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The role of eosinophils in regulation of T- and B-cell responses during enteric nematode infection

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AAM	Alternatively activated macrophages
AID	Activation induced cytidine deaminase
APC	Antigen presenting cell
Arg-1	Arginase-1
BCR	B-cell receptor
BMDC	Bone marrow dendritic cells
CCR	Chemokine receptor
CD	Cluster of differentiation
Ch	Constant heavy chain
CSR	Class switch recombination
d.p.i	Days post infection
DC	Dendritic cell
EAR	Eosinophil associated ribonucleases
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EPO	Eosinophil peroxidase
FcR	Constant fragment receptor
FDC	Follicular dendritic cell
FLT-3L	fms-related tyrosine 3 ligand
GC	Germinal centre
GM-CSF	Granulocyte-macrophage colony stimulating factor
HES	*H. polygyrus* excretory secretory products
HEV	High endothelial venules
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-R	Interleukin receptor
ILC2	Innate lymphoid cells
L1-L4	Larval stage 1-4
mAb	Monoclonal antibody
MBP	Major basic protein
mLN	Mesenteric lymph node
NKT	Natural killer T cell
p.i.	Post infection
pLN	Peripherpheral lymph node
PP	Peyer's patch
Relm-α	Resistin like molecule alpha
Relm-β	Resistin like molecule beta
RT-qPCR	Real time quantitative polymerase chain reaction
SCF	Stem cell factor
SHM	Somatic hyper mutation
siLP	Small intestinal lamina propria
TCR	T cell receptor

TFH	T follicular helper cell
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR	Toll like receptor
Tg	Transgenic
TNFα	Tumor necrosis factor alpha
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The story of the eosinophil is an intriguing one. They are present in all vertebrate species (Lee et al. 2012) and the rarity of people lacking eosinophils (Gleich et al. 2013), would suggest they have an important role in organism homeostasis, yet their functions in health and disease remain poorly understood.
For many years it seemed the only conceivable role of the eosinophil was that of killing parasites and raking havoc in the asthmatic lung. However, though their numbers increase dramatically during both helminth infections and allergic asthma and although they can cause damage to helminth larval stages *in vitro* (Butterworth et al. 1975; Kazura and Aikawa 1980) evidence of their absolute *in vivo* requirement to free hosts of parasites is still lacking (Meeusen & Balic 2000). Moreover, their accountability for asthma-related pulmonary pathology came into question after three consecutive clinical trials using anti IL-5 monoclonal antibodies; mepolizumab (Leckie et al. 2000; Flood-Page et al. 2007) and reslizumab (Kips et al. 2003), failed to improve asthma symptoms and lung function in patients, despite significantly decreasing blood and sputum eosinophilia. Furthermore the few reports on patients suffering with severe asthma and at the same time lacking eosinophils, (Gleich et al. 2013) exemplifies how little we still understand about this cell.

In addition to their association with helminth infections, eosinophils are implicated in control of both viruses (Phipps et al. 2007) and bacteria (Linch et al. 2009), in particularly pulmonary pathogens. Further functions of eosinophils that have been demonstrated *in vitro*, but of which the *in vivo* relevance is still to be elucidated, include release of extracellular DNA traps (Yousefi et al. 2008), and antigen presentation of parasitic antigens to T-cells (Padigel et al. 2007).
In the absence of inflammation and disease, eosinophils are often cited as “rare granulocytes”, but this is a matter of location. In the absence of parasite infection or eosinophilic disorders, eosinophils make up only 1-3% of circulating white blood cells in humans (Lee et al. 2012). However, they are a prominent part of the healthy gastrointestinal tract (ca. 10%). Furthermore, eosinophils are present in the thymus (Throsby et al. 2000), adipose tissue (Wu et al. 2011), mammary glands (Gouon-Evans et al. 2002) and uterus, where their numbers cycle in an estrogen dependent manner (Rothenberg & Hogan 2006). Their role in the thymus and the female reproductive system are so far only touched upon by speculation but some of their functions in the gastrointestinal tract are slowly being elucidated. For example, it has now repeatedly been shown that eosinophils in the intestinal lamina propria support IgA class switching (V.T. Chu et al. 2014; Jung et al. 2015) thus contributing to gut homeostasis. Moreover, in their absences, development of Peyer’s patches- immune organs which line the small intestine- is severely impaired, suggesting that eosinophils are involved in normal development and/or maintenance of the gut immune system (V.T. Chu et al. 2014; Jung et al. 2015). Finally, eosinophils are increasingly being recognized for their potential in tissue repair (Heredia et al. 2013; Goh et al. 2013), which sheds a new light on their conceivable activity during infection with tissue migrating helminths or during tissue destructive allergic responses (Humbles et al. 2004).
Thus, it is an exciting time to be an eosinophil enthusiast. At no time previous has so many new potential roles for this granulocyte been recognized.

