ ex-Foxp3 cells developed in a lympho-complete mouse, we crossed existing fate-mapping and fluorescent cytokine reporter strains *Foxp3*$^{YFP\text{-}Cre}$ (Rubtsov et al., 2008), 4get (Mohrs et al., 2001) and *R26R*^{FP635} mice (Coomes et al., 2015) to generate *Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635} fate-reporter mice. Using this mouse, we were able to track cells actively transcribing *Il4* (*Il4*-GFP^{+}) and *Foxp3* (*Foxp3*-YFP-Cre^{+}FP635^{+}) and cells that had previously expressed *Foxp3* (*Foxp3*-YFP-Cre^{-}FP635^{+}). Thus, we could also identify whether any *Il4*-GFP^{+} cells had previously expressed *Foxp3* (*Il4*-GFP^{+}FP635^{+}) *in vivo* in a lympho-complete mouse. We infected *Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635} mice with *H. polygyrus,* drug-treated them and subsequently gave them a secondary infection (Hp 2°). At the same time, we infected naïve *Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635} mice with a primary *H. polygyrus* infection *(*Hp 1°) (model; **Fig. 4.11 A**). We harvested the mice at day 7 post-infection, when we had previously seen a strong regulatory to effector shift in the CD4+ populations (**Fig. 4.2**). We saw a small reduction of *Foxp3*-YFP-Cre^{+}*Il4*-GFP^{-} Treg cells in Hp 2° mice compared to Hp 1° mice (**Fig. 4.11 B**) as previously described using the double-reporter mice (**Fig. 4.2**). This decrease correlated with an increase in the

proportion and total number of *Il4*-GFP$^+$ Th2 cells (**Fig. 4.11 C**), as illustrated in representative FACS plots of the spleen (**Fig. 4.11 B**), confirming previously described results using the double-reporter mice (**Fig. 4.2**). As expected, all *Foxp3*-YFP-Cre$^+$*Il4*-GFP$^-$ Treg cells were fate-marked and expressed FP635+ (**Fig. 4.11. B**), highlighting the efficiency of the Cre-mediated recombination. Furthermore, a significant proportion of *Il4*-GFP+ cells were FP635$^+$ (10%) (*Il4*-GFP$^+$FP635$^+$) demonstrating that ex-Foxp3 *Il4*-GFP$^+$ cells develop in wild-type mice. Both the proportion and absolute number of *Il4*-GFP$^+$FP635$^+$ cells increased in Hp 2° mice, with up to 20-25% of all the memory Th2 cells deriving from a *Foxp3*-expressing past (**Fig. 4.11 D**). Furthermore, *Il4*-GFP$^+$FP635$^+$ cells expressed similar levels of CD25, GITR and CD103 as *Il4*-GFP$^+$ cells (**Fig. 4.11 E**) and lower than *Foxp3*-YFP$^+$*Il4*-GFP$^-$ cells, suggesting that they were more functionally similar to Th2 cells than Treg cells. Intracellular CTLA-4 levels were marginally elevated on all cells and not different between groups (**Fig. 4.11 F).**

4.2.4.2. ex-Foxp3 *Il4*-GFP$^+$FP635$^+$ cells exhibit Th2 effector function whilst maintaining characteristics of their Treg past

Unlike the ex-*Foxp3*-RFP$^+$*Il4*-GFP$^+$ cells isolated following adoptive transfer in the previous section, FACS-purified fate-marked *Il4*-GFP$^+$FP635$^+$ from Hp 2° mice secreted high levels of IL-4, IL-13, IL-5 and IL-2 protein following re-stimulation *in vitro,* to similar levels as FACS-purified *Il4*-GFP$^+$ Th2 cells (**Fig. 4.12 A**). IL-4, IL-13, IL-5 or IL-2 were undetectable in the supernatants of FACS-purified and re-stimulated *Foxp3*-YFP-Cre$^+$*Il4*-GFP$^-$ Treg cells (**Fig. 4.12 A**). We designed an assay to test whether T cell-derived IL-4 and IL-13 could alternatively activate macrophages (AAMΦ) *in vitro,* a process known to be dependent on either of these cytokines (Junttila et al., 2008). These cellular interactions are also thought to occur around the larvae during a secondary infection with *H. polygyrus* and were shown to be required for larval killing and immunity to secondary infection (Anthony et al., 2006). We FACS-purified *Il4*-GFP$^+$FP635$^+$ cells from Hp 2° mice and co-cultured the cells with bone marrow derived

macrophages (BMDMs) *in vitro* overnight. Following this, we washed away the $CD4^+$ T cells and measured *Arg1* and *Retnla* expression in macrophages. Both *Il4*-GFP^+ and *Il4*-GFP^+FP635^+ cells induced *Arg1* and *Retnla* expression in BMDMs, to a better extent than recombinant IL-4 and IL-13, frequently used to differentiate AAMΦ *in vitro* (**Fig. 4.12 B**). As expected, *Foxp3*-YFP-Cre^+*Il4*-GFP^- Treg cells did not induce detectable levels of *Arg1* or *Retnla* expression (**Fig. 4.12 B**). Following the observation that ex-Foxp3 *Il4*-GFP^+FP635^+ were functionally similar to Th2 cells, we wanted to determine the methylation status of the *Foxp3* locus and again hypothesised that *Il4*-GFP^+FP635^+ cells had re-methylated their *Foxp3* locus, similarly to previously described *Il4*-GFP^+ cells (**Fig. 4.9**). We FACS-purified *Foxp3*-YFP-Cre^+*Il4*-GFP^- Treg, *Il4*-GFP^+ Th2 and ex-Foxp3 *Il4*-GFP^+FP635^+ cells and performed bisulphite modification and methylation sequencing of the *Foxp3* CNS2 and negative control region, as previously described (**Fig. 4.9**). As expected, the majority of *Foxp3*-YFP-Cre^+*Il4*-GFP^- Treg cells (98%) had a demethylated *Foxp3* CNS2 and maintained a methylated control region (grey bars, **Fig. 4.12 C**), as we had previously shown in FACS-purified *Foxp3*-RFP^+ cells from double-reporter mice (**Fig. 4.8**). In addition, a high proportion of *Il4*-GFP^+ cells (90%) had a methylated *Foxp3* CNS2, in accordance with the lack of *Foxp3* expression (white bars, **Fig. 4.12 C**). Interestingly, a high proportion of *Il4*-GFP^+FP635^+ cells (75-80%) also had a methylated *Foxp3* CNS2 (black bars, **Fig. 4.12 C**), in contrast to the methylation status of converted cells from the adoptive transfer system. There was a significant difference in the proportion of methylated *Il4*-GFP^+FP635^+ compared to *Il4*-GFP^+ cells, highlighting a potential heterogeneity in the populations and suggesting a small proportion of ex-Foxp3 *Il4*-GFP^+FP635^+ cells retained epigenetic memory (**Fig. 4.12 C**). To test whether this heterogeneity reflected the ability of ex-Foxp3 *Il4*-GFP^+FP635^+ cells to re-express *Foxp3,* we cultured ex-Foxp3 *Il4*-GFP^+FP635^+ in inducible Treg (iTreg) polarising conditions *in vitro* and measured *Foxp3*-YFP expression. We FACS-purified *Foxp3*-YFP-Cre^+, *Il4*-GFP^+ and *Il4*-GFP^+FP635^+ cells and cultured them in the presence of TGF-β and IL-2 with TCR stimulation for 7 days. Firstly, we observed a significant proportion of ex-Foxp3 *Il4*-

GFP$^+$FP635$^+$ cells lost *Il4*-GFP expression (black bars; **Fig. 4.12 D**) compared to *Il4*-GFP$^+$ cells cultured in the same conditions (white bars; **Fig. 4.12 D**) suggesting they were more unstable**.** Furthermore, a higher proportion of cultured *Il4*-GFP$^+$FP635$^+$ cells re-expressed *Foxp3*-YFP in comparison to cultured *Il4*-GFP$^+$ cells (**Fig. 4.12 D**). Taken together, this data demonstrated that ex-Foxp3 *Il4*-GFP$^+$FP635$^+$ cells that had previously expressed *Foxp3* could function as Th2 effector cells *in vitro,* similar to *Il4*-GFP$^+$ cells. In addition, these data highlighted heterogeneity in the methylation status of the cells as well as the ability of cells to re-express *Foxp3*, suggestive of true plasticity, albeit only in a small proportion of cells. These data also suggest that ex-Foxp3 *Il4*-GFP$^+$FP635$^+$ cells may be a heterogeneous population of cells displaying some properties of their Treg past.

4.2.5. Investigating a role for IL-4 signalling for the development of ex-Foxp3 *Il4*-GFP$^+$FP635$^+$ cells

IL-4 and IL-4Rα are critically required for the functional expulsion of *H. polygyrus* (Urban et al., 1991). Importantly, anti-IL-4 treatment administered around the time of a secondary *H. polygyrus* infection inhibited worm expulsion (Urban et al., 1991), suggesting IL-4 was required for functional immune responses in a secondary infection rather than uniquely in the generation of Th2 memory cells. Using two separate approaches, we have shown that *Il4*-GFP$^+$ cells can differentiate from *Foxp3*-expressing cells in the context of a secondary infection with *H. polygyrus*. We therefore hypothesised that IL-4 was required for the development and expansion of ex-Foxp3 *Il4*-GFP$^+$FP635$^+$ cells. We aimed to test this in two ways: 1) through antibody blockade of IL-4 during a secondary infection and 2) by specifically deleting *Il4ra* in Foxp3-expressing cells.

4.2.5.1. IL-4 signals through STAT6 in CD25^{high}*Foxp3*-RFP^{+}cells and promotes their conversion to *Il4*-GFP^{+} cells *in vitro*

Before testing whether IL-4 was contributing to the conversion of HpTR to *Il4*-GFP^{+} cells, we first wanted to determine whether Treg cells were responsive to IL-4. As previously described, CD25^{high}*Foxp3*-RFP^{+} cells express *Il4ra* to similar levels as naïve T cells (**Fig. 4.8a**). To test the signalling capacity of IL-4Rα on CD25^{high}*Foxp3*-RFP^{+} cells, we FACS-purified CD25^{high}*Foxp3*-RFP^{+} (HpTR) or naïve T cells (nT) from *Il4*-GFP*Foxp3*-RFP double-reporter mice. We stimulated the FACS-purified cells with recombinant IL-4 for 15min *in vitro* (model; **Fig. 4.13 A**) and measured the phosphorylation of STAT6 (Kaplan et al., 1996; Takeda et al., 1996). IL-4 triggered the phosphorylation of STAT6 (pSTAT6) to a similar extent in nT cells and HpTR cells (**Fig. 4.13 B**). Interestingly, we saw a basal level of pSTAT6 in the control HpTR cells that were cultured in media alone (**Fig. 4.13 B**), possibly reflecting the IL-4-rich environment in the spleen and MLN where HpTR cells were purified from. Following this observation, we wanted to test the impact of IL-4 signalling on the conversion of HpTR cells. We FACS-purified CD25^{high}*Foxp3*-RFP^{+} (HpTR) or naïve T cells (nT) from *Il4*-GFP*Foxp3*-RFP double-reporter mice and cultured the cells in Th2 polarising conditions *in vitro* for 7 days (model; **Fig. 4.13 A**). After 7 days, the majority of nT cells had up-regulated *Il4*-GFP as shown in the representative FACS plots (**Fig. 4.13 C**). In addition, a high proportion (75-80%) of the cultured HpTR cells lost *Foxp3*-RFP expression with a substantial fraction (35-40%) up-regulating *Il4*-GFP (**Fig. 4.13 C and D**) indicating that IL-4 can drive conversion of HpTR to *Il4*-GFP^{+} cells *in vitro*.

4.2.5.2. Foxp3^{+} cells are dispensable for the development of Th2 responses and functional immunity to *H. polygyrus*

In light of the observation that HpTR cells were actively phosphorylating STAT6 in a *H. polygyrus* infection, and that IL-4 could promote Treg to Th2 conversion *in vitro*,

we wanted to test the impact of IL-4 signalling in Treg cells on the development of memory Th2 responses in a secondary *H. polygyrus* infection.

Our first broad approach was to determine whether *Foxp3*-expressing cells contributed to the Th2 response. Because *Foxp3*-deficient mice develop severe lymphoproliferative disease (Brunkow et al., 2001), we made use of "deletion of T regulatory cell" BAC transgenic (DEREG) mice that express a diphtheria toxin receptor (DTR) DTR-eGFP fusion protein under the control of the *Foxp3* promoter. This allows for the identification of *Foxp3*-expressing cells using GFP, as well as the temporary and selective depletion of *Foxp3*-expressing cells upon diphtheria toxin injection (Lahl et al., 2007). We hypothesised that the selective depletion of Foxp3-expressing cells at the time of *H. polygyrus* challenge infection would impair Th2 responses by depleting a potential pool of ex-Foxp3 Th2 cells. We infected DEREG mice with *H. polygyrus,* drug-treated them and subsequently gave them a secondary infection (*Hp* 2°). We pre-treated mice with two rounds of diphtheria toxin before infection and once on the day of secondary infection (DEREG Hp 2° + Dtx) and left another group untreated (DEREG Hp 2°) (model; **Fig. 4.14 A**). In addition, we harvested treated or untreated naïve DEREG mice. We harvested the mice 7 days post-infection for FACS analysis and 14 days post-infection to measure intestinal worm burden. The proportion of *Foxp3*-GFP^{+}CD25^{+} cells was significantly reduced in DEREG Hp 2° + Dtx mice as shown in representative FACS plots of the MLN (**Fig. 4.14 B**). Furthermore, *Foxp3*-GFP^{+}CD25^{+} depletion was effective in both naïve and infected mice (**Fig. 4.14 C**). Following this, we measured the impact of Treg depletion on the functional Th2 response by assessing functional worm expulsion on day 14 post-infection. As previously described, drug-cured mice expel a challenge infection with *H. polygyrus*. Indeed, adult worms almost completely failed to mature in WT Hp 2° mice and control DEREG Hp 2° mice which were not given diphtheria toxin (white bars; **Fig. 4.14 D**) compared to mice given a primary infection (grey bars; WT Hp 1°) (**Fig. 4.14 D**). Worm expulsion was not compromised in DEREG Hp 2° + Dtx mice (black bar; **Fig. 4.14 D**). Indeed, depletion of *Foxp3*-expressing cells resulted in a significant increase in the

proportion of $CD4^+IL\text{-}4^+$ or $CD4^+IL\text{-}13^+$ cells as measured by intra-cellular cytokine staining (**Fig. 4.14 E**), suggesting that on a population level, a dominant functional role of $Foxp3^+$ cells was to limit Th2 expansion, rather than contribute to the Th2 pool.

The second approach we used to test the impact of IL-4 signalling in Treg cells on functional immunity to *H. polygyrus* followed the observation that IL-4 could stimulate the conversion of Treg cells to Th2 cells *in vitro.* We hypothesised that IL-4 signalling could drive the conversion of Treg to Th2 cells and therefore contribute to the memory Th2 response. To test this, we used a mixed bone marrow approach to specifically delete *Il4ra* in *Foxp3*-expressing cells. We used lethally irradiated T-cell deficient *Tcra*$^{-/-}$ recipient mice and bone marrow from WT mice, *Il4ra*$^{-/-}$ mice and Scurfy (*Foxp3*$^{-/-}$) pups that are deficient in Treg cells and develop fatal lymphoproliferative disease within a few weeks of birth (Fontenot et al., 2003). We reconstituted irradiated *Tcra*$^{-/-}$ mice with either WT, *Il4ra*$^{-/-}$, WT:*Foxp3*$^{-/-}$ (20%:80%) or *IL4ra*$^{-/-}$:*Foxp3*$^{-/-}$ (20%:80%) bone marrow. In the latter two groups, $Foxp3^+$ Treg cells originated from either the WT or *Il4ra*$^{-/-}$ compartments. Although at this stage we couldn't test the impact of IL-4 signalling on Treg conversion, using this chimeric system we could test whether the deletion of IL-4Rα on Treg cells would have any impact on the development of memory Th2 cells and functional expulsion of *H. polygyrus*. Following reconstitution, we infected the chimeric mice with *H. polygyrus,* drug-treated them and subsequently gave them a secondary infection. We harvested the mice 14 days post-infection for FACS and adult worm counts (**Fig. 4.15 A**). There were no significant differences in the proportion of $Foxp3^+CD25^+$ cells in the spleen of the different groups, and none of the mice developed wasting disease in the time-frame of the experiment, crudely demonstrating that the IL-4Rα was not required for the development or function of Treg cells (**Fig. 4.15 B**). To test whether IL-4Rα expression on Treg cells contributed to the Th2 response, we measured IL-4 production by CD4+ T cells by intra-cellular cytokine staining. The development of $CD4^+IL\text{-}4^+$ cells was significantly impaired in mice fully reconstituted with *Il4ra*$^{-/-}$ bone marrow, compared to

WT reconstituted mice (**Fig. 4.15 C**), as previously described in *Stat6*$^{-/-}$ mice (Perona-Wright et al., 2010) and in mice treated with anti-IL-4Rα antibody following secondary infection (Urban et al., 1991; Urban et al., 1995). Restricting IL-4Rα deficiency to Foxp3-expressing cells (*IL4ra*$^{-/-}$:*Foxp3*$^{-/-}$) did not significantly impact the development of CD4^{+}IL-4^{+} cells compared to the WT control mice (WT:Foxp3$^{-/-}$) (**Fig. 4.15 C**). As a consequence, immunity to *H. polygyrus* as assessed by luminal worm counts was impaired in complete *Il4ra*$^{-/-}$ mice but not in mice with a selective deficiency in IL-4Rα expression on Foxp3-expressing cells (**Fig. 4.15 D**). These data suggest that IL-4Rα expression on Treg cells is not required for the development, or control, of Th2 memory responses and does not impact the expulsion of *H. polygyrus.*

4.2.5.3. Generation of a conditional IL-4Rα-deficient mice to study the role of IL-4 signalling on Treg conversion *in vivo*

The above experiments suggest IL-4 signalling in Foxp3-expressing cells had no impact on the development of Th2 cells or functional expulsion of *H. polygyrus.* However, given that we knew a high proportion of *Il4*-GFP^{+} cells seen in a secondary *H. polygyrus* infection originated from a Foxp3-expressing past (**Fig. 4.11**), we wanted to test whether IL-4 signalling was required for the generation of *Il4*-GFP^{+}FP635^{+} cells. Our first approach was to treat fate-reporter mice with anti-IL-4 monoclonal antibody during the course of a secondary infection and monitor the development of *Il4*-GFP^{+}FP635^{+} cells (model, **Fig. 4.15 E**). Preliminary data suggests that treatment of fate-reporter mice with anti-IL-4 over the course of the secondary challenge did not reduce the proportion or number of *Il4*-GFP^{+} Th2 cells in the spleen or MLN **(Fig. 4.15 F**) or the proportion and total number of ex-Foxp3 *Il4*-GFP^{+} cells (**Fig. 4.15 G**).

Our second approach is to directly test the role of IL-4 on *Foxp3*-expressing cells by generating a conditional knockout mouse. We have crossed our fate-reporter *Foxp3*$^{YFP\text{-}Cre}$*Il4*-GFP*R26R*^{FP635} mice with an *Il4ra*$^{fl/fl}$ mouse harbouring a conditional *Il4ra* allele. Using this mouse, we will determine whether IL-4 signalling on *Foxp3*-

expressing cells is required for the development of ex-Foxp3 *Il4*-GFP^{+}FP635^{+} cells and whether IL-4-responsive Treg cells contribute or regulate Th2-mediated expulsion of *H. polygyrus*.

4.3. Discussion

Helminths have evolved to subvert host immune responses by stimulating the expansion of Treg cells (Grainger et al., 2010) and establishing chronic infections in the host. Despite helminth-elicited immune-modulation, functional immunity to intestinal helminths can occur, and is dependent on the development of a polarised type-2 response, orchestrated by CD4+ Th2 cells (Urban et al., 1991; Urban et al., 1991). As we have shown, expulsion of the intestinal helminth *H. polygyrus* is associated with a shift from a regulatory to a Th2 effector response. Furthermore, recent work has demonstrated that *Foxp3*$^{+}$ Treg cells can lose *Foxp3* expression in the context of inflammation or infection and convert to pathogenic T helper cells (Coomes et al., 2013). In this chapter, we tested the hypothesis that Treg cells convert to Th2 cells during secondary infection with *H. polygyrus* and contribute to protective immune responses to *H. polygyrus*. We hypothesise that IL-4 signalling contributed to Treg to Th2 conversion and finally, we will ask whether Treg to Th2 conversion is required for protective immune responses to *H. polygyrus.*

Using double-reporter mice we found that a proportion of *Foxp3*-RFP^{+}CD25^{high} Treg cells could lose *Foxp3* expression and up-regulate *Il4*-GFP following a secondary infection with *H. polygyrus*. Furthermore, using novel fate-reporter mice we identified ex-Foxp3 *Il4*-GFP^{+} cells *in vivo* that expanded during the development of protective immune responses. Finally, we generated novel fate-reporter mice with a specific deletion of the high affinity IL-4 receptor chain (IL-4Rα) on *Foxp3*-expressing cells to test whether IL-4 was required for Treg to Th2 conversion and whether ex-Foxp3 *Il4*-GFP^{+} cells contribute to protective immunity to *H. polygyrus.*

4.3.1. Promoting effector responses for improved anti-helminth immunity

H. polygyrus establishes chronic infections in C57BL/6 hosts, correlating with the early expansion (Rausch et al., 2008) and activation (Finney et al., 2007) of Treg cells that limit the development of a protective Th2 response (Rausch et al., 2009). Indeed, the early onset and strength of the immune response correlates with natural resistance to the parasite in genetically resistant hosts. Transient depletion of Treg cells in susceptible C57BL/6 hosts early following infection resulted in increased Th2 responses, in particular in the Peyer's patches (Mosconi et al., 2015) and increased pathology (Rausch et al., 2009), suggesting that a major function of Treg cells in the context of a primary infection is to limit host damage by suppressing the development of otherwise protective Th2 responses. Following drug-treatment, mice become resistant to re-infection and mount a protective immune response against the developing larvae upon re-infection, ultimately resulting in larval killing (Reynolds et al., 2012). Immunity to secondary infection correlated with the development of a faster and stronger Th2 response (Anthony et al., 2006) as well as with reduced Treg expansion (Liu et al., 2010) highlighting the dynamic interplay between immune balance and immunity. Using *Il4*-GFP*Foxp3*-RFP reporter mice, we described increased proportions of both *Foxp3*-RFP$^+$ Treg and *Il4*-GFP$^+$ Th2 cells following primary infection, confirming previously published results on the individual responses using either *Il4*-GFP reporter mice (Mohrs et al., 2005) or *Foxp3* intranuclear staining (Rausch et al., 2008). Upon secondary infection, we observed a shift in the ratio of Th2 to Treg cells to favour an effector response, correlating with increased type-2 responses and functional expulsion of *H. polygyrus,* as previously described (Reynolds et al., 2012). Collectively, these results demonstrate that a dominant Th2 response correlates with immunity to *H. polygyrus.* In support of this, treatment of mice with a complex of IL-4 and anti-IL-4 antibody (IL-4c) to enhance IL-4 signalling was shown to promote the expulsion of *H. polygyrus* during a chronic infection by mimicking the expansion of IL-4-secreting Th2 cells (Urban et al., 1995; Herbert et al., 2009). Similarly, IL-2c treatment has been shown to activate CD25-expressing cells including Treg (Boyman et al., 2006) and T

helper cells (Tang et al., 2008) and promote immunity to *N. brasiliensis* (Roediger et al., 2013) and *H. polygyrus* (chapter 3) in *Rag*-deficient mice. Although low-dose IL-2c has been shown to preferentially expand Treg cells in naïve mice and in the context of disease (Klatzmann and Abbas, 2015), high doses of IL-2c were shown to expand pathogenic Th1 cells and Treg cells, with a net result of exacerbated autoimmune progression (Tang et al., 2008). This suggests that treatment of mice with different doses of IL-2c can influence the Treg to T effector ratio in favour of an effector response. Indeed, we tested whether the treatment of mice with variable doses of IL-2c could impact the balance of effector cells over regulatory cells and promote immunity to *H. polygyrus* by stimulating Th2 expansion. We found that a high dose IL-2c treatment promoted both Th2 expansion and Treg expansion resulting in the maintenance of a balanced Treg to Th2 ratio, suggesting that immunity to *H. polygyrus* would not be altered. Given that IL-2c treatment expanded the absolute number of Th2 cells, it would be interesting to test whether additional treatments, or an altered treatment regimen could promote immunity despite the expansion of Treg cells. Conversely, it would be interesting to treat mice with low-dose IL-2c during a memory response and to restore Treg numbers to determine whether Treg expansion limits the protective Th2 responses. The generation of IL-2c using different IL-2 antibody clones (Spangler et al., 2015) or the recent generation of IL-2 variants (Mitra et al., 2015) are thought to confer differing signalling potential to IL-2 and could be used to test preferential expansion of specific populations. However given the impact of IL-2c on the expansion of many CD25-expressing cells such as Treg, T effector and ILC2s, therapeutic applications of IL-2c will be limited and must be carefully controlled.

4.3.1.1. Reporter mice: useful tool for studying Treg specialisation and conversion

It has become clear that there is transcriptional heterogeneity between populations of Treg cells isolated from distinct locations (Feuerer et al., 2009; Feuerer et al., 2010). In

particular, Tregs express unique genes that are required for their maintenance and suppressor function in the tissue, such as *Pparg* in adipose tissue Treg cells (Cipolletta et al., 2012) and *Il1rl1* (IL-33R) on colon-, spleen-, and adipose-tissue-derived Treg cells (Matta et al., 2014; Schiering et al., 2014; Molofsky et al., 2015; Vasanthakumar et al., 2015). Furthermore, the transcriptional specialisation of Tregs correlates with the ability of Tregs to specifically suppress helper cell subsets (Cretney et al., 2013). For example the micro-RNA miR-182 was highly expressed in Treg cells isolated from the liver of *S. mansoni* infected mice and required for Treg suppression of Th2 cells, but not Th1 cells, *in vivo* (Kelada et al., 2013). Furthermore, Treg expression of the Th2-related TF *Irf4* was required for their selective inhibition of Th2 inflammation (Zheng et al., 2009). In addition, many studies have described the spontaneous development of type-2 inflammation, upon reduced levels of *Foxp3* (Wan and Flavell, 2007; Wang et al., 2010) or loss of *Foxp3* expression in mice deficient in *Foxp3*-stabilising genes*Traf6* (Muto et al., 2013), *Itch* (Jin et al., 2013), *Cbfb* (Kitoh et al., 2009), *Bcl6* (Sawant et al., 2012) and *Bach2* (Roychoudhuri et al., 2013). Together, these data suggest that Treg cells specialise to suppress different Th responses.

Using *Il4*-GFP*Foxp3*-RFP mice, we identified double-positive (DP) *Il4*-GFP^{+}*Foxp3*-RFP^{+} cells that expressed similar levels of lineage-defining TFs (*Foxp3* and *Gata3*) as Treg and Th2 cells, suggesting DP cells may represent a unique subset of Treg cells. In support of this hypothesis, we observed that DP cells, compared to Th2 or Treg cells, expressed higher levels of *Il10,* which is a dominant mechanism of Treg-mediated immune suppression (Rubtsov et al., 2008). DP cells may represent a population of specialised regulatory Tregs with the ability to potently control Th2 responses. Further transcriptional profiling of DP cells would provide insight into potential molecular mechanisms, required for the specialisation of *H. polygyrus*-elicited Th2 cells including TF or miRNA usage, and identify novel therapeutic targets for limiting Treg suppression and improving Th2 responses.

Previous studies have shown that Treg cells lose *Foxp3* expression (Komatsu et al., 2009), convert to Tfh cells (Tsuji et al., 2009) and/or acquire the potential to

produce effector cytokines including TNFα, IL-17 (Duarte et al., 2009) and IFN-γ (Feng et al., 2011) following adoptive transfer into T-cell deficient mice. Furthermore, $CD4^+$ cells with attenuated levels of *Foxp3* have been shown to selectively secrete type-2 cytokines (Wan and Flavell, 2007; Wang et al., 2010) and preferentially express Th2-related TFs such as *Gata3, Irf4* as well as secrete IL-4 (Muto et al., 2013). Finally, *Foxp3*-RFP^+ cells were shown to co-express *Il4*-GFP (Wang et al., 2010) and secrete IL-4 (Duarte et al., 2009) following adoptive transfer into $Rag2^{-/-}$ mice. Following the adoptive transfer of HpTR cells to $Tcra^{-/-}$ mice, we also identified cells co-expressing *Il4*-GFP^+ and *Foxp3*-RFP^+, suggesting that DP cells identified in *Il4*-GFP*Foxp3*-RFP mice may be of Treg origin. In addition, we found that the majority of transferred HpTR cells lost *Foxp3* expression and a proportion of these cells upregulated *Il4*-GFP in systemic, local and gut associated lymphoid organs demonstrating that Treg to Th2 conversion can occur in the context of a secondary immune response to *H. polygyrus*. Given the role of B cells in supporting Th2 differentiation during memory responses to *H. polygyrus* (Wojciechowski et al., 2009), the development of single positive *Il4*-GFP^+ cells in our model may be attributed to the presence of B cells in $Tcra^{-/-}$ recipient mice, not seen following Treg transfer to B- and T-cell deficient $Rag2^{-/-}$ recipients (Wang et al., 2010).

4.3.1.2. Transcriptional re-wiring of ex-Foxp3 cells

Transcriptional reprogramming of Treg cells has been described following the loss of *Foxp3* expression (Williams and Rudensky, 2007), perhaps as a result of the associated loss of the complex network of TFs bound to *Foxp3* (Rudra et al., 2012). Indeed, we observed dramatic changes in the transcriptional profile of HpTR→*Il4*-GFP^+ cells that had lost *Foxp3* expression. It is interesting to speculate that some of the genes upregulated in HpTR→*Il4*-GFP^+ cells could be direct targets of *Foxp3* normally suppressed during the differentiation program of Treg cells. For example *Foxp3* was shown to directly bind to the *Il4* locus (Kwon et al., 2008) and *Il2* locus (Williams and

Rudensky, 2007) to repress their expression, and both *Il4* and *Il2* were expressed in HpTR→*Il4*-GFP$^+$ cells, suggesting loss of *Foxp3* released a 'molecular brake'. Furthermore, we found that HpTR→*Il4*-GFP$^+$ cells had downregulated *Bach2,* a transcription factor required for the maintenance of Treg stability *in vivo* and a direct repressor of genes required for Th2 differentiation (Roychoudhuri et al., 2013). Together, these data suggest that the loss of *Foxp3* has resulted in the spontaneous acquisition of a Th2-phenotype, and loss of Treg associated markers such as *Ctla4* (Walker and Sansom, 2015) and *Il10* (Rubtsov et al., 2008). However we also observed significant changes in the transcriptional profile of cells that had maintained *Foxp3* expression following adoptive transfer. This suggests that a fraction of transcriptional changes following adoptive transfer may also be caused by their response to the lympho-deficient environment in addition to the loss of *Foxp3* expression. Indeed, transferred Tregs are likely to be subject to increased homeostatic proliferation and limiting concentrations of IL-2, both of which which are important for Treg stability and are altered in lymphodeficient hosts (Fontenot et al., 2005). Furthermore, TCR activation was shown to modulate the transcriptional profile of Treg cells *in vivo* (Levine et al., 2014). It is therefore conceivable that adoptively transferred HpTR cells lose *Foxp3* expression following changes in TCR activation with the appropriate antigen in their new host environment. To minimise the impact of lymphodeficiency, we could co-transfer naïve T cells with HpTR cells into lymphodeficient hosts, which was previously shown to limit Treg instability through by providing a source of IL-2 (Duarte et al., 2009) or transfer congenically marked HpTR cells into lymphocomplete *Il4*-GFP*Foxp3*-RFP mice.

4.3.1.3. Influence of tissue environment and antigen on Treg instability

Tissue microenvironments have been show to influence Treg stability (Murai et al., 2010). Indeed, adoptively transferred Tregs were most unstable in the Peyer's patches, (Hirota et al., 2013) where they were also found to preferentially convert to

Tfh cells (Tsuji et al., 2009) suggesting that gut-specific cues may promote the instability of Tregs. Consistent with these results, we observed that adoptively transferred Tregs were most unstable and acquired the highest levels of *Il4*-GFP expression in the Peyer's patches. This was surprising given the recent finding that Treg cells and not Th2 cells accumulate in the PP following *H. polygyrus* infection suggesting the PP environment supports immune regulation (Mosconi et al., 2015). Interestingly, a subset of myeloid-derived DCs enriched in the PP has been shown to preferentially prime IL-4 production by naïve T cells when compared to myeloid DCs from non-mucosal sites (Iwasaki and Kelsall, 2001) and could also be promoting increased Treg to Th2 conversion following *H. polygyrus* infection. In addition to testing the influence of the organ of migration on Treg plasticity, we tested the ability of Tregs isolated systemically or from tissue draining MLNs to convert to *Il4*-GFP$^+$ cells. MLN-derived Tregs formed the dominant population of CD4$^+$ T cells in the PP following co-transfer to *H. polygyrus* infected T-cell deficient recipients, suggesting they had a competitive advantage over splenic Treg cells. In addition, a higher proportion of MLN-derived cells converted to *Il4*-GFP$^+$ cells in all organs analysed, suggesting they were inherently more plastic at a population level, which may be a consequence of their increased proliferation, or reflect their proximity to the site of infection and antigen.

Treg instability has been observed both at steady state as well as in the context of infection (Wohlfert and Belkaid, 2010) suggesting Treg to Th2 conversion following *H. polygyrus* infection could either be antigen-independent, or dependent on the presence of pathogen-derived signals, including signals from resident intestinal bacteria. Indeed, Tregs have been recently shown to accumulate in PP adjacent to *H. polygyrus* larvae (Mosconi et al., 2015), and *H. polygyrus* excretory/secretory proteins are known to directly stimulate Treg proliferation (Mosconi et al., 2015) and induction *in vitro* (Grainger et al., 2010). We tested whether *H. polygyrus* derived antigens could influence Treg conversion to *Il4*-GFP$^+$ cells and found that Treg cells originating from either *H. polygyrus* infected or naïve double-reporter mice had a similar ability to convert when transferred to either to either naïve or *H. polygyrus* infected recipients,

suggesting the conversion of Treg to Th2 cells following adoptive transfer does not depend on *H. polygyrus* antigen. To test whether *H. polygyrus* antigens can promote Treg to Th2 conversion we could culture Tregs in the presence of HES and measure the loss of *Foxp3*-RFP and acquisition of *Il4*-GFP expression, similarly to the experiments performed testing the role of IL-4 on Treg to Th2 conversion. In addition, bacterial-derived metabolites have recently been shown to directly promote Treg development in the intestine (Arpaia et al., 2013; Furusawa et al., 2013). It is conceivable that microbiota-derived signals influence Treg conversion, especially in the PP where specialised M cells are permissive to antigen transfer (reviewed in (Siebers and Finlay, 1996)). Indeed, expression of activation markers on Treg cells accumulating in the PP following *H. polygyrus* infection were decreased following antibiotic treatment (Mosconi et al., 2015) suggesting microbial factors drive *H. polygyrus* elicited Tregs. To test whether microbiota-derived signals are required for Treg to Th2 conversion following adoptive transfer, we could pre-treat *Tcra*$^{-/-}$ recipients with antibiotics to temporarily deplete intestinal bacteria, or transfer Treg cells to germ-free mice. Taken together, these results suggest that the tissue microenvironment can influence the stability of Treg cells. Kaede transgenic mice express the photo convertible Kaede protein that converts green fluorescence to red fluorescence following violet light exposure, enabling the visualisation of the migration of red fluorescent cells throughout the mouse (Tomura et al., 2008). It would be interesting to expose the MLN of Kaede^{+} Treg recipients to violet light and track the migration of MLN-derived photo-converted red cells to different organs. Furthermore it would be interesting to purify the Kaede^{+} MLN-derived cells following their migration to different organs and compare their transcriptional profile to MLN-resident Tregs to identify markers correlating with their migration and adaptation to different tissues which could be useful for targeting the migration of Treg cells to particular organs following adoptive transfer or in the context of Treg therapy.

The adoptive transfer of Tregs has proved to be very useful for revealing the potential for Treg cells to lose *Foxp3* expression. Given the differences in cellular

composition and cytokine environment between lymphodeficient hosts and wild-type hosts, the Treg instability following transfer into lymphodeficient hosts may not reflect the situation in lymphocomplete mice.

4.3.2. Identification of functional Treg conversion *in vivo* using novel fate-reporter mice

To study Treg plasticity in a more physiological context, recent studies have made use of fate-mapping strategies that enable the identification of ex-Foxp3 cells *in vivo.* These studies have been entirely performed in models of autoimmune disease and inflammation (Bailey-Bucktrout et al., 2013; Komatsu et al., 2014; Noval Rivas et al., 2015). Ex-Foxp3 cells have been shown to secrete IFN-γ in the context of type-1 diabetes (Zhou et al., 2009) and EAE (Bailey-Bucktrout et al., 2013) as well as IL-17 in the synovial fluid of arthritic joints (Komatsu et al., 2014). Furthermore, a recent study showed that transgenic Treg cells overexpressing the IL-4Rα could convert to Th2 cells following intestinal food allergy, however it is unclear whether this was true of wild-type Treg cells (Noval Rivas et al., 2015). The development of ex-Foxp3 cells in the context of an infection remains unknown. Furthermore, the function and requirement for ex-Foxp3 T helper cells in the pathogenicity of disease has not been tested. We generated novel fate-reporter mice by crossing Foxp3-fate reporter mice with *Il4*-GFP mice and characterised the *Foxp3*- and *Il4*-expressing cells following primary and secondary infection. We identified a substantial proportion of *Il4*-GFP$^+$ cells that had previously expressed *Foxp3* during memory immune responses to *H. polygyrus*. The proportion and number of ex-Foxp3 *Il4*-GFP$^+$ cells expanded during the secondary immune response to *H. polygyrus*. Furthermore, they were secreting Th2 cytokines and could promote the activation of alternative macrophages *in vitro* and lost their Treg phenotype, suggesting they were functional Th2 memory cells. Taken together these results suggest *Il4*-GFP$^+$ ex-Foxp3 cells could be participating in the functional immune response to secondary infection with *H. polygyrus* by promoting type-2 effector

mechanisms known to be required for immunity to *H. polygyrus* (Anthony et al., 2006). Interestingly, we found that a proportion of *Il4*-GFP$^+$ ex-Foxp3 cells retained a demethylated locus, correlating with unstable maintenance of *Il4*-GFP$^+$ expression *in vitro* and correlating with an increased potential to re-express *Foxp3 in vitro.* Taken together this data suggests that the majority of *Il4*-GFP$^+$ ex-Foxp3 cells have converted to Th2 effector cells however a minority have maintained a demethylated *Foxp3* locus and the ability to re-express *Foxp3,* which may represent a sub-population of truly plastic Treg cells that are able to function as effector cells whilst maintaining the potential to re-express *Foxp3.*

It would be interesting to further characterise the heterogeneity of the *Il4*-GFP$^+$ ex-Foxp3 population. To do this, we could perform genome-wide RNA sequencing on cells isolated from diverse tissues and time-points following infection and compare their transcriptional profiles. Furthermore, it would be interesting to study the heterogeneity within a unique population of *Il4*-GFP$^+$ ex-Foxp3 cells by performing RNA sequencing on a single-cell level. This technique was recently used to identify a discrete sub-population of steroidogenic Th2 cells within *N. brasiliensis*-elicited *Il13*-GFP$^+$ cells with a unique immunoregulatory function (Mahata et al., 2014). In a similar way, single-cell RNA sequencing would allow the identification of sub-populations of *Il4*-GFP$^+$ cells with more effector or regulatory properties.

4.3.3. Dynamicity of DNA methylation at the *Foxp3* locus

The *Foxp3* locus is composed of three conserved non-coding elements (CNS1-3). Both the CNS1 and CNS3 were shown to be required for *Foxp3* expression and Treg development (Zheng et al., 2010). TCR signalling in thymic CD4$^+$ T cells led to the induction of the transcriptional activator c-rel and its positioning on the *Foxp3* CNS3 resulting in *Foxp3* expression (Zheng et al., 2010). Furthermore, CNS1-deficient mice exhibited reduced proportions of iTreg cells in the MLN and gut associated lymphoid tissue suggesting CNS1 was required for peripheral *Foxp3* induction (Zheng et al.,

2010). In contrast, CNS2 was not required for *Foxp3* induction but for the maintenance of *Foxp3* expression in peripheral Treg cells and following adoptive transfer to T cell deficient mice (Zheng et al., 2010). The CpG islands on the CNS2 of the *Foxp3* locus were shown to be specifically demethylated in CD25^{+}*Foxp3*-expressing cells (Floess et al., 2007) and the maintenance of demethylated CpG islands on the *Foxp3* CNS2 was required for Treg stability (Ohkura et al., 2012; Okada et al., 2014). Thus, the level of methylation of the *Foxp3* CNS2 has been used to predict the function and stability of Treg cells.

We performed bisulphite sequencing (Clark et al., 1994) to characterise the methylation status of the *Foxp3* CNS2 region in ex-Foxp3 cells following adoptive transfer or isolated from fate-reporter mice infected with *H. polygyrus*. There are two distinctive quantitative advantages to this method. Firstly, we can interrogate modified sequences at a nucleotide level and characterise methylation patterns on individual CpG islands including heterogeneity between CpG clones islands. Secondly, we can characterise the heterogeneity of the population of cells by cloning separate PCR products, which in theory represent individual cells within a population, and get a representation of the methylation status of the whole Treg population.