In this thesis I summarize my own efforts in trying to understand the eosinophil. What I have found place the eosinophil in an evolutionary sensible position in which it contributes to homeostasis as well as regulation of the immune response to a parasite which has evolved together with its murine host for millennia.

1.2 EOSINOPHIL BIOLOGY

1.2.1 Eosinophil development

Eosinophils develop in the bone marrow from pluripotent hematopoietc stem cells. In mice, common myeloid progenitors (CMP's) develop into granulocyte macrophage progenitors (GMP's), which in turn give rise to basophils, monocytes, neutrophils and eosinophils. In humans eosinophils develop directly from CMPs while GMPs give rise to the other granulocytes (Lee et al. 2012). Several transcription factors regulate eosinophil poiesis, including PU.1, C/EBP, FOG-1 and GATA-1 (Hogan et al. 2008). In mice, GATA-1 expression in GMP's distinguishes the development of eosinophils from other granulocytes. Indeed, deletion of a high affinity binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage (Yu et al. 2002) (see "eosinophil deficient mouse models").
A number of cytokines are central for eosinophil development, most notably GM-CSF, IL-3 and IL-5 (Hogan et al. 2008). GM-CSF and IL-3 are multi-potent and regulate the development of several cell types whereas IL-5 specifically supports eosinophils and does not augment the development of other granulocytes, but enhances eosinophil development and maturation, regulates eosinophil exit from the bone marrow (Kouro & Takatsu 2009) and is partly responsible for the high eosinophilia seen in parasitic disease, allergies and eosinophilic disorders (Simon et al. 2010). Importantly however, IL-5 and IL-5 receptor alpha (IL-5Rα) deficient mice still have a basal pool of bone marrow eosinophils (Kopf et al. 1996) and surprisingly, these are able to migrate into blood and tissues during helminth infection (Takamoto et al. 1997), demonstrating that IL-5 is not essential for eosinophil differentiation or migration but most likely plays an enhancing role in eosinophil development and trafficking.

1.2.2 Eosinophil granule proteins

When looking at an eosinophil through the microscope, its polymorphic nucleus and granules are clearly observable. Although the possibility of several distinct granules was debated in the early literature, the current consensus is that only one type of granule exists, namely the eosinophil specific granules, also know as secondary granules. Early literature often cites "primary granules", which can be found in developing eosinophils and "small granules". These are now believed to be precursors and vesicular derivates of the secondary granules respectively (Lee et al. 2012).
The granules are made up of a crystalline core that contains almost exclusively major basic protein (MBP)- the most abundant eosinophil granule protein by mol (Abu-Ghazaleh et al. 1992). The matrix surrounding the core contains the other eosinophil granule proteins; Eosinophil associated ribonucleases (EAR's) and Eosinophil peroxidase (EPO), the latter of which is the most eosinophil specific of all the granule proteins and is also the most abundant by mass (Abu-Ghazaleh et al. 1992). The matrix also contains an impressive range of pre-formed cytokines, which are selectively released upon eosinophil stimulation. Interestingly, the granule matrix is compartmentalized with membranes, suggesting that trafficking and secretion of specific mediators can be carried out in response to distinct stimuli (Spencer et al. 2014). The eosinophil granules have both overlapping and distinct functions.

1.2.2.1 *MBP*

Two homologues of MBP exists in humans and mice (Plager et al. 2006; Macias et al. 2000). MBP-1 is most abundant and also present in low levels in basophils whereas MBP-2, far less cationic than MBP-1, is expressed exclusively in eosinopihls (Plager et al. 2006). MBP is cytotoxic to helminths and bacteria, but also to host cells, causing tissue damage. The cytotoxic properties of MBP come from its interaction with biological membranes, in which it alters the surface charge, thereby increasing membrane permeability (Hogan et al. 2008). Eosinophils appear to protect themselves from this cytotoxicity by transcribing a neutralizing acidic pro sequence which is only cleaved once MBP has reached the granules (Hogan et al. 2008).