We found that the majority of our donor HpTR cells had a demethylated *Foxp3* CNS2, similar to *Foxp3*-YFP^{+} Tregs isolated form fate-reporter mice and *Foxp3*-RFP^{+} cells isolated from naïve double-reporter mice, as previously reported (Okada et al., 2014). Although the majority of HpTR cells used as donor Tregs in our adoptive transfer model were exhibiting characteristics of bona fide Treg cells, we found they were unstable and lost *Foxp3* expression following adoptive transfer, suggesting methylation does not confer stability to Treg cells when transferred into a lymphodeficient environment. A previous study described the re-methylation of *Foxp3* CNS2 in ex-Foxp3 cells generated *in vitro* or *in vivo* following their adoptive transfer into lymphopenic mice (Feng et al., 2014). We quantified the methylation of HpTR cells that had lost *Foxp3* expression but did not upregulate *Il4* (HpTR→DN) and found that they had re-methylated their *Foxp3* locus. In contrast, cells that had lost *Foxp3*

expression and acquired *Il4*-GFP expression maintained a demethylated *Foxp3* locus. This suggests HpTR→*Il4*-GFP$^+$ cells may have developed directly from HpTR precursors rather than going through a double-negative stage. Furthermore, it supports the idea that HpTR→*Il4*-GFP$^+$ originated from HpTR cells displaying a demethylated *Foxp3* CNS2 and not from potentially contaminating and uncommitted or *Foxp3*$^-$ cells within the transferred HpTR pool. These data also suggest that HpTR→*Il4*-GFP$^+$ cells form a unique population of plastic cells with epigenetic memory of their Treg past. Indeed, we found that HpTR→*Il4*-GFP$^+$ but not HpTR→DN cells were able to suppress the proliferation of naïve T cells *in vitro,* confirming previous studies showing *Foxp3* methylation correlating with Treg suppressor function (Feng et al., 2014). However, their ability to suppress effector T cells was reduced compared to cells that had maintained *Foxp3* expression, suggesting that HpTR→*Foxp3*-RFP$^+$ cells had additional suppressor mechanisms. For example *Il10*, known to be an important mechanism of Treg suppression *in vivo* (Rubtsov et al., 2008), was highly expressed in HpTR→*Foxp3*-RFP$^+$ cells but undetectable in HpTR→*Il4*-GFP$^+$ cells. These results differ from previous methylation analysis showing the majority of ex-Foxp3 cells isolated from fate-reporter mice had a demethylated *Foxp3* locus (Bailey-Bucktrout et al., 2013; Feng et al., 2014; Komatsu et al., 2014). Using our fate-reporter system, we also found that the vast majority of ex-Foxp3 *Il4*-GFP$^+$ cells isolated from fate-reporter mice displayed a re-methylated *Foxp3* CNS2 in contrast to HpTR→*Il4*-GFP$^+$ cells, suggesting that signals present in wild-type mice promote the remethylation of *Foxp3* and potentially more stable conversion of Treg to *Il4*-GFP–expressing cells. Alternatively, it is conceivable that ex-Foxp3 *Il4*-GFP$^+$ originate from DN cells displaying a methylated locus in both systems, whereas HpTR→*Il4*-GFP$^+$ originate directly from *Foxp3*-expressing cells with a demethylated locus. *Foxp3* methylation is thought to correlate with stability of *Foxp3* expression, and only a small proportion of ex-Foxp3 *Il4*-GFP$^+$ cells could re-express *Foxp3* following their culture in Treg conditions *in vitro,* correlating with the small proportion of cells displaying a demethylated *Foxp3* locus. We hypothesise that HpTR→*Il4*-GFP$^+$ displaying a demethylated *Foxp3* locus would

readily convert back to *Foxp3*-expressing cells, however this remains to be tested. Finally, it would be interesting to interrogate other Treg specific demethylated regions (TSDRs) known to be specifically demethylated in Treg cells such as the *Il2ra* and *Ctla4* loci to see whether changes in methylation patterns on the *Foxp3* locus are reflected throughout and whether reduced *Il2ra* and *Ctla4* mRNA expression correlates with changes in methylation at these sites, in contrast to the *Foxp3* locus.

Together, these results confirm discordance between *Foxp3* mRNA expression and *Foxp3* DNA methylation, and instead, highlight the correlation between *Foxp3* methylation and Treg suppressor function, suggesting the accessibility of the *Foxp3* locus is required for the binding of other TFs important for Treg function. Although the adoptive transfer of Tregs into lymphodeficient hosts has been invaluable for tracking and studying the fate of Treg cells, our results suggest that the lymphodeficient environment may not provide appropriate signals for the conversion of Treg to Th2 cells, such as B- and T-cell derived cytokines.

4.3.4. Investigating the role of cytokine signalling for Treg instability

$CD4^+$ T cells are dependent on cytokine signalling for their development, maintenance and differentiation. As a result, modulations in the cytokine environment can influence the balance in T helper cell subsets (Dardalhon et al., 2008; Veldhoen et al., 2008) as well as the stability of differentiated cells (Hegazy et al., 2010). For example IL-2 (Setoguchi et al., 2005) and IL-10 (Murai et al., 2009) are required for the development and maintenance of the Treg lineage *in vivo,* whereas IL-4 and IL-6 promote instability of *Foxp3* expression (Feng et al., 2014). Indeed, IL-4 has been shown to inhibit *Foxp3* expression *in vitro* (Chapoval et al., 2010), *in vivo* (Noval Rivas et al., 2015) and limit Treg suppressor function during allergic lung inflammation (Dorsey et al., 2013). Furthermore, STAT6, phosphorylated downstream of the IL-4Rα, has been shown to directly bind to DNA methyl transferase *Dnmt1* (Feng et al., 2014) required for the demethylation of the *Foxp3* locus and maintenance of *Foxp3*

expression (Lee et al., 2001). As a consequence, IL-4 is thought to directly inhibit *Foxp3* expression by recruiting *Dnmt1* to the *Foxp3* locus, resulting in CNS2 re-methylation (Feng et al., 2014). Taken together, this data demonstrates that IL-4 signalling promotes Treg instability. We found that Tregs isolated from a primary *H. polygyrus* infection expressed a functional IL-4Rα and were actively phosphorylating STAT6 *ex vivo*. Furthermore, we found that Treg cells lost *Foxp3* expression following IL-4 stimulation *in vitro*, confirming studies describing a dominant inhibitory effect of IL-4 on the function of Treg cells (Mantel et al., 2007; Wei et al., 2007; Feng et al., 2014).

Emerging evidence suggests that IL-4 is required both for Treg instability as well as Treg to Th2 conversion. Indeed, Treg-specific overexpression of *Il4ra* and enhanced IL-4 signalling was shown to result in Treg acquisition of a Th2 phenotype however it was unclear whether wild-type Tregs expressing could convert to Th2 cells in this system (Noval Rivas et al., 2015). IL-4 and IL-1β were identified as two of the highest predicted regulators of converted HpTR→*Il4*-GFP$^+$ cells, and have previously been shown to be significantly increased early following *H. polygyrus* infection (Urban et al., 1991; Zaiss et al., 2013). Interestingly, IL-4 was also predicted to be upstream of the transcriptional profile of donor HpTR cells. Given that HpTR cells were isolated from a polarised type-2 environment following *H. polygyrus* infection, this may suggest that IL-4 is actively signalling in HpTR and driving the expression of a unique set of IL-4-related genes such as miR-182, previously shown to be upregulated following IL-4 stimulation of Treg cells *in vitro* (Kelada et al., 2013). We also found that IL-4 stimulation led to the conversion of Treg to *Il4*-GFP$^+$ cells *in vitro* suggesting that IL-4 signalling might be upstream of Treg conversion to Th2 cells in our study. In light of the recent evidence showing that strong IL-4 signalling was required for Treg reprogramming in the context of oral allergy (Noval Rivas et al., 2015), it is conceivable that there is a quantitative threshold of IL-4 signalling that dictates Treg specialisation or conversion. To test whether IL-4 signalling was required for Treg to Th2 conversion during *H. polygyrus* infection, we have generated fate-reporter mice with a Treg-

specific deficiency in IL-4Rα that will allow us to measure the impact of IL-4Rα-deficiency on the development of ex-Foxp3 *Il4*-GFP$^+$ cells *in vivo* and their expansion following secondary *H. polygyrus* infection.

Increased reprogramming of Treg to Th2 cells has been shown to correlate with exacerbated Th2-mediated disease following oral challenge with ovalbumin (OVA) (Noval Rivas et al., 2015). Similarly, increased proportions of ex-Foxp3 *Il4*-GFP$^+$ cells following secondary *H. polygyrus* infection correlated with increased type-2 responses and functional immunity to *H. polygyrus.* However the functional relevance of Treg to Th2 conversion on disease outcome or immunity in both these models remains unclear. We hope to test the role of Treg to Th2 conversion for functional expulsion of *H. polygyrus* using the IL-4Rα-deficient fate-reporter mouse. While we haven't had the opportunity to characterise the *Il4ra*$^{-/-}$ fate-reporter mice, we can speculate about functional outcomes following secondary infection with *H. polygyrus* (**Figure 4.16**).

IL-4-deficient mice (Finkelman et al., 1997) have impaired type-2 responses and compromised worm expulsion following secondary infection with *H. polygyrus.* IL-4 is required for the generation of a functional type-2 response following primary infection (Urban et al., 1991). Furthermore, mice treated with anti-IL-4 antibody at the time of secondary challenge had reduced type-2 responses and worm expulsion (Urban et al., 1991) suggesting IL-4 is required at the time of secondary infection. However whether IL-4 is required for de novo differentiation of Th2 cells during a secondary challenge is unclear. We will test this hypothesis by measuring the number of *Il4*-GFP$^+$ cells that have originated from a Treg past in the presence or absence of the IL-4Rα. We found that anti-IL-4 antibody treatment at the time of secondary challenge did not impair the differentiation of Th2 cells or ex-*Foxp3 Il4*-GFP$^+$ cells, suggesting that the activation of memory Th2 cells is IL-4-independent. Previous studies showing impaired type-2 responses following anti-IL-4 treatment were most likely blocking the wide variety of IL-4 target cells and downstream effector pathways but not the re-activation of memory Th2 cells (Urban et al., 1991).

We hypothesise that IL-4 could be required for the generation of *Il4*-GFP$^+$ ex-Foxp3 cells in a primary infection. Consequently, Treg-specific IL-4Rα deficiency may reduce the conversion of *Foxp3*$^+$ cells to *Il4*-GFP$^+$ cells and impair the development of memory responses following primary infection, thereby resulting in the impaired development of *Il4*-GFP$^+$ ex-Foxp3 memory cells seen following secondary infection with *H. polygyrus* (**Figure 4.16 A**). Since we have shown that a significant proportion of *Il4*-GFP$^+$ cells originated from a *Foxp3*-expressing past during a secondary infection, we hypothesise that Treg-specific IL-4Rα deficiency would reduce the magnitude of the Th2 population (**Figure 4.16 A**) and impair functional worm expulsion. Our studies and others have shown that IL-4 signalling promotes *Foxp3* instability in mature Treg cells *in vitro* (Noval Rivas et al., 2015). Therefore an alternative hypothesis is that IL-4Rα signalling could be reducing *Foxp3* expression in mature Treg cells *in vivo*. It is therefore conceivable that IL-4Rα deficiency in Treg cells could lead to enhanced Treg differentiation, resulting in increased suppression of Th2 cells, and impaired memory Th2 responses (**Figure 4.16 B**). Preliminary data using a chimeric system suggested that *Il4ra*-deficiency in Treg cells does not significantly alter the ratio of Treg and Th2 cells in a secondary infection with *H. polygyrus*.

Alternatively, given that IL-4 was predicted to be upstream of the transcriptional profile of HpTR it is conceivable that IL-4 is required for the specialisation of HpTR cells and their ability to specifically suppress Th2 cells. Indeed, IL-4 was shown to induce *cmaf* and mir-182 expression and confer nTreg cells with the ability to suppress Th2 cells *in vitro* (Kelada et al., 2013). Furthermore, *Irf4* bound by STAT6 following IL-4 signalling (Gupta et al., 1999) was shown to confer HpTR with the specific ability to suppress Th2 cells (Zheng et al., 2009). Thus, inhibition of IL-4 signalling in HpTR cells compromise the ability of HpTR cells to specifically suppress Th2 cells and result in the enhanced development of Th2 responses (**Figure 4.16 C**), similarly to what we have seen following the transient depletion of Treg cells in DEREG mice during secondary

infection (**Fig. 4.13**). Of note, persistent Th2 responses may result in Th2-mediated immunopathology, such as the development of fibrosis.

Alternatively, reduced type-2 responses following the inhibition of Treg to Th2 conversion may result in impaired downstream type-2 responses and limit the expulsion of *H. polygyrus*, as seen following combined IL-4 and IL-4Rα antibody treatment during memory immune response (Urban et al., 1991). We will fully characterise the type-2 responses such as the activation of alternative macrophages, the stimulation of antibody class-switching to protective IgG1 and IgE, and worm expulsion (**Figure 4.16**). Preliminary results from the generation of bone-marrow chimeras indicate that functional expulsion of *H. polygyrus* may not be compromised in the absence of IL-4 signalling on Treg cells (**Fig 4.15**).

It would be interesting to determine the temporal characteristics of conversion and determine whether Treg cells convert during a primary infection or a secondary infection. One way to do this would be to adoptively transfer congenically marked *Foxp3*-YFP^{+}FP635^{+} Treg cells isolated from fate-reporter mice into wild-type hosts either in a primary or secondary infection and compare their conversion to *Il4*-GFP-expressing cells. Alternatively, a recent study employed a tamoxifen inducible fate mouse model to study Th17 to Tr1 conversion (Gagliani et al., 2015). By selectively inducing *Il17*-Cre at baseline or during inflammation, the authors could show that Th17 cells could reprogram to IL-10^{+} Tr1 cells during inflammation rather than proliferate from cells which had developed in steady-state (Gagliani et al., 2015). To identify whether ex-Foxp3 Th2 cells are generated *de novo* during memory Th2 responses to *H. polygyrus* or proliferate from a cell that was generated during a primary infection, it would be useful to use tamoxifen inducible *Foxp3*-Cre fate-reporter system. To date, the only conditional *Foxp3* fate reporter mice harbours a DNA sequence which encodes a triple fusion protein containing eGFP, Cre and ERT2 (*Foxp3*-eGFP-Cre-ERT2) (Rubtsov et al., 2010), limiting our ability to cross it to *Il4*-GFP mice. To test *Foxp3* to *Il4*-GFP conversion we would need to generate knock-in mice harbouring an

IRES site followed by a triple fusion sequence containing YFP fluorescent protein instead of GFP.

4.3.5. Advantages and limitations of transcriptional reporter mice

Il4-GFP expression reflects *Il4* transcription into mRNA but not translation into IL-4 protein (Mohrs et al., 2005). To better understand the relationship between mRNA and protein, Mohrs et al generated a double-reporter mouse by crossing *4get* (*Il4*-GFP) mice (Mohrs et al., 2001) with KN2 mice in which the expression of surface huCD2 faithfully reports IL-4 protein secretion (Mohrs et al., 2005). This previous study found that the correlation of *Il4*-GFP mRNA and huCD2 expression was highly dependent on the tissue and disease model (Mohrs et al., 2005). In the context of a *H. polygyrus* infection, they showed that *Il4*-GFP expression correlated with IL-4 protein in $CD4^+$ T cells isolated from the gut associated lymphoid tissues (MLN, PP and LP) (Mohrs et al., 2005). In concordance with these results, we found the highest proportion of converted HpTR→*Il4*-GFP^+ cells in the MLN and PP, which suggested they were likely to be secreting IL-4 protein. Indeed, transferred HpTR cells expressed IL-4 protein *ex vivo* as assessed by flow cytometry, however IL-4 protein was undetectable following the re-stimulation of purified HpTR→*Il4*-GFP^+ cells compared to nT→*Il4*-GFP^+ cells, suggesting there may be post-transcriptional or post-translational regulatory mechanisms inhibiting the secretion of IL-4 in ex-Foxp3 cells. Conversely, we found that ex-*Foxp3 Il4*-GFP^+ cells isolated from fate-reporter mice secreted large amounts of IL-4 protein suggesting that these inhibitory mechanisms were absent in a lymphocomplete context, although it still remains unclear to what extent *Il4*-GFP expression correlates with protein in this context. To test this, we would ideally cross the *Foxp3*-RFP reporter or *Foxp3* fate-reporter mice with $KN2^{fl/wt}$ mice to determine the correlation of *Il4*-GFP expression with IL-4 protein production in ex-*Foxp3* cells. Alternatively, we could perform an IL-4 ELISPOT assay on purified ex-*Foxp3 Il4*-GFP^+ cells re-stimulated with P+I. For example, if we plated 1000 *Il4*-GFP-expressing cells,

we could count the number of spots, illustrating single-cell IL-4 secretion, and in theory calculate the ratio of *Il4*-GFP-expressing cells with the number of spots. An additional method to test this, which has been used for correlating IL-17 production and reporter expression in IL-17 fate-reporter mouse (Hirota et al., 2011), IL-9 reporter mice (Wilhelm et al., 2011) and IL-22 fate-reporter mice (Ahlfors et al., 2014) would be to use a modified intra-cellular cytokine staining protocol, which would allow us to retain intra-cellular GFP expression and co-stain for *Il4*-GFP and IL-4 protein. Although this would be useful for correlating *Il4*-GFP and IL-4 protein at a single-cell level, it may be harder to detect cells secreting low levels of cytokine and therefore may underrepresent the true levels of IL-4 protein.

A possible limitation of *Foxp3* fate-reporter systems comes from evidence that $CD4^+$ T cells can transiently up-regulate *Foxp3* expression following their activation. Indeed, human (Pillai et al., 2007; Tran et al., 2007; Wang et al., 2007; Dorsey et al., 2013) and murine (Miyao et al., 2012) naïve T cells have been shown to transiently express *Foxp3* upon activation *in vitro*, however it's unclear to what extent this happens *in vivo* during inflammation or infection (Hori, 2011; Miyao et al., 2012). *In vivo,* a small proportion of $CD4^+$ T cells have been shown to express *Foxp3* and low levels of the fate-reporter (Miyao et al., 2012). Furthermore, these cells were shown to have a partially methylated *Foxp3* locus and to lose *Foxp3* expression upon transfer into *Rag1*$^{-/-}$ recipients co-transferred with bulk $CD4^+$ T cells suggesting they were not committed Treg cells and were more unstable *in vivo* (Miyao et al., 2012). Based on these results, the authors defined a *Foxp3* heterogeneity model in which a small proportion of the $Foxp3^+$ pool of Tregs express unstable *Foxp3* expression and represent cells that were never fully committed Treg cells. This suggests that a proportion of *Foxp3*-expressing cells identified throughout our work may only be transiently expressing *Foxp3.* Alternatively, the identification of cells expressing *Foxp3* reporter but not fate-marked could also suggest that the Cre recombinase was inefficient and that the cells had not had the time to properly excise the loxP sites either side of the stop cassette inserted into the *Rosa26* locus (Ohkura et al., 2012), which

we do not observe in our model. Furthermore, in our hands, the majority of HpTR cells, *Foxp3*-YFP$^+$FP635$^+$ cells and converted HpTR→*Il4*-GFP$^+$ cells had a demethylated *Foxp3* locus, suggesting they were not transiently expressing *Foxp3.* If a significant proportion of activated effector cells were transiently expressing *Foxp3,* we would expect to see a large proportion of *Foxp3*-RFP cells isolated from a *H. polygyrus* infection (HpTR) have a methylated *Foxp3* CNS2. In contrast to HpTR→*Il4*-GFP$^+$ cells, the majority of ex-Foxp3 *Il4*-GFP$^+$ cells in fate-reporter mice had re-methylated their *Foxp3* locus. This could either be due to *de novo* methylation in ex-Foxp3 *Il4*-GFP$^+$ cells (as previously discussed). Alternatively, it suggests they could have originated from cells that transiently expressed *Foxp3* and were consequently expressing FP635 without displaying characteristic *Foxp3* CNS2 demethylation. Instead, we found that a small but significant fraction of the ex-Foxp3 *Il4*-GFP$^+$ cells displayed a demethylated *Foxp3* locus compared to *Il4*-GFP$^+$ cells, correlating with their ability to re-express *Foxp3.* It is conceivable that these cells represent a population of truly plastic ex-Treg Th2 cells. We could study the role of IL-4 signalling on methylation *in vivo* by comparing the methylation status of IL-4Rα-sufficient and *Il4ra*$^{-/-}$ Treg cells.

In conclusion, we have shown that *Foxp3*-expressing cells can convert to *Il4*-GFP$^+$ cells *in vivo* in complete mice or following adoptive transfer into T-cell deficient mice. Furthermore, we have shown these cells are functional in the context of secondary immune responses to *H. polygyrus.* We have developed a fate-reporter mouse with a Treg specific deletion of the IL-4Rα to test the requirement of IL-4 signalling for Treg to Th2 conversion and to test its impact on functional immune responses to *H. polygyrus*.

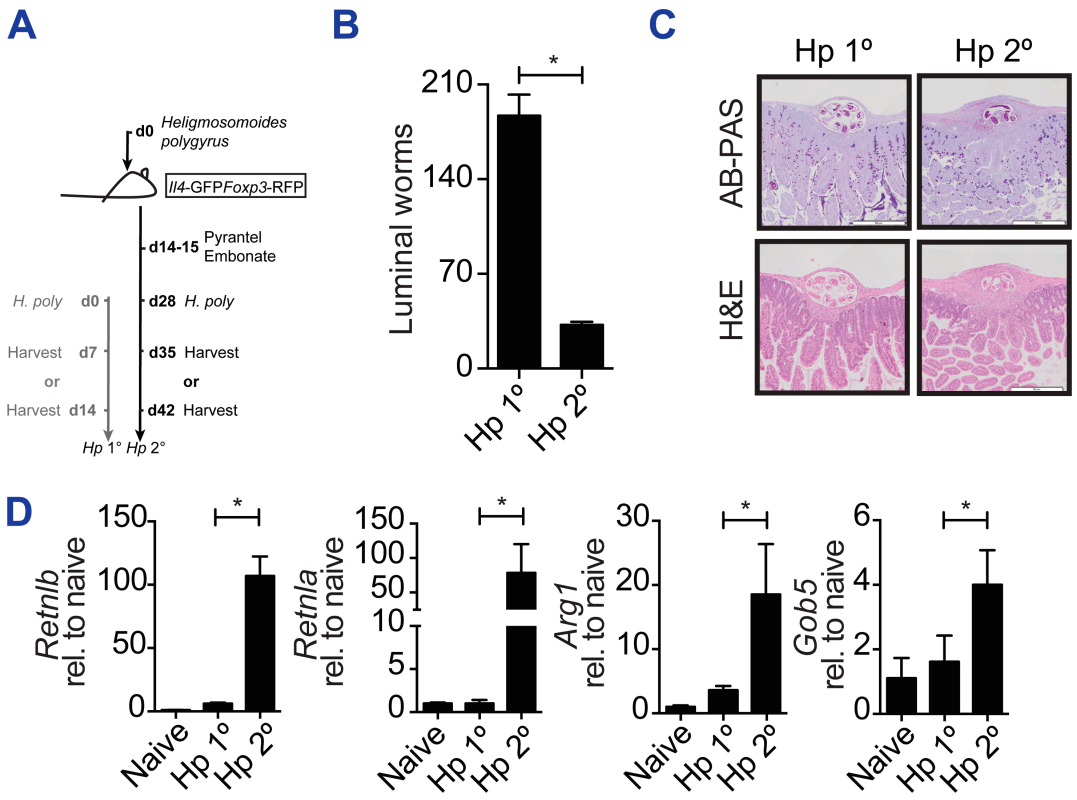


Figure 4.1. Secondary infection with *H. polygyrus* correlates with increased type-2 responses and larval killing. (A) Schematic representation of the experimental model. *Il4*-GFP*Foxp3*-RFP double-reporter mice were infected with 200 *H. polygyrus* larvae. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. Naïve *Il4*-GFP*Foxp3*-RFP mice were given a primary (Hp 1°) infection at the same time-point. All groups were harvested 7 days post-infection for FACS analysis and 14 days post-infection for worm counts. **(B)** Adult worms were counted in the intestinal lumen of Hp 1° and Hp 2° mice 14 days post-infection. **(C)** AB-PAS and H&E stained sections of the small intestine at day 7 post infection in Hp 1° and Hp 2° mice infection. The images depict larvae trapped in the intestinal wall. **(D)** *Retnlb, Retnla, Arg1,* and *Gob5* gene expression in the small intestine of Hp 1° and Hp 2° mice 7 days post-infection, expressed as fold change relative to naïve mice. Data is representative of 2-3 independent experiments with 3-5 mice per group. Statistical test applied Mann-Whitney, two-tailed, * denotes $p<0.05$.

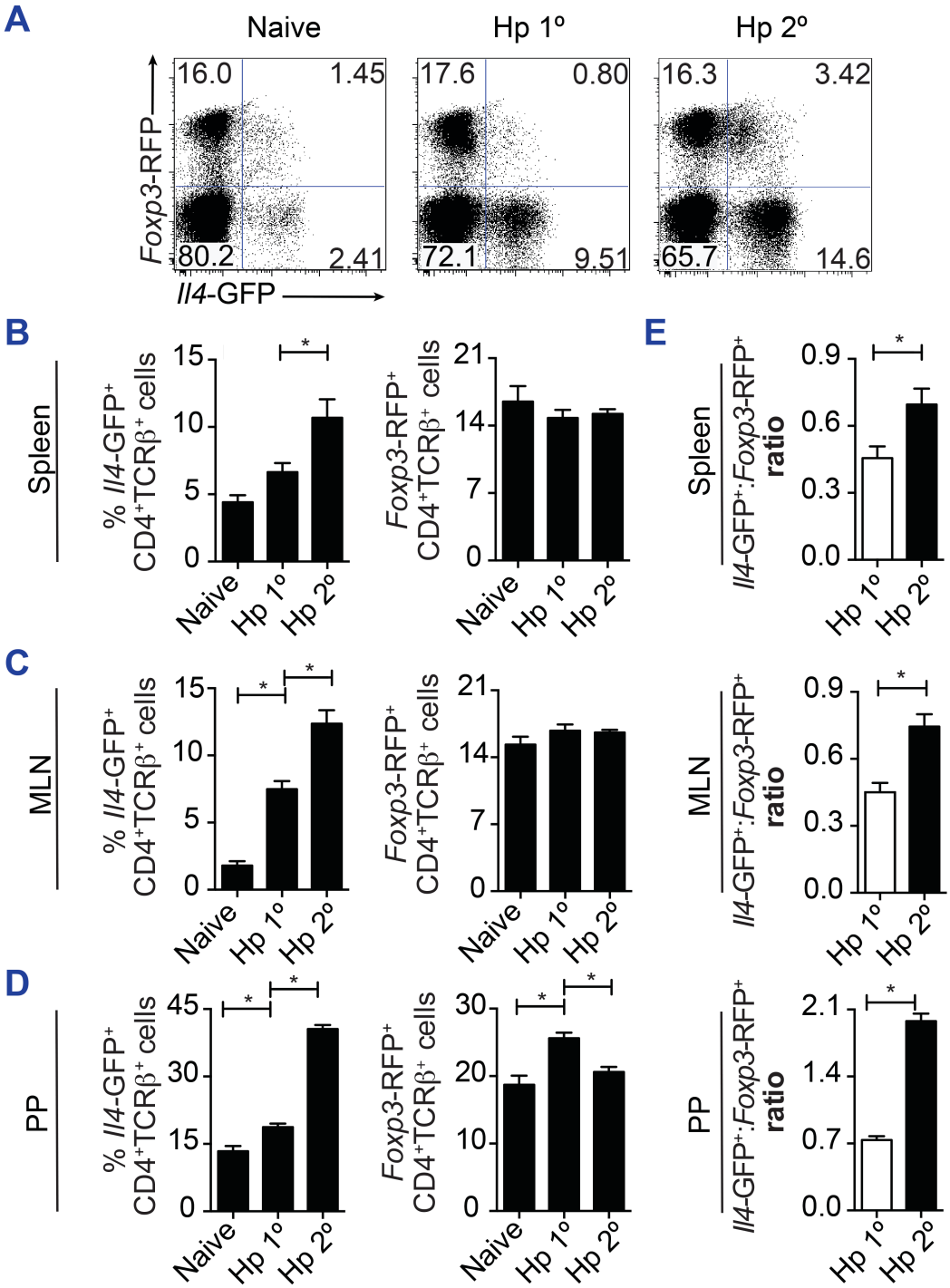

A
Naive
Hp 1º
Hp 2º
16.0
1.45
80.2
2.41
17.6
0.80
72.1
9.51
16.3
3.42
65.7
14.6
Foxp3-RFP
Il4-GFP
B
Spleen
% Il4-GFP+ CD4+TCRβ+ cells
Foxp3-RFP+ CD4+TCRβ+ cells
Il4-GFP+:Foxp3-RFP+ ratio
C
MLN
D
PP
E
Naive
Hp 1º
Hp 2º

Figure 4.2. Shift from a regulatory to an effector immune response during secondary infection with *Heligmosomoides polygyrus*. *Il4*-GFP*Foxp3*-RFP reporter mice were infected with 200 *H. polygyrus* larvae. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. Naïve *Il4*-GFP*Foxp3*-RFP mice were given a primary (Hp 1°) infection at the same time-point. The mice were harvested 7 days post-infection for FACS analysis. **(A)** Representative FACS dot plot showing *Il4*-GFP and *Foxp3*-RFP expression in the mesenteric lymph nodes (MLN) of naïve, Hp 1º and Hp 2º mice. Graphical representation of the frequency of *Il4*-GFP^{+}*Foxp3*-RFP^{-} and *Il4*-GFP^{-}*Foxp3*-RFP^{+} cells (of the CD4^{+}TCRβ^{+} cells) in the spleen (**B),** mesenteric lymph nodes (MLN) **(C)** and Peyer's patches (PP) **(D)** of naïve, Hp 1º and Hp 2º mice. **(E)** Ratio of the frequency of *Il4*-GFP^{+} cells over *Foxp3*-RFP^{+} cells in the spleen, MLN and PP. Data is representative of 3 independent experiments with 3-5 mice per group. Statistical test applied Mann-Whitney, two-tailed, * denotes $p<0.05$.

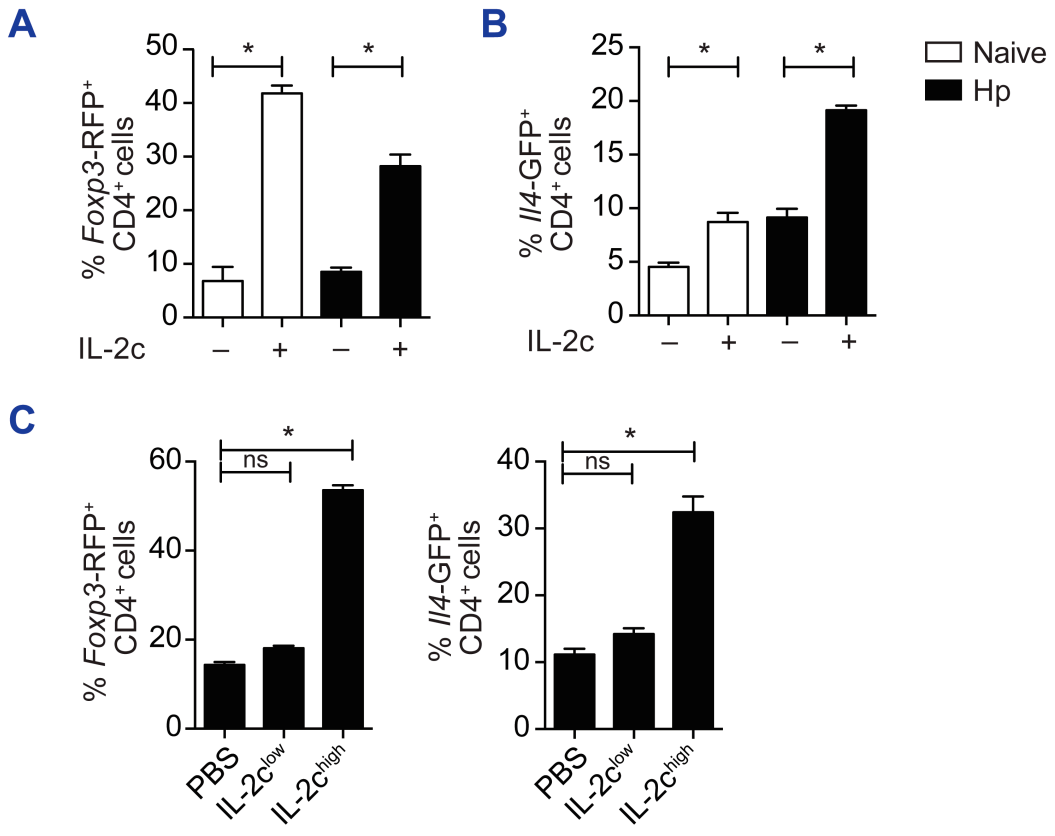


Figure 4.3. IL-2c treatment expands Treg and Th2 cells. *Il4*-GFP*Foxp3*-RFP reporter mice were infected with 200 *H. polygyrus* larvae. Naïve or *H. polygyrus*-infected mice were treated with IL-2c or PBS at days 12, 14 and 16 post-infection and harvested at d21. **(A)** Graphical representation of the frequency of *Il4*-GFP^{-}*Foxp3*-RFP^{+} and **(B)** *Il4*-GFP^{+}*Foxp3*-RFP^{-} cells (of the CD4^{+} cells) in the spleen of naïve (white bars), or infected mice (black bars). *H. polygyrus*-infected mice were treated with either a high (IL2c^{high}) or a low (IL-2c^{low}) dose of IL-2c at days 12, 14 and 16 post-infection and harvested at d21. **(C)** Graphical representation of the frequency of *Il4*-GFP^{-}*Foxp3*-RFP^{+} and *Il4*-GFP^{+}*Foxp3*-RFP^{-} cells (of the CD4^{+} cells) in the spleen. CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{+}CD25^{high} (HpTR) Treg cells were FACS-purified from the spleen and MLN of *Il4*-GFP*Foxp3*-RFP reporter mice infected with *H. polygyrus* for 14 days and transferred to TCRα-deficient mice (*Tcra*$^{-/-}$). Recipient mice were treated with IL-2c or PBS at days 12, 14 and 16 post-infection and harvested at d21. **(C)** Representative FACS dot plots showing *Foxp3*-RFP, *Il4*-GFP and CD25 expression pre- and post-purification.

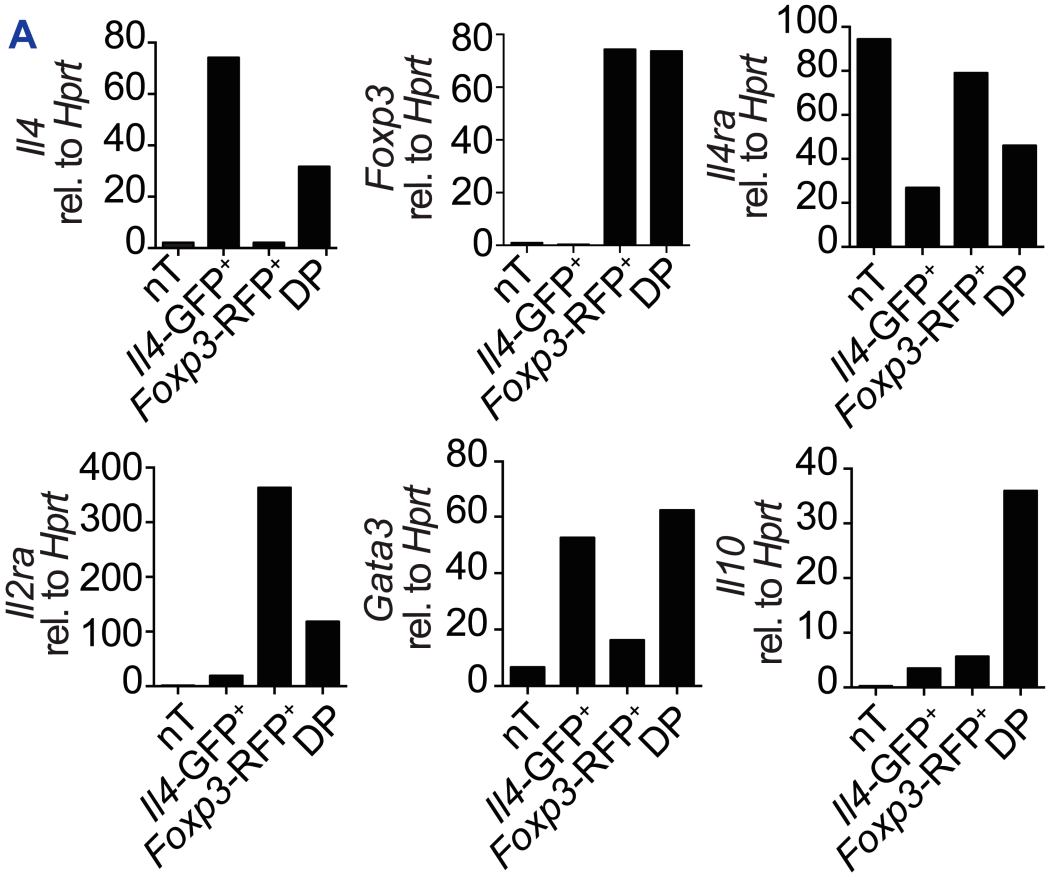


Figure 4.4. The *Il4*-GFP*Foxp3*-RFP reporter faithfully reports *Il4* and *Foxp3* expression. **(A)** *Il4, Foxp3, IL4ra, Il2ra, Gata3* and *Il10* expression in FACS-purified $CD4^+TCR\beta^+CD25^-CD44^{low}$ naïve T cells (nT), $CD4^+TCR\beta^+$*Il4*-GFP^+*Foxp3*-RFP^- Th2 cells (*Il4*-GFP+), $CD4^+TCR\beta^+$*Il4*-GFP^-*Foxp3*-RFP^+ Treg cells (*Foxp3*-RFP^+) and double-positive $CD4^+TCR\beta^+$*Il4*-GFP^+*Foxp3*-RFP^+ (DP) cells expressed relative to *Hprt* (x100). Data is representative of 3 independent experiments. Cells were pooled from 3-4 mice.

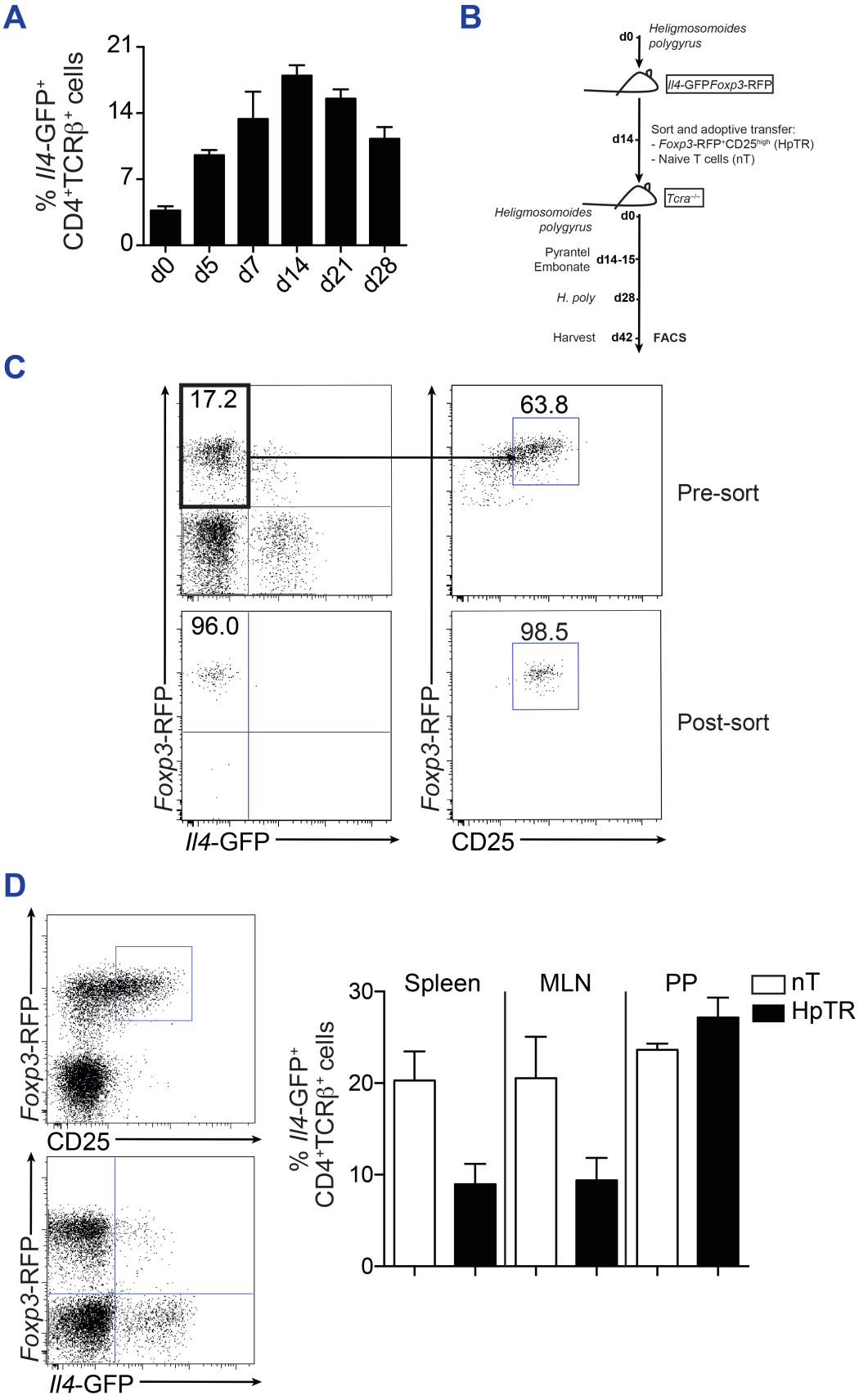

A
% Il4-GFP+ CD4+TCRβ+ cells
21
14
7
0
d0
d5
d7
d14
d21
d28
B
d0
Heligmosomoides polygyrus
Il4-GFPFoxp3-RFP
d14
Sort and adoptive transfer:
- Foxp3-RFP+CD25high (HpTR)
- Naive T cells (nT)
Tcra-/-
Heligmosomoides polygyrus
d0
Pyrantel Embonate
d14-15
H. poly
d28
Harvest
d42
FACS
C
17.2
63.8
96.0
98.5
Pre-sort
Post-sort
Foxp3-RFP
Il4-GFP
CD25
D
Foxp3-RFP
CD25
Foxp3-RFP
Il4-GFP
% Il4-GFP+ CD4+TCRβ+ cells
30
20
10
0
Spleen
MLN
PP
nT
HpTR

Figure 4.5. A proportion of adoptively transferred *Foxp3*-RFP^{+}CD25^{high} Treg cells lose *Foxp3* expression and express *Il4*-GFP. (A) Naïve *Il4-GFPFoxp3*-RFP mice were infected with *H. polygyrus* and harvested at days 0, 5, 7, 14, 21 and 21 post-infection. Graphical representation of the frequency of *Il4*-GFP^{+}*Foxp3*-RFP^{-}cells (of the CD4^{+}TCRβ^{+} cells) in the spleen over the time-course. **(B)** Schematic representation of the adoptive transfer experimental set-up. CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{+}CD25^{high} (HpTR) Treg cells were FACS-purified from the spleen and MLN of *Il4*-GFP*Foxp3*-RFP reporter mice infected with *H. polygyrus* for 14 days. CD4^{+}TCRβ^{+}CD25^{-}CD44^{low} (nT) naïve T cells were FACS sorted from the spleen and MLN of naïve *Il4*-GFP*Foxp3*-RFP mice. HpTR cells or nT were transferred to TCRα-deficient mice (*Tcra*$^{-/-}$). Recipient mice were infected with *H. polygyrus,* drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. The mice were harvested 14 days post-infection for FACS analysis (d42). **(C)** Representative FACS dot plots showing *Foxp3*-RFP, *Il4*-GFP and CD25 expression pre- and post-purification. **(D)** Representative FACS dot plots showing CD25, *Il4*-GFP and *Foxp3*-RFP expression in the mesenteric lymph nodes (MLN) of *Tcra*$^{-/-}$ HpTR recipients at day 42 post-transfer. **(E)** Frequency of *Il4*-GFP^{+}*Foxp3*-RFP^{-} cells (of CD4^{+}TCRβ^{+} cells) in the spleen, MLN and Peyer's patches (PP) of nT (white bars) or HpTR (black bars) recipients d42 post-transfer. Data are representative of at least 5 independent experiments with 3-5 mice per group. Statistical test applied Mann-Whitney, two-tailed, * denotes $p<0.05$.