In addition to its direct cytotoxic effects, MBP can stimulate secretion of tissue remodelling factors, induce dermal permeability, alter smooth muscle function (Rothenberg & Hogan 2006), activate complement pathways and provoke mediator release from mast cells, neutrophils and basophils (Hogan et al. 2008). Mature eosinophils appear to have lost the ability to transcribe MBP mRNA suggesting that all the MBP protein an individual cell will ever make is transcribed during eosinophil development (Hogan et al. 2008).

1.2.2.2 *EPO*

As mentioned above, EPO is the most eosinophil specific of all the granule proteins and the most abundant by mass, making up ca. 25% of the total eosinophil protein content (Carlson et al. 1985). EPO is made up of two subunits - one light chain (15kDa) and one heavy chain (52kDa), covalently linked by a haeme co-factor. The haeme group is essential in mediating EPOs main function, namely catalyzing the oxidation of halides (specifically chloride bromide and iodide) and pseudohalides (thyiocaynate) by hydrogen peroxide, ultimately resulting in the formation of bactericidal reactive oxygen species and nitrogen metabolites (Rothenberg & Hogan 2006).
Like MBP, EPO is toxic to helminths (Jong et al. 1981; Hamann et al. 1990) and bacteria (Borelli et al. 2003) but also to mammalian cells (Hogan et al. 2008).

1.2.2.3 *EARs*

The EAR's are a collection of rapidly diversifying genes, which in humans codes the eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). Mice have at least 12 EAR's, not all of which are eosinophil specific (Yamada et al. 2015) and slightly less than half of these are granule proteins (Lee et al. 2012).
The function of the human EARs ECP and EDN are best characterised and include bacterial and mammalian cytotoxicity via membrane pore formation (Young et al. 1986), induction of mast cell degranulation (ECP) and antiviral immunity (EDN) (Hogan et al. 2008).

1.2.2.4 *Cytokines*

In addition to cationic proteins, the granule matrix also contains pre-formed stores of a large variety of cytokines (over 30 have been identified in human eosinophils), chemokines and growth factors (Spencer et al. 2014). Although eosinophils do not secrete large amounts of cytokines, they can do so very rapidly, due to the ready-to-go nature of their cargo (Spencer et al. 2009). Eosinophils have been shown to differentially secrete cytokines associated with Th2, Th1 and regulatory functions (e.g. IL-4, IL-12 and TGF-β), making them perfectly poised to orchestrate early events during a diverse set of immune responses (Spencer et al. 2009).

1.2.2.5 *Eosinophil modes of granule release*

Four distinct mechanism are proposed for selective release of eosinophil granule content; exocytosis of individual granules, compound exocytosis (fusion of several granules prior to fusion with plasma membrane and release of the accumulated content), piecemeal degranulation (gradual release of granule contents via vesicles) and cytolysis (release of intact granules due to rupture of plasma membrane) (Spencer et al. 2014). Importantly, cytolysis does not necessarily lead to release of granule contents, as granules are surrounded by their own membranes and intact granules can be found extracellularly in tissues of eosinophil associated inflammation (Lacy and Moqbel 2013). Such granules have a fascinating partial autonomy and can respond to stimuli via surface bound receptors such as the IFN gamma receptor (IFNγR) (Neves et al. 2008).

1.3 EOSINOHIL SURFACE RECEPTORS

Eosinophils express a plethora of surface molecules including receptors for cytokines, chemokines, prostaglandin, leukotrine, immunoglobulins and complement factors as well as

receptors involved in antigen presentation and co-stimulation (Driss, Legrand & Capron 2013). In the following section, only those receptors with direct importance for this thesis due to their use in detection or depletion of eosinophils or in interpretation of presented results are introduced.

1.3.1 Siglec-F

The most specific eosinophil surface receptor is the inhibitory sialic acid binding immunoglobulin like type lectin Siglec-F and its human homologue Siglec-8, both of which preferentially bind the sialic acid 6′sulfo-sialyl-Lewis X (Zhang et al. 2007). Siglec-F ligation induces eosinophil apoptosis and is thought to control eosinophil responses in a negative feedback loop. Thus, inflamed lung tissue increases expression of Siglec-F ligands (Zhang et al. 2007), presumably to reduce the potentially damaging effects of infiltrating eosinophils. Due to its apoptotic effects on eosinophils, Siglec-F has been used to deplete eosinophils *in vivo* (Chu et al. 2011; V.T. Chu et al. 2014).
Furthermore, although alveolar macrophages have been shown to express Siglec-F (Hussell & Bell 2014), it is a very useful marker for detecting eosinophils by flow cytometry, especially when dealing with tissues other than the lung.