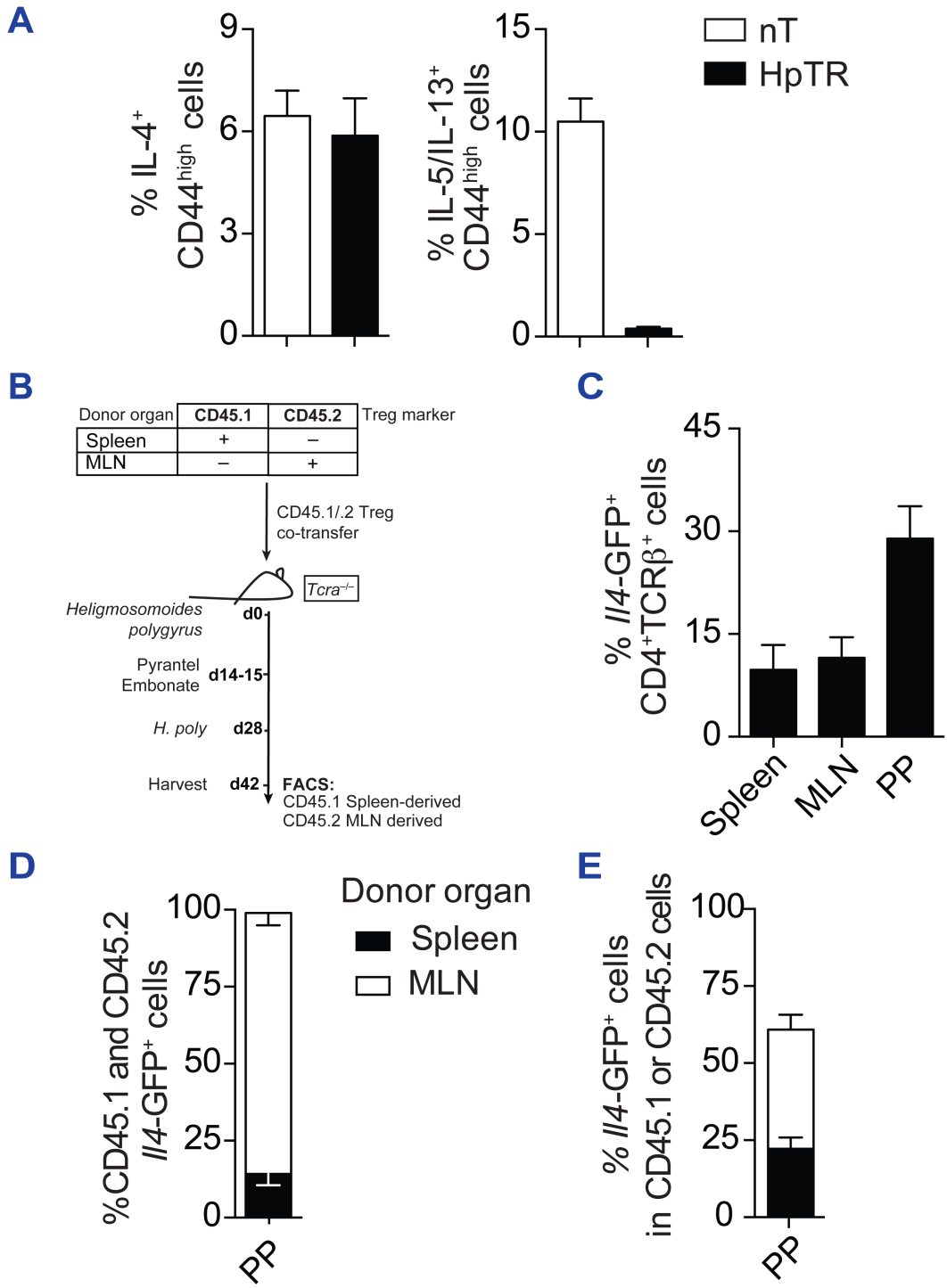

A
% IL-4+ CD44high cells
9
6
3
0
% IL-5/IL-13+ CD44high cells
15
10
5
0
nT
HpTR
B
Donor organ
CD45.1
CD45.2
Treg marker
Spleen
+
–
MLN
–
+
CD45.1/.2 Treg co-transfer
Tcra–/–
Heligmosomoides polygyrus
d0
Pyrantel Embonate
d14-15
H. poly
d28
Harvest
d42
FACS:
CD45.1 Spleen-derived
CD45.2 MLN derived
C
% Il4-GFP+ CD4+TCRβ+ cells
45
30
15
0
Spleen
MLN
PP
D
%CD45.1 and CD45.2 Il4-GFP+ cells
100
75
50
25
0
PP
Donor organ
Spleen
MLN
E
% Il4-GFP+ cells in CD45.1 or CD45.2 cells
100
75
50
25
0
PP

Figure 4.6. MLN-derived *Foxp3*-RFP^{+}CD25^{high} Treg cells convert to the highest extent in the Peyer's patches. (A) Adoptive transfer of HpTR cells was performed as described previously (Fig. 4.4 B). Frequency of IL-4+ and IL-5+IL-13+ CD4^{+}CD44^{high} cells in the spleen and mesenteric lymph nodes (MLN) of CD4^{+}TCRβ^{+}CD25^{-}CD44^{low} (nT) (white bars) and CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{+}CD25^{high} (HpTR) (black bars) recipients as measured by intra-cellular cytokine staining. Data are representative of 2 independent experiments with 4 mice per group. **(B)** Schematic representation of the modified adoptive transfer experimental set-up. Congenically marked spleen (CD45.1) and MLN (CD45.2) HpTR cells were purified and co-transferred to TCRα-deficient mice. Recipient mice were infected with *H. polygyrus,* drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. The mice were harvested 14 days post-infection for FACS analysis (d42). **(C)** Frequency of *Il4*-GFP^{+}*Foxp3*-RFP^{-} cells (of CD4^{+}TCRβ^{+} cells) in the spleen, MLN and Peyer's patches (PP) of HpTR recipients d42 post-transfer. **(D)** Frequency of CD45.1^{+} (spleen-derived) or CD45.2^{+} (MLN-derived) cells (within the *Il4*-GFP^{+} population) in the PP of HpTR recipients d42 post-transfer. **(E)** Frequency of *Il4*-GFP^{+} cells within the spleen-derived (black bar) or MLN-derived (white bar) HpTR populations in the PP of HpTR recipients d42 post-transfer. Data are representative of 2 independent experiments with 5 mice.

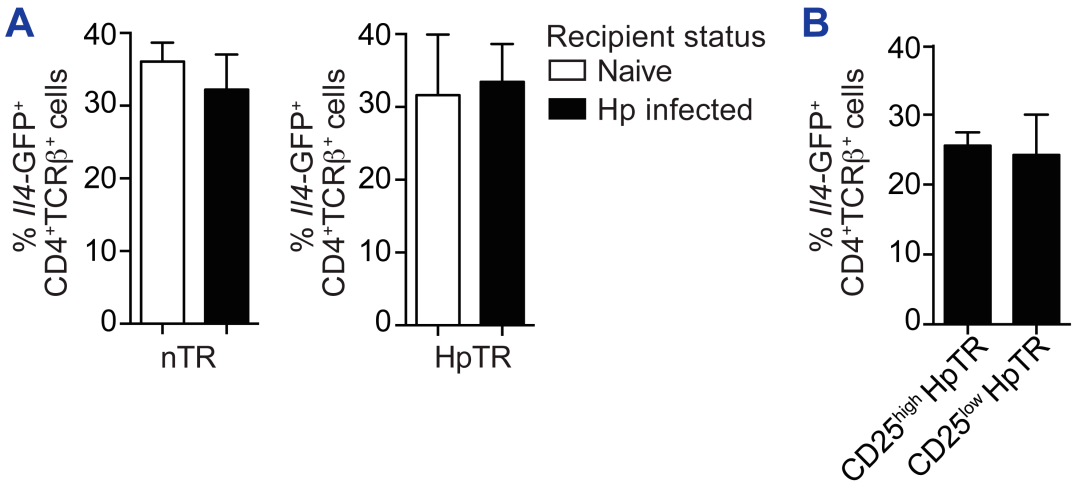
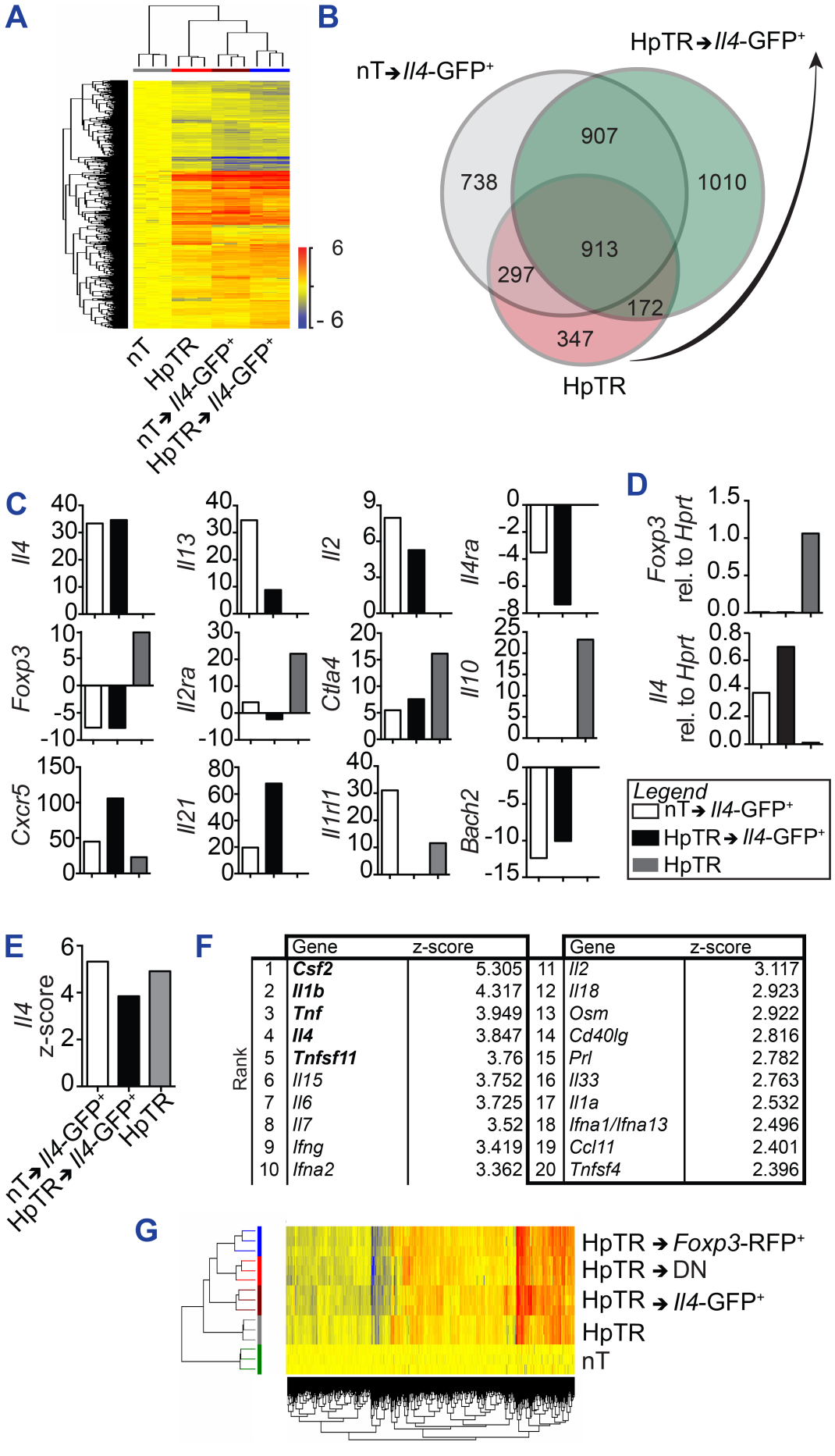


Figure 4.7. *Foxp3*-RFP^{+} cells convert to *Il4*-GFP^{+} cells in the absence of antigen and of high levels of CD25 expression. (A) CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{+}CD25^{high} cells were purified from naïve (nTR) or *H. polygyrus* infected (HpTR) *Il4*-GFP*Foxp3*-RFP donor mice and transferred to TCRα-deficient mice. One group of recipient mice were left uninfected (naïve; white bars). Another group were infected with *H. polygyrus,* drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28 (Hp infected; black bars). The mice were harvested 14 days post-infection for FACS analysis (d42). Frequency of *Il4*-GFP^{+}*Foxp3*-RFP^{-} cells (within the CD4^{+}TCRβ^{+} population) in the Peyer's patches (PP) of nTR and HpTR recipients d42 post-transfer. **(B)** CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{+}CD25^{high} or CD4^{+}TCRβ^{+}*Il4*-GFP^{-}*Foxp3*-RFP^{+}CD25^{low} cells were purified from *H. polygyrus* infected *Il4*-GFP*Foxp3*-RFP donor mice and transferred to TCRα-deficient mice. Recipient mice were infected with *H. polygyrus,* drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28 (Hp infected; black bars). The mice were harvested 14 days post-infection for FACS analysis (d42). Frequency of *Il4*-GFP^{+}*Foxp3*-RFP^{-} cells (within the CD4^{+}TCRβ^{+} population) in the Peyer's patches (PP) of CD25^{high} and CD25^{low} HpTR recipients d42 post-transfer.



Rank	Gene	z-score	Rank	Gene	z-score
1	***Csf2***	5.305	11	*Il2*	3.117
2	***Il1b***	4.317	12	*Il18*	2.923
3	***Tnf***	3.949	13	*Osm*	2.922
4	***Il4***	3.847	14	*Cd40lg*	2.816
5	***Tnfsf11***	3.76	15	*Prl*	2.782
6	*Il15*	3.752	16	*Il33*	2.763
7	*Il6*	3.725	17	*Il1a*	2.532
8	*Il7*	3.52	18	*Ifna1/Ifna13*	2.496
9	*Ifng*	3.419	19	*Ccl11*	2.401
10	*Ifna2*	3.362	20	*Tnfsf4*	2.396

Figure 4.8. HpTR→*Il4*-GFP$^+$ cells are transcriptionally re-wired. CD4$^+$TCRβ$^+$CD25$^-$CD44^{low} (nT) naïve T cells were FACS sorted from the spleen of naïve *Il4*-GFP*Foxp3*-RFP mice. CD4$^+$TCRβ$^+$*Il4*-GFP$^-$*Foxp3*-RFP$^+$CD25^{high} (HpTR) cells were FACS-purified from the spleen and mesenteric lymph nodes (MLN) of *H. polygyrus* infected mice day 14 post-infection. The adoptive transfer of HpTR cells was performed as described previously (Fig. 4.4 B). Adoptively transferred CD4$^+$TCRβ$^+$*Il4*-GFP$^+$*Foxp3*-RFP$^-$ cells were FACS-purified from nT (nT→ *Il4*-GFP) or HpTR (HpTR→ *Il4*-GFP) recipient mice at day 42 post-transfer. Gene expression profiling was performed using microarray, and analysed using Genespring. (**A)** Heatmap depicting significantly expressed mRNA transcripts ($p<0.05$) in FACS-purified HpTR, nT→ *Il4*-GFP$^+$ and HpTR→ *Il4*-GFP$^+$ cells expressed as fold change relative to nT cells (> 2.0). Each heatmap lane represents the 3 biological replicates. (**B)** Venn diagram showing comparative analysis of common and unique mRNA transcripts between nT, HpTR, nT→ *Il4*-GFP$^+$ and HpTR→ *Il4*-GFP$^+$ cells. **(C)** Graphical representation of microarray data showing fold change expression of selected Th2- and Treg-related mRNA transcripts. Data is representative of 1 experiment with 3 biological replicates per group. For each biological replicate, cells were sorted from 3-4 mice. **(D)** Validation of *Foxp3* and *Il4* expression by real-time PCR. **(E)** Graph depicting the ingenuity pathways analysis (IPA) of the *Il4* signalling pathway predicting the role for IL-4 signalling upstream of the transcriptional profile of nT→*Il4*-GFP$^+$, HpTR→*Il4*-GFP$^+$ and HpTR cells. **(F)** Table listing the top 20 genes predicted to be upstream of the transcriptional profile of HpTR→*Il4*-GFP$^+$ cells as determined by IPA. **(G)** Heatmap depicting significantly expressed mRNA transcripts ($p<0.05$) in FACS-purified HpTR, HpTR→*Il4*-GFP$^+$, HpTR→DN and HpTR→*Foxp3*-RFP$^+$ cells expressed as fold change relative to nT cells (> 2.0). Each heatmap lane represents the 3 biological replicates. Data is representative of 2 experiments with 3-4 biological replicates per group.

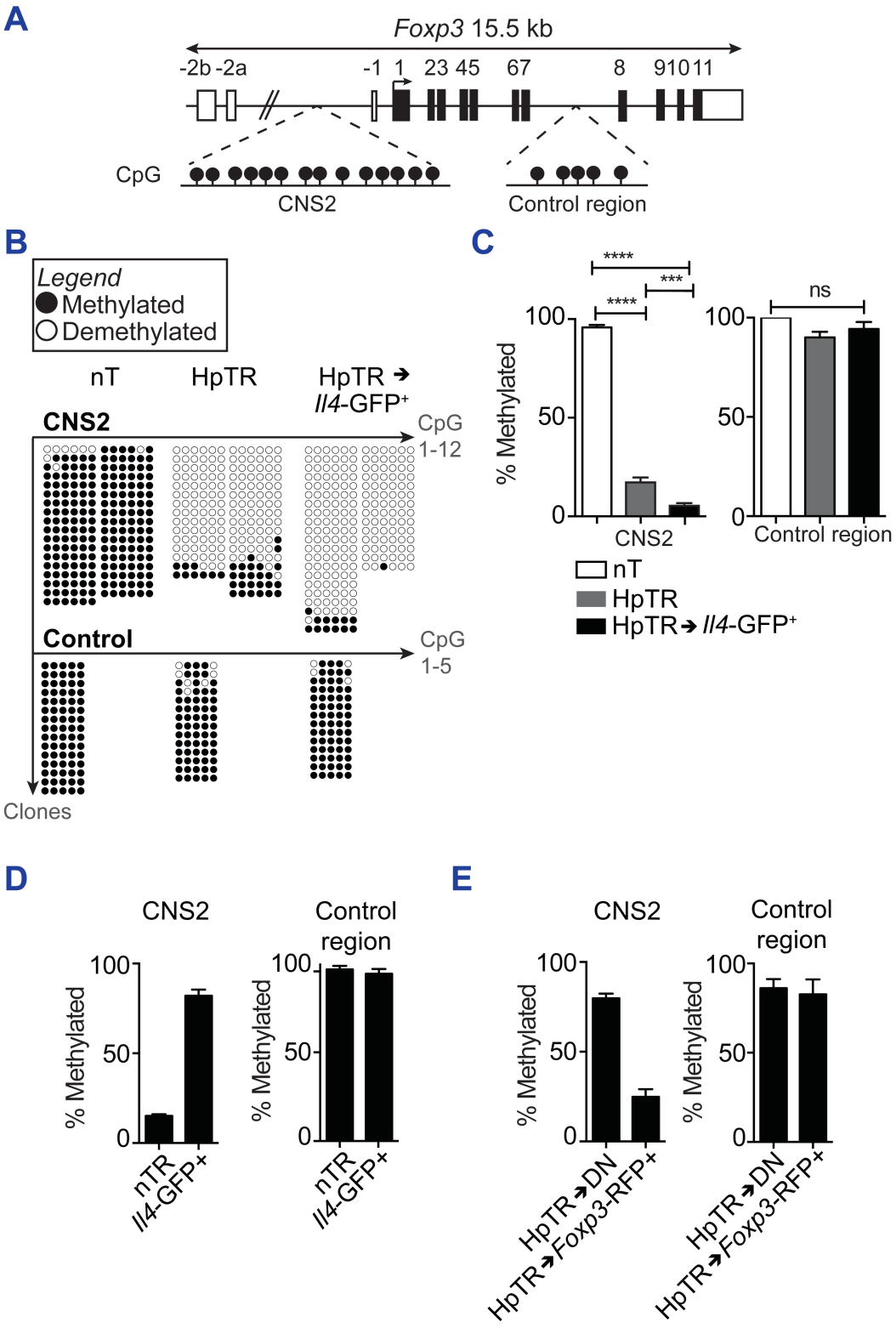

A
Foxp3 15.5 kb
-2b -2a
-1 1 23 45 67 8 91011
CpG
CNS2
Control region
B
Legend
Methylated
Demethylated
nT
HpTR
HpTR → Il4-GFP+
CNS2
CpG 1-12
Control
CpG 1-5
Clones
C
****
***
****
ns
100
50
0
% Methylated
CNS2
Control region
nT
HpTR
HpTR → Il4-GFP+
D
CNS2
Control region
% Methylated
nTR Il4-GFP+
E
CNS2
Control region
% Methylated
HpTR→DN HpTR→Foxp3-RFP+

Figure 4.9. HpTR→*Il4*-GFP$^+$ cells maintain a demethylated *Foxp3* locus. (A) Schematic representation of the *Foxp3* locus depicting 12 CpG islands within the conserved non-coding sequence 2 (CNS2) of intron 1 and 5 CpG islands within intron 7. Bisulphite modification of DNA was performed followed by the amplification of the CNS2 and control region of the *Foxp3* locus. Amplified products were cloned and sequenced to determine their methylation status. **(B)** Diagram generated using QUMA (Kumaki et al., 2008) representing methylated (full circles) and demethylated (empty circles) CpG sites on the amplified CNS2 (1-12) and control region (1-5) of nT, HpTR and HpTR→*Il4*-GFP$^+$ replicate clones (14-21 clones per sample). **(C)** Graphical representation of the frequency of methylated CpG islands in the CNS2 and control region of nT(white bars), HpTR (grey bars) and HpTR→*Il4*-GFP$^+$ (black bars) cells. Data is pooled from 1-2 independent experiments. Cells were pooled from 5 recipient mice for experiments. **(D)** Graphical representation of the frequency of methylated CpG islands in the CNS2 and control region of nTR and *Il4*-GFP$^+$ cells. Data is pooled from 1-2 independent experiments. Cells were pooled from 5 recipient mice for experiments. **(E)** Graphical representation of the frequency of methylated CpG islands in the CNS2 and control region of HpTR→DN and HpTR→*Foxp3*-RFP$^+$ (black bars) cells. Data is pooled from 1-2 independent experiments. Cells were pooled from 5 recipient mice for experiments. All experiments were done in collaboration with Manolis Gialitakis.

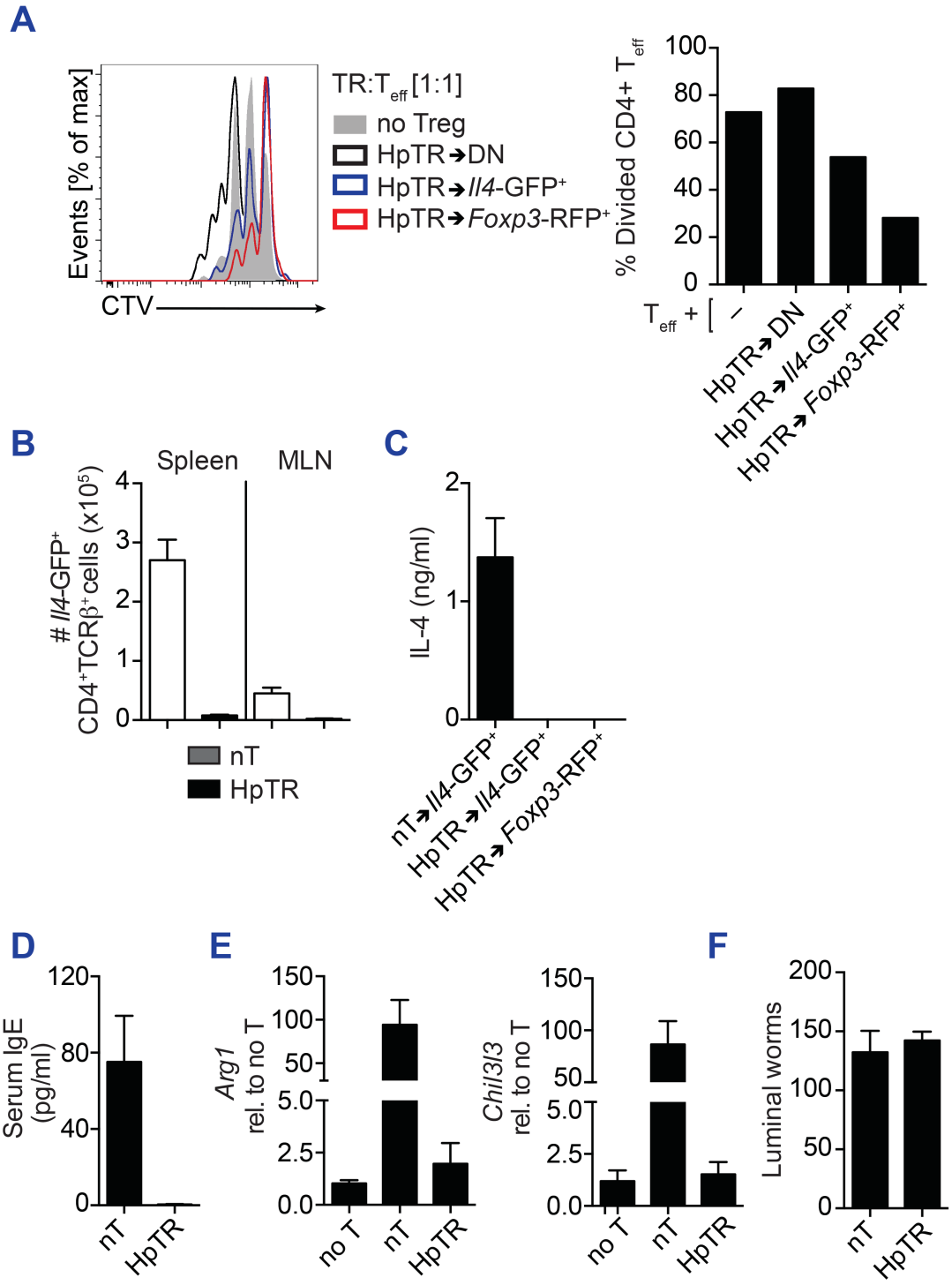

A
Events [% of max]
CTV
TR:T$_{eff}$ [1:1]
no Treg
HpTR➔DN
HpTR➔*Il4*-GFP$^+$
HpTR➔*Foxp3*-RFP$^+$
% Divided CD4+ T$_{eff}$
100
80
60
40
20
0
T$_{eff}$ + [–
HpTR➔DN
HpTR➔*Il4*-GFP$^+$
HpTR➔*Foxp3*-RFP$^+$
B
Spleen
MLN
Il4-GFP$^+$ CD4$^+$TCRβ$^+$ cells (x10^5)
4
3
2
1
0
nT
HpTR
C
IL-4 (ng/ml)
2
1
0
nT➔*Il4*-GFP$^+$
HpTR➔*Il4*-GFP$^+$
HpTR➔*Foxp3*-RFP$^+$
D
Serum IgE (pg/ml)
120
80
40
0
nT
HpTR
E
Arg1 rel. to no T
150
100
50
5.0
2.5
0.0
no T
nT
HpTR
Chil3/3 rel. to no T
150
100
50
5.0
2.5
0.0
no T
nT
HpTR
F
Luminal worms
200
150
100
50
0
nT
HpTR

Figure 4.10. The adoptive transfer model is insufficient to test effector function of HpTR→*Il4*-GFP$^+$ cells. Treg suppression assay setup: nT (T_{eff}) cells were isolated from naïve C57BL/6 mice. The adoptive transfer of HpTR cells was performed as described previously (Fig. 4.4 B). HpTR→DN, HpTR→*Il4*-GFP$^+$ and HpTR→*Foxp3*-RFP$^+$ cells were FACS-purified 42 days post-transfer and cultured at a 1:1 ratio with T_{eff} cells for 2.5 days. Proliferating T_{eff} cells were measured by cell tracker violet (CTV) staining. **(A)** Representative FACS histogram plot depicting CTV dilutions in the cultured cells and graphical representation of CTV dilutions. Data is representative of 1 experiment. Cells were pooled from 4 mice. **(B)** Absolute cell number of *Il4*-GFP$^+$ cells in the spleen and MLN of nT (grey bars) or HpTR (black bars) recipients day 42 post-transfer. Data are representative of at least 3 independent experiments. **(C)** nT→*Il4*-GFP$^+$, HpTR→*Il4*-GFP$^+$ and HpTR→*Foxp3*-RFP$^+$ cells were FACS-purified from nT or HpTR recipients day 42 post-transfer. Purified cells were re-stimulated with P+I *in vitro* for 24h. IL-4 protein production as assessed by Flow Cytomix. Data are representative of 3 independent experiments. Cells were pooled from 3-4 mice. **(D)** Total IgE levels in the serum of nT or HpTR recipients day 42 post-transfer. **(E)** *Arg1* and *Chil3l3* expression in the small intestine of nT or HpTR recipients day 42 post-transfer expressed as fold change relative to *Tcra*$^{-/-}$ mice having received no T cells. **(F)** Intestinal worm count in nT or HpTR recipients day 42 post-transfer (day 14 post-infection, see model (Fig. 4.4 B).

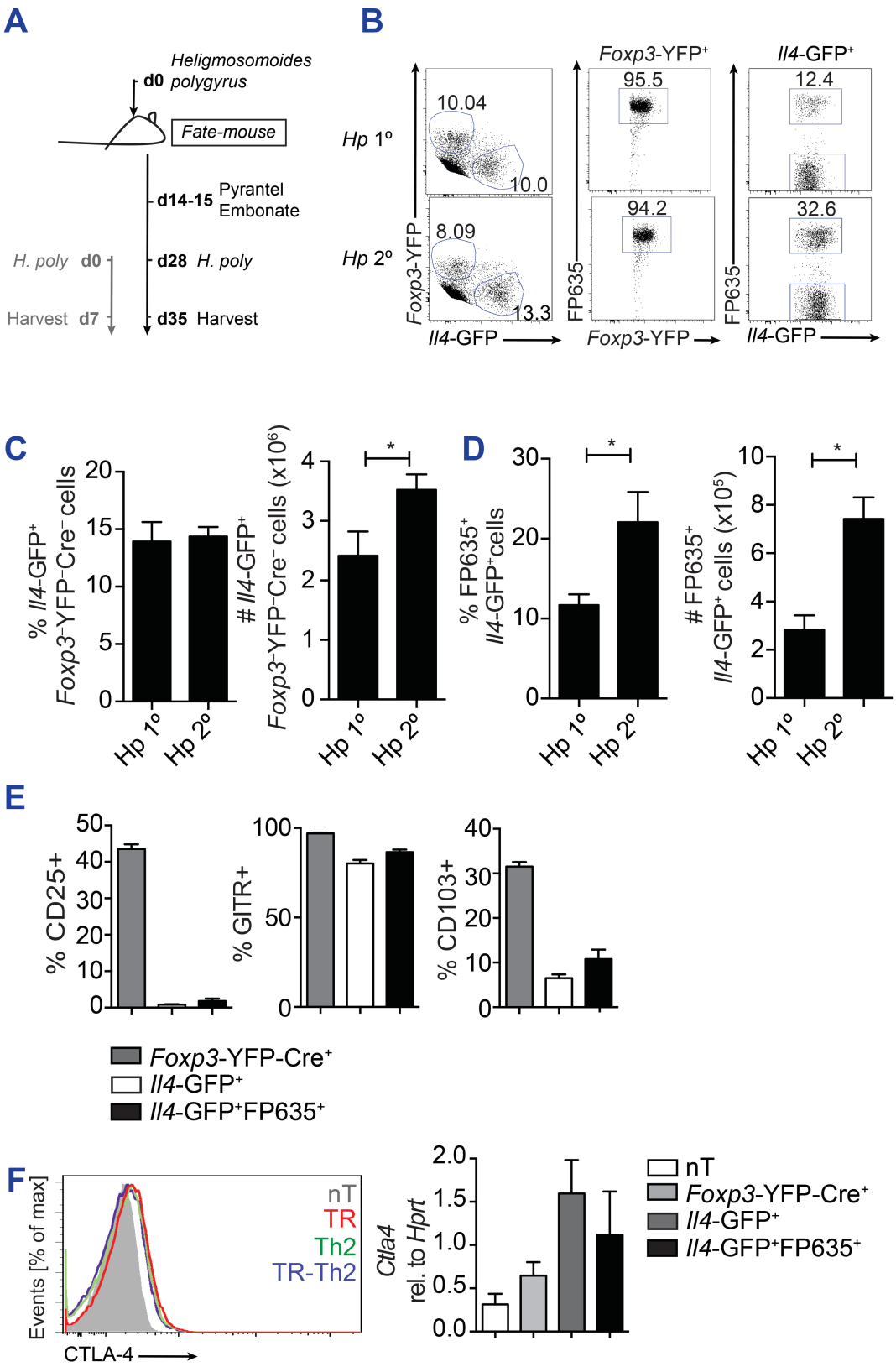

A
Heligmosomoides polygyrus
d0
Fate-mouse
d14-15 Pyrantel Embonate
H. poly d0
d28 H. poly
Harvest d7
d35 Harvest
B
Foxp3-YFP+
Il4-GFP+
Hp 1°
Hp 2°
10.04
10.0
8.09
13.3
95.5
94.2
12.4
32.6
Foxp3-YFP
Il4-GFP
FP635
C
% Il4-GFP+ Foxp3-YFP-Cre- cells
# Il4-GFP+ Foxp3-YFP-Cre- cells (x10^6)
Hp 1°
Hp 2°
D
% FP635+ Il4-GFP+cells
# FP635+ Il4-GFP+ cells (x10^5)
E
% CD25+
% GITR+
% CD103+
Foxp3-YFP-Cre+
Il4-GFP+
Il4-GFP+FP635+
F
Events [% of max]
CTLA-4
nT
TR
Th2
TR-Th2
Ctla4 rel. to Hprt

Figure 4.11. Ex-Foxp3 *Il4*-GFP^{+}FP635^{+} cells develop *in vivo* and expand following secondary infection with *H. polygyrus*. (A) Schematic representation of the experimental setup. *Foxp3*$^{YFP\text{-}Cre}$*Il4*^{GFP}*R26R*^{FP635} fate-reporter mice were infected with 200 *H. polygyrus* larvae. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. Naïve *Foxp3*$^{YFP\text{-}Cre}$*Il4*^{GFP}*R26R*^{FP635} mice were given a primary (Hp 1°) infection at the same time-point. All groups were harvested 7 days post-infection for FACS analysis. **(B)** Representative FACS dot plots showing expression of *Il4*-GFP against *Foxp3*-YFP-Cre (within the CD4^{+}TCRβ^{+} population) and expression of *Il4*-GFP against FP635 in the spleen of Hp 1° and Hp 2° mice. **(C)** Graphical representation of the frequency and absolute number of *Foxp3*-YFP-Cre^{-}*Il4*-GFP^{+} cells in the spleen of Hp 1° and Hp 2° mice. (**D)** Graphical representation of the frequency and absolute number of *Foxp3*-YFP-Cre^{-}*Il4*-GFP^{+}FP635^{+} cells in the spleen of Hp 1° and Hp 2° mice. Data are pooled from 2 independent experiments with 3-4 mice per group. **(E)** Graphical representation of the frequency of CD25-, GITR- and CD103- expressing cells within the *Foxp3*-YFP-Cre^{+} (grey bars), *Il4*-GFP^{+} (white bars) and *Il4*-GFP^{+}FP635^{+} (black bars) populations. Data is representative of 1 experiment with 4 mice per group. **(F)** Representative FACS histogram showing CTLA-4 surface expression on FACS purified populations and CTLA-4 mRNA expression in FACS-purified *Foxp3*-YFP-Cre^{+} (light grey bar), *Il4*-GFP^{+} (dark grey bar) and *Il4*-GFP^{+}FP635^{+} (black bars) populations. Data are representative of 1-2 independent experiments with 3-4 mice per group.

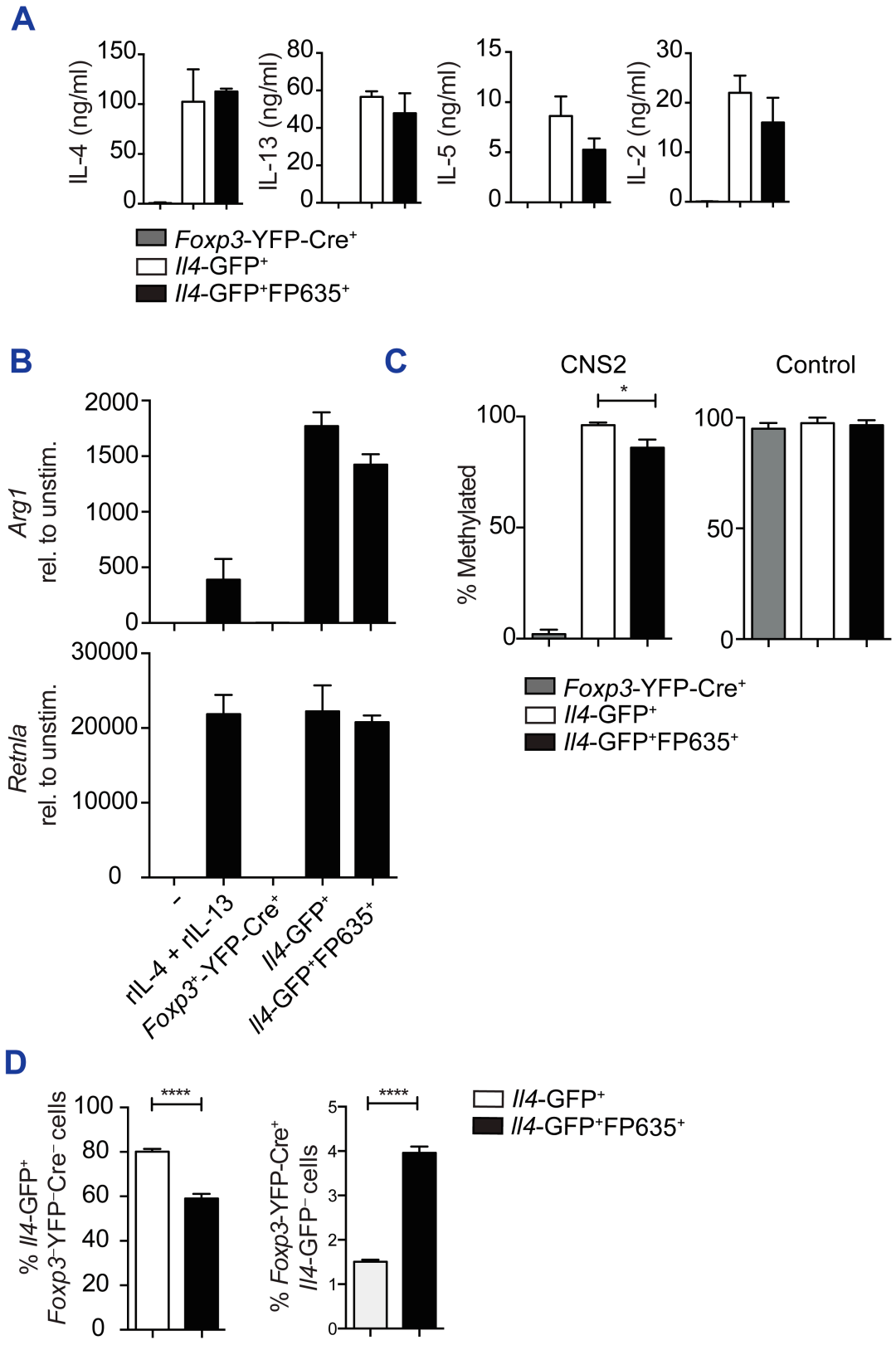

A
IL-4 (ng/ml)
IL-13 (ng/ml)
IL-5 (ng/ml)
IL-2 (ng/ml)
Foxp3-YFP-Cre+
Il4-GFP+
Il4-GFP+FP635+
B
Arg1 rel. to unstim.
Retnla rel. to unstim.
-
rIL-4 + rIL-13
Foxp3+-YFP-Cre+
Il4-GFP+
Il4-GFP+FP635+
C
CNS2
Control
% Methylated
*
Foxp3-YFP-Cre+
Il4-GFP+
Il4-GFP+FP635+
D
% Il4-GFP+ Foxp3-YFP-Cre- cells
% Foxp3-YFP-Cre+ Il4-GFP- cells
****
****
Il4-GFP+
Il4-GFP+FP635+

Figure 4.12. Ex-Foxp3 *Il4*-GFP^{+}FP635^{+} cells are functional Th2 effector cells and maintain molecular elements of their Treg past. *Foxp3*$^{YFP\text{-}Cre}$*Il4*^{GFP}*R26R*^{FP635} fate-reporter mice were infected with 200 *H. polygyrus* larvae. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. Naïve *Foxp3*$^{YFP\text{-}Cre}$*Il4*^{GFP}*R26R*^{FP635} mice were given a primary (Hp 1°) infection at the same time-point. *Foxp3*-YFP-Cre^{+} (grey bars), *Il4*-GFP^{+} (white bars) and *Il4*-GFP^{+}FP635^{+} (black bars) cells were FACS-purified from Hp 2° fate-reporter mice and re-stimulated with P+I *in vitro* for 24 hours. **(A)** IL-4 protein in the cell-culture supernatants as assessed by Flow Cytomix. **(B)** FACS-purified *Foxp3*-YFP-Cre^{+}, *Il4*-GFP^{+} and *Il4*-GFP^{+}FP635^{+} cells were cultured with bone marrow derived macrophages (BMDMs) in the presence of TCR stimulation for 24 hours. BMDMs were cultured in media or with recombinant IL-4 and IL-13 as a control. *Arg1* expression was measured in co-cultured BMDMs. Data are representative of 2 independent experiments. T cells were sorted from 3-4 mice and pooled for *in vitro* assays. **(C)** Graphical representation of the frequency of methylated CpG islands in the CNS2 and control region of *Foxp3*-YFP-Cre^{+} (grey bars), *Il4*-GFP^{+} (white bars) and *Il4*-GFP^{+}FP635^{+} (black bars) cells FACS-purified from Hp 2° fate-reporter mice. Data is pooled from 2 independent experiments. Cells were pooled from 4 mice per experiment. Experiments were done in collaboration with Manolis Gialitakis. **(D)** Graphical representation of the frequency of *Il4*-GFP and *Foxp3*-YFP-Cre expression following *in vitro* culture of FACS-purified *Il4*-GFP^{+} (white bars) and *Il4*-GFP^{+}FP635^{+} (black bars) cells in Treg polarising conditions (IL-2 and TGF-β) for 7 days.

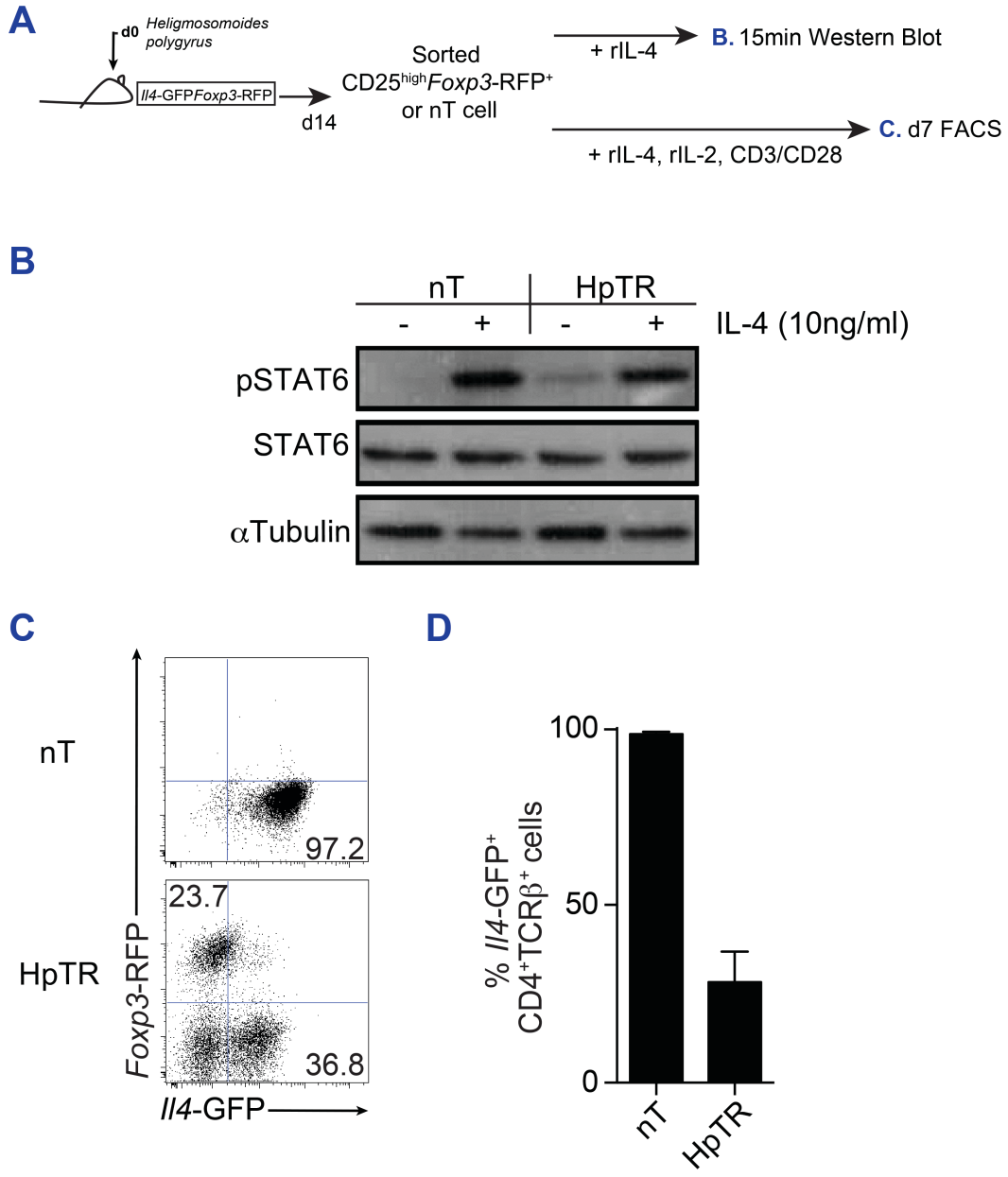


Figure 4.13. IL-4 signals through STAT6 in CD25^{high}*Foxp3*-RFP^{+} cells and promotes their conversion to *Il4*-GFP^{+} cells *in vitro*. **(A)** Schematic representation of the experimental setup. *Il4*-GFP*Foxp3*-RFP mice were infected with 200 *H. polygyrus* larvae. CD4^{+}TCRβ^{+}CD25^{high}*Foxp3*-RFP^{+} (HpTR) cells were FACS-purified at day 14 post-infection. CD4^{+}TCRβ^{+}CD25^{-}CD44^{low} (nT) cells were FACS-purified from naïve reporter mice. Purified nT and HpTR cells were stimulated with recombinant IL-4 at 37°C for 15min or with media as a control followed by protein extraction. **(B)** Western blot representing pSTAT-6, STAT6 and alpha-tubulin protein levels in cultured cells. Data is representative of 3 independent experiments. Cells were pooled from 3-4 mice. Purified nT and HpTR cells were cultured in Th2 polarising conditions (IL-4, IL-2 and anti-IFN-γ) for 7 days. **(C)** Representative FACS dot plots showing the expression of *Il4*-GFP and *Foxp3*-RFP in nT and HpTR cultures following polarisation. **(D)** Graphical representation of the frequency of *Il4*-GFP^{+} cells in nT and HpTR cultures following polarisation. Data are pooled from 3 independent experiments. Cells were sorted from 1-4 mice.

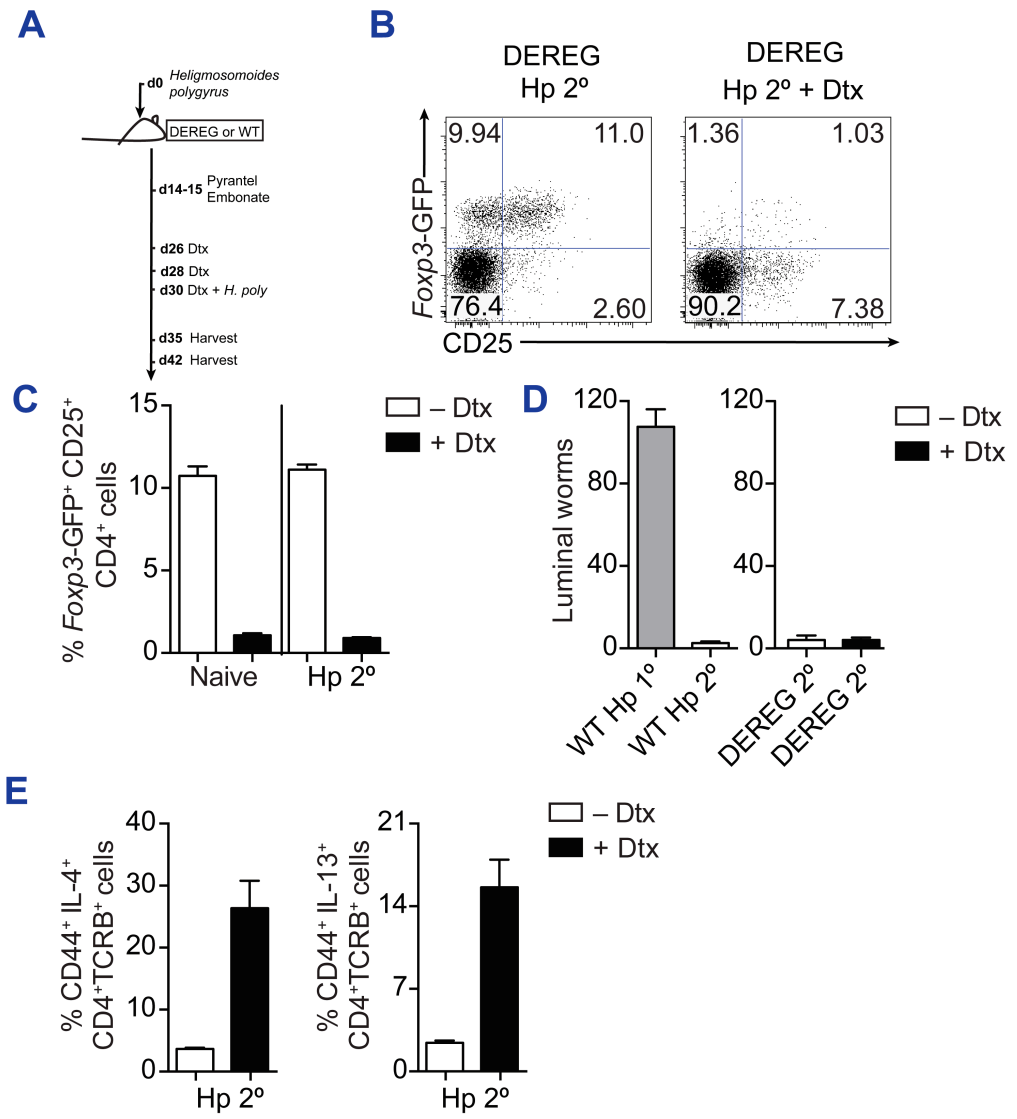


Figure 4.14. Foxp3$^+$ cells are dispensable for the development of Th2 responses and functional immunity to *H. polygyrus*. (A) Schematic representation of the experimental setup. "Depletion of regulatory T cell" (DEREG) mice were infected with 200 *H. polygyrus* larvae. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. One group of mice was treated with diphtheria toxin (Dtx; black bars) at days 26, 28 and 30 (Hp 2°+Dtx). All groups were harvested 7 days post-infection for FACS analysis and 14 days post-infection for worm counts. **(B)** Representative FACS plots showing the expression of *Foxp3*-GFP and CD25 in the MLN of Hp 2° and Hp 2°+ Dtx mice 7 days post-infection. **(C)** Graphical representation of the frequency of *Foxp3*-GFP$^+$CD25$^+$ cells in the MLN of naïve and Hp 2° DEREG mice treated with Dtx (black bars) or with PBS (white bars) 7 days post-infection. **(D)** Intestinal worm count in WT Hp 1° and Hp 2° mice and in Dtx-treated (black bars) and PBS-treated (white bars) Hp 2° DEREG mice day 14 post-infection. **(E)** Graphical representation of the frequency of CD44$^+$IL-4$^+$ and CD44$^+$IL-13$^+$ (within the CD4$^+$TCRβ$^+$ population) in the MLN of naïve and Hp 2° DEREG mice treated with Dtx (black bars) or with PBS (white bars) 7 days post-infection. Data is representative of 1 experiment with 5 mice per group.

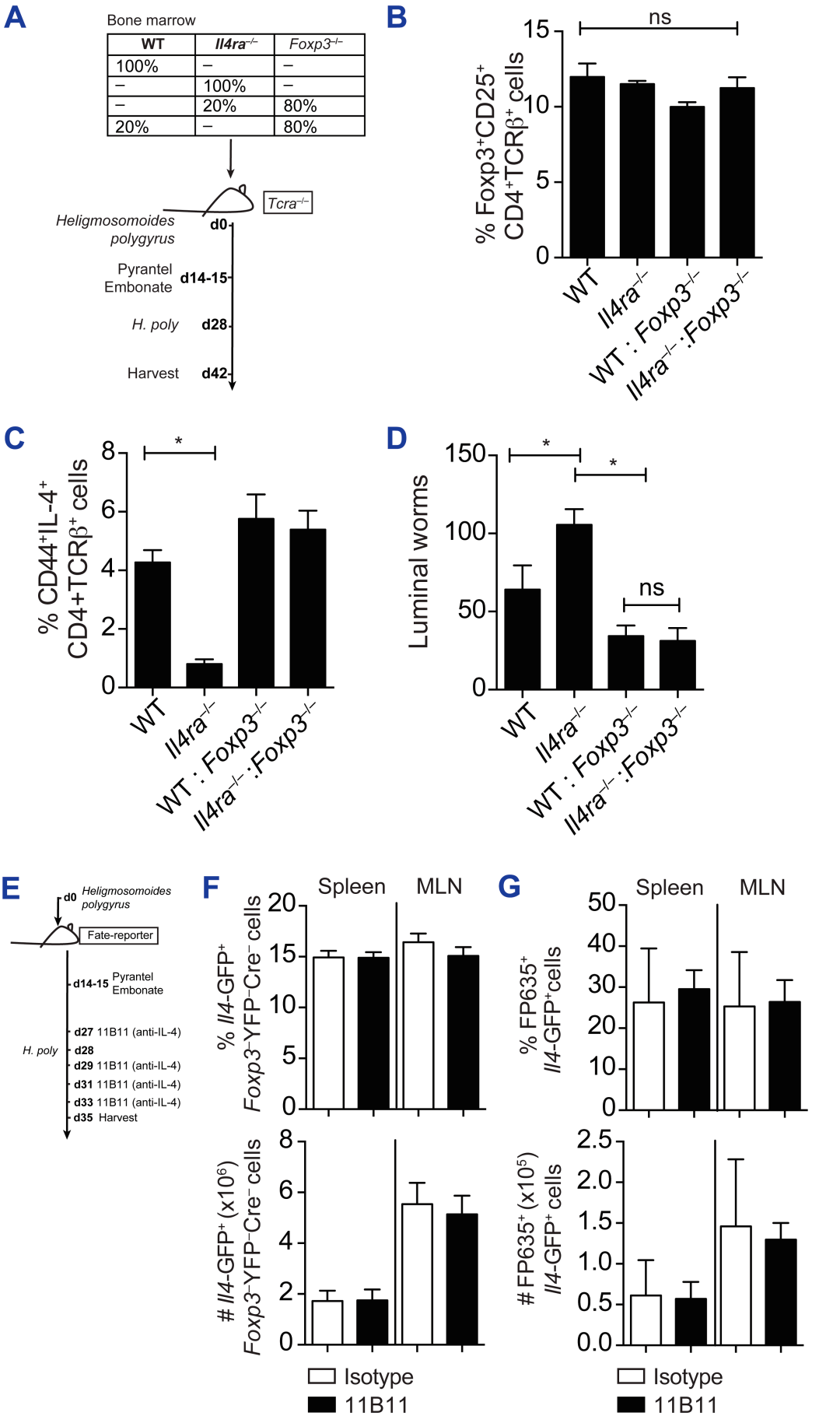

A
Bone marrow
WT
Il4ra−/−
Foxp3−/−
100%
–
–
–
100%
–
–
20%
80%
20%
–
80%
Tcra−/−
Heligmosomoides polygyrus
d0
Pyrantel Embonate
d14-15
H. poly
d28
Harvest
d42
B
% Foxp3+CD25+ CD4+TCRβ+ cells
ns
WT
Il4ra−/−
WT : Foxp3−/−
Il4ra−/− :Foxp3−/−
C
% CD44+IL-4+ CD4+TCRβ+ cells
*
D
Luminal worms
*
*
ns
E
d0 Heligmosomoides polygyrus
Fate-reporter
d14-15 Pyrantel Embonate
H. poly
d27 11B11 (anti-IL-4)
d28
d29 11B11 (anti-IL-4)
d31 11B11 (anti-IL-4)
d33 11B11 (anti-IL-4)
d35 Harvest
F
Spleen
MLN
% Il4-GFP+ Foxp3−YFP−Cre− cells
# Il4-GFP+ (x10^6) Foxp3−YFP−Cre− cells
Isotype
11B11
G
Spleen
MLN
% FP635+ Il4-GFP+cells
# FP635+ (x10^5) Il4-GFP+ cells
Isotype
11B11

Figure 4.15. IL-4 is required for development but not re-activation of Th2 cells. (A) Schematic representation of the experimental setup. *Tcra*$^{-/-}$ mice were sub-lethally irradiated and reconstituted with T-depleted WT, *Il4ra*$^{-/-}$ or a mix of WT(20%):*Foxp3*$^{-/-}$(80%) or *Il4ra*$^{-/-}$ (20%):*Foxp3*$^{-/-}$(80%). Chimeric mice were infected with 200 *H. polygyrus* larvae 7 weeks after reconstitution. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. All groups were harvested 14 days post-infection for FACS analysis and worm counts. **(B)** Graphical representation of the frequency of Foxp3^{+}CD25^{+} cells in the MLN of chimeric mice 14 days post-infection. **(C)** Graphical representation of the frequency of CD44^{+}IL-4^{+} cells in the spleen 14 days post-infection. **(D)** Intestinal worm counts 14 days post-infection. Data is representative of 1 experiment with 3-5 mice per group. **(E)** Graphical representation of experimental model. *Foxp3*$^{YFP\text{-}Cre}$*Il4*^{GFP}*R26R*^{FP635} fate-reporter mice were infected with 200 *H. polygyrus* larvae. Mice were drug-cured with the anthelminthic Pyrantel Embonate (PE) at days 14 and 15 post-infection and given a secondary infection (Hp 2°) at day 28. Naïve *Foxp3*$^{YFP\text{-}Cre}$*Il4*^{GFP}*R26R*^{FP635} mice were given a primary (Hp 1°) infection at the same time-point. Mice were treated with 11B11 (anti-IL-4 antibody) or isotype control on days 1, 3 and 5 of the secondary challenge. **(F)** Graphical representation of the frequency and absolute number of *Foxp3*-YFP-Cre^{-}*Il4*-GFP^{+} cells in the spleen and MLN of isotype (white bars) or antibody-treated (black bars) mice. Data is representative of 1 independent experiment with 4 mice per group. **(G)** Graphical representation of the frequency and absolute number of *Foxp3*-YFP-Cre^{-}*Il4*-GFP^{+}FP635^{+} cells in the spleen and MLN of isotype (white bars) or antibody-treated (black bars) mice. Data is representative of 1 independent experiment with 4 mice per group.

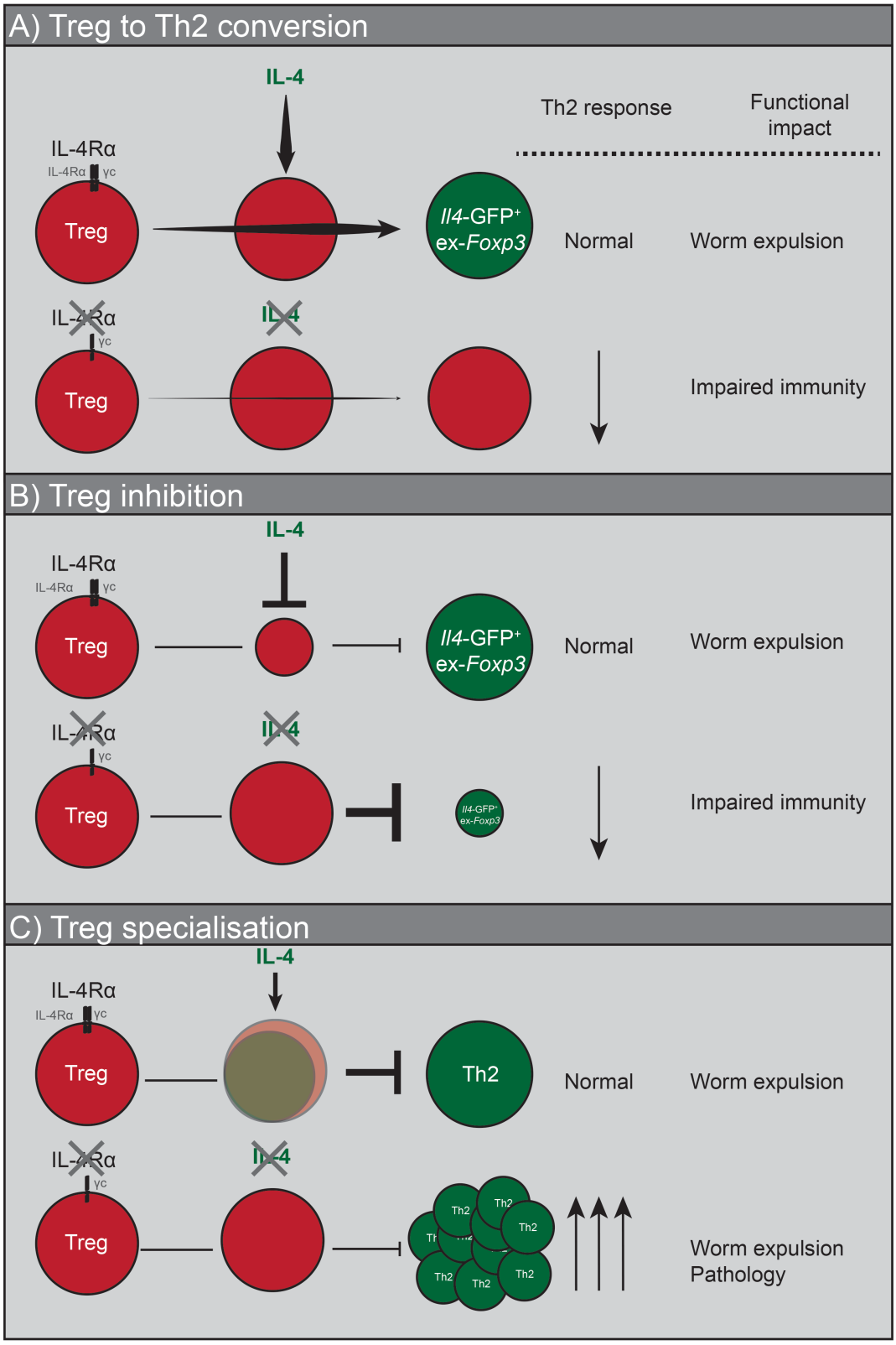


Figure 4.16. Illustration of the impact of IL-4 signalling on Treg cells during a secondary infection with *H. polygyrus*. (A) IL-4 signalling may promote Treg to Th2 conversion. **(B)** IL-4 signalling may be required to limit the development of Treg cells. **(C)** IL-4 signalling may be required for Treg suppression of Th2 cells.

Chapter 5: General discussion

Intestinal helminth infections modulate host immune responses and promote an immunoregulatory environment, primarily limiting anti-helminth immune mechanisms, and subsequently preventing immune-mediated pathology. This modified immune response results in the long-term survival of the parasite and chronic infection. In humans, the chronicity of intestinal helminth infections has a significant impact on the health, development and morbidity of the host. Using laboratory models of infection, it has been shown that favouring the development of type-2 responses correlates with the functional expulsion of the parasite, either in genetically resistant hosts or following therapeutic intervention. It is within this context that we have identified a new cellular source and target of IL-4 that contributes to the development of Th2 cells, type-2 immunity and anti-helminth immune mechanisms.

5.1. Immunoregulation of type-2 responses by intestinal helminths

Both ILC2s and their Th2 counterparts play important roles in anti-helminth immunity by secreting canonical cytokines required for the activation of downstream effector responses, ultimately resulting in the expulsion of the parasite. Given their functional similarities, understanding the relative contribution of ILC2s and their Th2 cell counterparts to protective immune responses, as well as the interplay between the two cells has become an increasingly important objective in immunology. Although ILC2s are sufficient to drive anti-helminth immunity in a limited number of helminth infections, it is becoming clear that ILC2s also provide important differentiation cues for Th2 cells (Oliphant et al., 2014). However, the precise mechanisms of how ILC2s relay cues to the adaptive immune compartment remains unclear. Importantly, the role of ILC2s during anti-helminth immunity has mostly been studied in the context of infections with the lab-adapted helminth *N. brasiliensis,* and it remains unclear whether ILC2s play a role during an infection with other helminth parasites that reside in different tissues and require different immune mechanisms. Previous studies have

shown ILC2s expand following infection with the intestinal helminth *H. polygyrus* (Mackley et al., 2015) in parallel with the development of Th2 cells (Filbey et al., 2014), however the contribution of ILC2s to the initiation of type-2 responses was not addressed. In chapter 3, we identified that ILC2s promote the differentiation of Th2 cells *in vitro* and are important for the development of Th2 cells *in vivo* following *H. polygyrus* infection. The magnitude of Th2 responses correlates with natural immunity to *H. polygyrus* (Filbey et al., 2014) suggesting that strategies designed to amplify ILC2s could indirectly promote Th2 responses and enhance immunity to infection. In the context of infections with complex intestinal helminths, immune responses need to be tightly regulated to limit excessive damage caused by inflammation. Many studies have characterised the ability of intestinal helminths to promote a regulatory environment for their survival and chronic establishment (McSorley et al., 2013). In particular, the establishment of *H. polygyrus* infection correlates with the early parasite-driven (Grainger et al., 2010) expansion of $Foxp3^+$ Treg cells (Finney et al., 2007; Rausch et al., 2008), and negatively correlates with the development of functional Th2 responses (Rausch et al., 2009).

Although Treg cells have been shown to directly (Huber et al., 2011) and indirectly (Fiorentino et al., 1991) suppress T helper cells, it remains unclear whether they play a role in regulating ILC2s. However recent transcriptional profiling of intestinal $KLRG1^+$ ILC2s and $CD127^+NKP46^+$ ILC1s found that they expressed significantly higher levels of *Il10ra* than other ILC subsets (Robinette et al., 2015) suggesting they could be directly regulated by Treg-derived IL-10 as has been previously shown for the control of T helper cells (Huber et al., 2011). An indirect role for *H. polygyrus* suppression of ILC2s is emerging instead. *H. polygyrus* ES was shown to negatively regulate IL-33R expression in mouse epithelial cell lines (Buck et al., 2014) and IL-33 release from the lung epithelium, leading to reduced ILC2 numbers (McSorley et al., 2014). Similarly, the secretion of IL-1β by *H. polygyrus*-elicited innate cells was shown to inhibit IL-33 and IL-25 release from the intestinal epithelium and limit ILC2 expansion (Zaiss et al., 2013). In a separate model, the secretion of the fatty acid maresin-1

(MaR1) in allergic lungs was shown to directly inhibit ILC2 cytokine production and promote resolution of lung inflammation (Krishnamoorthy et al., 2015), suggesting that other tissue-derived factors can limit ILC2 effector function. In addition, ILC2s have been described to express high levels of KLRG1, an inhibitory c-type lectin shown to negatively regulate *Gata3* expression and IL-5 and IL-13 secretion by human dermal ILC2s (Salimi et al., 2013). Together, these results provide evidence of potential regulatory pathways for the control of ILC2s, which suggests that *H. polygyrus* and other intestinal helminths may have evolved to subvert immune responses by limiting the expansion of Th2 cells as well as ILC2s. Indeed, it was recently reported that children infected with helminths have reduced proportions of ILC2s, which were restored following curative anthelminthic treatment (Nausch et al., 2015).

5.2. Enhancing type-2 immune responses by stimulating ILC2s and Tregs

Treg cells have recently been shown to lose their regulatory phenotype and contribute to Th responses in the context of inflammation, however whether Treg conversion contributes to protective immune responses in the context of an infection was unclear. In chapter 4, we tested whether Treg cells could convert to Th2 cells and play a protective role during memory Th2 responses. We found that Treg cells could convert to IL-4 expressing cells *in vitro* in response to IL-4 signalling and *in vivo* following adoptive transfer and identified that a significant proportion of functional Th2 cells originate from a *Foxp3*-expressing past *in vivo.* We are currently testing the requirement for IL-4 in promoting Treg to Th2 conversion *in vivo*. Based on preliminary results, IL-4 may be redundant for the re-activation of memory Th2 cells during a secondary infection with *H. polygyrus.* Instead, an early source of IL-4 may be required to promote the conversion of Treg to Th2 cells during a primary infection. Basophils, eosinophils, mast cells and $CCR2^{+}$ monocytes are redundant for the development of Th2 cells following a primary *H. polygyrus* infection (Smith et al., 2012) or vaccine-mediated immunity (Hewitson et al., 2015). In light of and our data showing ILC2-

derived IL-4 can promote Th2 differentiation, it is conceivable that ILC2s could be providing an important source of IL-4 in the tissue and promoting the conversion of Treg to Th2 cells during a primary infection.

Alternatively, ILCs may be important antigen presenting cells. Indeed, MHC Class II expression on ILC3s (Hepworth et al., 2013; Hepworth et al., 2015) and ILC2s (Neill et al., 2010) has been shown to be important for immune tolerance, T cell proliferation *in vitro* (Mirchandani et al., 2014) and functional expulsion of *N. brasiliensis* (Oliphant et al., 2014). Furthermore, previous studies found that ILC2s promote Th2 differentiation and proliferation in an MHC-dependent manner (Mirchandani et al., 2014; Oliphant et al., 2014). Finally, both ILC3s and ILC2s have the potential to migrate to the appropriate site for Th2 cell reactivation, as they were found to migrate to the MLN following primary *H. polygyrus* infection (Mackley et al., 2015). Taken together these studies suggest ILC2s could be providing antigen as well as IL-4 to support Treg to Th2 conversion (**Fig. 5.1**).

Current experimental strategies to modulate the ratio of Th2 and Treg cell either amplify or inhibit Treg and Th2 cells using cytokines and antibodies (Klatzmann and Abbas, 2015). In some settings, shifting the ratio of Treg to Th2 cells was shown to limit pathogenic Th2 responses. For example, *in vivo* expansion of Treg cells following the administration of IL-2c (Wilson et al., 2008) or the stimulation of the TNF-superfamily receptor DR3 (Schreiber et al., 2010) were shown to reduce allergic inflammation by reversing the ratio of Th2 to Treg cells. Conversely, in the context of helminth infections, therapeutic strategies aim to modify to promote the differentiation of Th2 cells for improved immunity to helminths. For example, depletion of Treg cells during a chronic infection with *H. polygyrus* (Rausch et al., 2009) and other intestinal helminths (Taylor et al., 2005) was shown to promote Th2 responses. Furthermore, enhanced Th2 responses following IL-4c treatment led to the expulsion of *H. polygyrus* (Urban et al., 1995; Herbert et al., 2009), however the targets of IL-4 following IL-4c treatment were unclear. Indeed, both $CD4^+$ cells (Kopf et al., 1993) and ILC2s (Motomura et al., 2014) are activated in response to IL-4 signalling. Thus, treatment

with recombinant IL-4 may directly, or indirectly (via ILC2s) stimulate the conversion of Treg to Th2 cells as well as *de novo* Th2 differentiation. By simultaneously increasing Th2 differentiation, originating from two independent sources, and concomitantly reducing the number of Treg cells, low-dose IL-4 treatment may be sufficient to amplify Th2 responses without mediating excessive pathology. Given the ability of an anti-helminth Th2 response to develop in the context of a strong regulatory environment, increasing numbers of studies are looking to identify helminth-associated molecular patterns that could be triggering the differentiation of protective immune responses (Loukas et al., 2011; Hotez et al., 2013) rather than the differentiation of Treg cells (Grainger et al., 2010). Indeed, preliminary data suggests that vaccination with helminth-derived excretory/secretory molecules, and in particular secreted venom-allergen-like proteins (VALS 1, 2 and 3), can stimulate potent immunity to *H. polygyrus* (Hewitson et al., 2015) suggesting that specific helminth antigens can drive protective immune responses. Furthermore, both IL-25R (IL-17RA) and IL-4Rα were required to promote sterile immunity to *H. polygyrus* after vaccination with HES (Hewitson et al., 2015). Hence, the authors suggested that IL-25-responsive cells, such as ILC2s, are required to drive Th2 differentiation and subsequent activation of antibody class-switching in B cells, culminating in sterile immunity (Hewitson et al., 2015). Therefore, enhancing early type-2 immune responses to *H. polygyrus* through the activation of ILC2s may be particularly relevant in the context of vaccine development.

In summary, we have identified new cellular targets for enhancing natural anti-helminth immune responses. Thus, we propose that strategies to promote Th2 differentiation could concomitantly expand ILC2s and promote Treg to Th2 conversion, shifting the balance of Treg and Th2 responses and ultimately improving anti-helminth immunity.

5.3. Evidence for Treg to Th2 conversion in natural immunity

An important continuation of this study will be to define unique characteristics of ex-Foxp3 Th2 cells in order to monitor their development in different models. For example, we could use a transcriptional approach to identify distinct surface receptor expression patterns combined with epigenetic analysis for the identification of markers of their Treg past. The significance of identifying markers unique to ex-Foxp3 Th2s would be two-fold. Firstly, they may provide mechanistic hints for the specific amplification or inhibition of the cells. In addition, they may serve as biomarkers for the identification of ex-Foxp3 Th2 cells *in vivo* in human patients.

Throughout this study we have infected mice with a single dose of 200 L3 larvae widely used in different laboratories (Reynolds et al., 2012), which may not represent infection rates in the wild. Furthermore, we studied functional immune responses in mice that developed immunity to re-infection following anthelminthic treatment (Urban et al., 1991). Although global deworming programs do exist, the global coverage of anthelminthic treatment for school children is estimated to be between 30-40%, and a high number of children are predicted to be chronically and repeatedly infected (WHO, 2015). Thus, both the dose and regimen of our infection model may not accurately model human infection. Instead, it would be interesting to study the role of ILC2s and Treg to Th2 conversion in the context of protective memory responses that develop following repeat trickle infections to *H. polygyrus* and to the human hookworm *Ancylostoma ceylanicum* (Brailsford and Behnke, 1992; Brailsford and Behnke, 1992). For example it would be interesting to cross our fate-reporter mice onto different genetic backgrounds that have varying degrees of resistance, and determine whether Treg to Th2 conversion correlates with natural immunity to *H. polygyrus* as well as increasing numbers of ILC2s (Filbey et al., 2014). Furthermore, infections with lower doses of *H. polygyrus* were shown to improve the rapidity of expulsion due to reduced immunomodulatory responses, suggesting that modifications in dose of *H. polygyrus* larvae inoculation may favour the development of Th2 cells over Treg cells, perhaps through Treg to Th2 conversion (Brailsford and Behnke, 1992)

or through the participation of ILC2s in 'trained immunity' as seen in monocyte populations following *Candida* infection (Netea, 2013). Together, these experiments would better model genetic variation and epidemiological diversity of human soil-transmitted helminth infections. Based on these results and following the identification of appropriate biomarkers, it would be interesting to determine whether the proportion of ex-Foxp3 Th2 cells correlates with increased protective immunity to intestinal helminths in humans, as observed following repeated infections with *S. mansoni* (Karanja et al., 2002) and infections with *Necator americanus* (Quinnell et al., 2004).

5.4. Lessons learnt and future applications

Throughout these studies we have identified that *H. polygyrus* elicits multiple mechanisms of Th2 differentiation that can be enhanced to promote immunity to infection. Although this would seem detrimental to the parasite's survival, Th2 cells also mediate important repair mechanisms that are crucial for the survival of the host. Indeed, it is increasingly thought that Th2 responses have evolved in response to helminth-infections to limit damage to the host in addition to immunity (Gause et al., 2013; Allen and Sutherland, 2014). Therefore it is conceivable that helminth-derived molecules may promote the conversion of Treg to Th2 cells to enhance promote tissue repair following infection. Indeed, we identified Foxp3^{+}IL-4^{+} cells that expressed high levels of the regulatory cytokine IL-10, and may be important for regulating damage-mediated inflammation.

Like in many immune responses, there may be a threshold at which IL-4-elicited specialised Treg cells convert to Th2 effector cells, which depending on the context may have protective or pathogenic roles. Treg cells may inadvertently adapt to the Th2 immune responses in strong polarising environments. Indeed, only high levels of IL-4 signalling were shown to promote Treg conversion to Th2 cells (Noval Rivas et al., 2015). Thus, promoting ILC2 expansion and Treg reprogramming may have a beneficial role for anti-helminth immune responses, however it may also have negative

consequences on the development of pathogenic Th2-mediated diseases such as asthma and allergic inflammation, the global prevalence of which is steadily increasing (Weinberg, 2011), correlating with increased hygiene (Maizels, 2005) and changes in diets (Julia et al., 2015). Indeed, ILC2s have been implicated in the pathogenesis of oral and airway allergies (Licona-Limon et al., 2013) and may be playing an important role in driving innate type-2 responses at mucosal barriers. Furthermore, Treg reprogramming to Th2 cells correlated with the development of inflammation following oral allergy (Noval Rivas et al., 2015), suggesting that reprogrammed Tregs may contribute to disease. Current immunotherapies are looking at adoptively transferring Treg cells to control inflammatory diseases such as allergic asthma (Miyara et al., 2014). Instability of cultured Tregs and their conversion to Th2, Th1 or Th17 cells *in vitro* pre-transfer or *in vivo* post-transfer could therefore exacerbate rather than regulate disease. Identifying mechanisms of Treg to Th2 conversion would therefore be vitally important for the inhibition of these pathways, resolution of disease, and enhancing the stability of Tregs. Finally, if ILC2-derived IL-4 provides an important source of IL-4 for Treg to Th2 conversion, inhibiting ILC2s in parallel with the inhibition of Treg to Th2 conversion may indirectly reduce Th2-mediated disease and inflammation. The generation of *Foxp3*$^{\text{YFP-Cre}}$*Il4*$^{\text{GFP}}$ *R26R*$^{\text{FP635}}$ fate-reporter mice will allow us to determine the prevalence of Treg to Th2 conversion in a diversity of settings where protective and pathogenic Th2 cells play a dominant role, including allergic inflammation, metabolic homeostasis and wound repair (Gause et al., 2013). Furthermore, if IL-4 stimulates Treg to Th2 conversion, we will be able to test the functional impact of inhibiting conversion by using *Foxp3*$^{\text{YFP-Cre}}$*Il4*$^{\text{GFP}}$ *R26R*$^{\text{FP635}}$*Il4ra*$^{\text{fl/fl}}$ mice.

In conclusion, we have identified a novel source and target of IL-4 in the context of immune responses to *H. polygyrus.* We have generated novel and sophisticated genetic tools to study the impact of Treg to Th2 conversion in diverse settings of immunity and disease. We hope these tools can help identify new targets to promote enhanced anti-helminth vaccine responses and to reduce type-2-mediated disease.

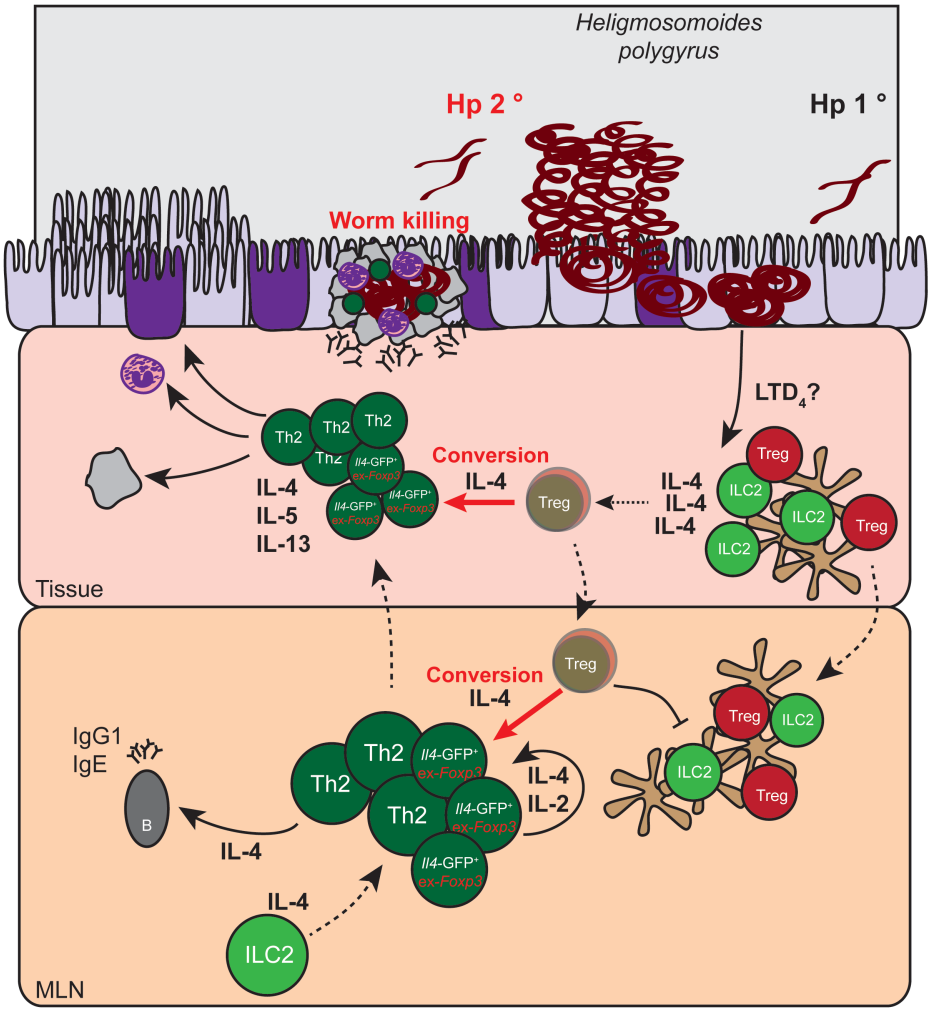


Figure 5.1. Working model: ILC2s promote the conversion of Treg to Th2 cells. Treg cells expand in response to a primary infection with *H. polygyrus* (Hp 1°). ILC2s expand concomitantly and provide a source of IL-4, which stimulates conversion of a proportion of tissue-resident Treg to Th2 cells. Furthermore, ILC2s and DCs migrate to the local MLN and promote the differentiation of nT cells in to Th2 cells, as well as further conversion of Treg to Th2 cells. Upon secondary infection (Hp 2°), re-activated Th2 and ex-Foxp3 Th2 cells expand and secrete high levels of IL-4, IL-13 and IL-5 that drive an early cascade of type-2 responses ultimately resulting in larval killing. Inhibition of Treg conversion to Th2 cells, or ILC2 secretion of IL-4 may impair the development of functional memory Th2 cells and immunity.

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Appendix

Publications

Victoria S. Pelly, Stephanie M. Coomes, Yashaswini Kannan, Isobel Okoye, Stephanie Czieso, Manolis Gialitakis, Frank Brombacher, Dominik Ruckerl and Mark S. Wilson. Foxp3^{+} cells adopt a Th2 phenotype, contributing to protective type-2 immunity against *Heligmosomoides polygyrus*. (manuscript under preparation).

Yashaswini Kannan, Yanda Li, Stephanie M. Coomes, Isobel S. Okoye, Victoria S. Pelly, Srividya Sriskantharajah, Eva Gückel, Lauren Webb, Stephanie Czieso, Nikolay Nikolov, Andrew S. MacDonald, Steven C. Ley* and Mark S. Wilson*. TPL-2 reduces severe allergic airway inflammation by inhibiting dendritic cell secretion of Ccl24. (submitted).

Stephanie M. Coomes, Yashaswini Kannan, Victoria S. Pelly, Jimena Perez-Lloret, Nikolay Nikolov, Werner Müller and Mark S. Wilson. IL-10 receptor signaling in Th2 cells restrains Th2-mediated allergic airway inflammation. (submitted).

Victoria S. Pelly, Yashaswini Kannan, Stephanie M. Coomes, Benedict Seddon, Dominik Ruckerl, Judith Allen, Andrew McKenzie and Mark S. Wilson. (2015). IL-4-producing ILC2s are required for differentiation of Th2 cells following helminth infection (under revision, Mucosal Immunology).

Stephanie M. Coomes, Victoria S. Pelly, Yashaswini Kannan, Isobel S. Okoye, Stephanie Czieso, Lewis J. Entwistle, Jimena Perez-Lloret, Nikolay Nikolov, Alexandre J. Potocnik, Judit Biró, Jean Langhorne, Mark S. Wilson. (2014) IFNγ and IL-12 Restrict Th2 Responses during Helminth/Plasmodium Co-Infection and Promote IFNγ from Th2 Cells. PLOS Pathogens *11*(7): e1004994

Isobel S. Okoye, Stephanie M. Coomes, Victoria S. Pelly, Stephanie Czieso, Venizelos Papayannopoulos, Tanya Tolmachova, Miguel C. Seabra, and Mark S. Wilson. (2014) MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. Immunity *41,* 89–10

Isobel S. Okoye, Stephanie Czieso, Eleni Ktistaki, Kathleen Roderick, Stephanie M. Coomes, Victoria S. Pelly, Yashaswini Kannan, Jimena Perez-Lloret, Jimmy L. Zhao, David Baltimore, Jean Langhorne, and Mark S. Wilson. Transcriptomics identified a critical role for Th2 cell-intrinsic miR-155 in mediating allergy and antihelminth

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Victoria S. Pelly, Stephanie M. Coomes, Mark S. Wilson. (2013). Plasticity within the $\alpha\beta^{+}CD4^{+}$ T-cell lineage: when, how and what for? Open Biology *3*: 120157.

Oral presentations

Cell Symposia: The Multifaceted Roles of Type 2 Immunity, Bruges, Belgium. December 10th – December 12th 2015. Title: Foxp3+ cells adopt a Th2 phenotype, contributing to protective type-2 immunity against *Heligmosomoides polygyrus*.

Woods Hole Immunoparasitology Conference (WHIP), Woods Hole, MA. April 27th – April 30th 2014. Foxp3+ cells adopt a Th2 phenotype, contributing to protective type-2 immunity against *Heligmosomoides polygyrus*.

Poster presentations

Keystone Symposia: Type 2 Immunity: Initiation, Maintenance, Homeostasis and Pathology, Santa Fe, NM. January 10th – January 15th 2013. Title: Investigating the functional relationship between Th2 and Treg cells during a helminth infection.