1.3.2 CCR3

Eosinophils constitutively express CCR3, mediating their trafficking to tissues by binding the chemokines CCL11 (eotaxin-1) and CCL24 (eostaxin-2) (as well as CCL26 (eotaxin-3) in humans), CCL3 (MIP-1α) and CCL5 (RANTES) (Rosenberg et al. 2013). CCR3 expression on eosinophils is further up-regulated in response to IL-5 (Radinger et al. 2013), thus providing a mechanism by which IL-5, in addition to its own eosinophil activating function, augments eosinophil responsiveness during inflammation. CCR3 expression is also essential for homeostatic trafficking of eosinophils to the gastrointestinal tract (Matthews et al. 1998), where eotaxin is constitutively expressed by epithelial cells (Rothenberg et al. 2001)
Although CCR3 was originally thought to be exclusively expressed by eosinophils, it has since been demonstrated on a range of other cell types including mast cells, basophils and a subset of human Th2 cells (Humbles et al. 2002). Nevertheless, CCR3 is useful in combination with Siglec-F and side scatter properties in identifying eosinophils by flow cytometry.

1.3.3 Fc receptors

Fc receptors (FcR) are important in host defence against diverse pathogens as they confer specificity on the innate immune system. Antibodies highly specific for a given pathogen can opsonise its surface after which innate cells, which on their own would only recognise conserved molecular patters, can be activated or inhibited by binding the available Fc part of the antibody.
Human eosinophils express a wide array of activating and inhibitory Fc receptors for all antibody isotypes and are able to kill pathogens *in vitro* by antibody dependent cell mediated cytotoxicity (ADCC) (Driss, Legrand & Capron 2013). Murine eosinohils differ from human eosinophils in that they express a more limited selection of FcR (Bruhns 2012). For example, whereas human eosinophils express the high affinity (FcγRI), low affinity (FcγRII) and inhibitory (FcγRIII) receptor for IgG, murine eosinophils only express FcγRII and FcRγIII (Bruhns 2012). Furthermore, although much effort has been made to detect receptors for IgE on murine eosinophils, it appears that mouse eosinophils do not express the high or low affinity receptor for IgE (Kita & Gleich 1997; de Andres et al. 1997)
The Fc alpha receptor 1 (FcαRI or CD89) is expressed on human eosinophils, but is not present in the murine genome. Mouse eosinophils also lack the alternative IgA receptors polymeric Ig receptor (PIGR) or secretory component (SC), both of which are expressed on human eosinophils (Decot et al. 2005). The only receptors expressed by mouse eosinophil that binds IgA are the transferring receptors 1 and 2 (Tfr1/Tfr2). IgA competes with transferrin (Tf) thereby inhibiting Tf-induced release of reactive oxygen and inflammatory eosinophil

proteins (Decot et al. 2005). Thus, IgA could have an anti inflammatory influence on mouse eosinophils.

1.4 EOSINOPHIL DEFICIENT MOUSE MODELS

1.4.1 IL-5 deficient mice

As IL-5 promotes eosinophil maturation, trafficking and activation (Kouro & Takatsu 2009), a vast proportion of the eosinophil literature deals with different models in which IL-5 is either depleted (anti-IL-5 antibodies, IL-$5^{-/-}$) or over expressed (rIL-5, IL-5 transgenic). This approach is associated with several drawbacks, the most apparent of which is the incomplete depletion of eosinophis (Kopf et al. 1996; Takamoto et al. 1997). Mice treated with anti-IL-5 and IL-$5^{-/-}$ mice have residual eosinophils in the bone marrow (Kopf et al. 1996) and these cells can infiltrate inflamed tissues independently of IL-5 (Takamoto et al. 1997). This is in agreement with the suggestion that IL-5 is not essential for eosinophil development, but rather enhances the effect of other mediators such as eotaxin (Mould et al. 1997).
Another obvious caveat are effects IL-5 may have in addition to those related to eosinophil survival and trafficking. Indeed, the original name for IL-5 was T-cell replacing factor (TRF) and it was described as a factor secreted by T-cells which stimulated antibody production from activated B-cells, but also supported B-cell maturation and survival (Takatsu et al. 1980). Thus, although manipulating IL-5 has produced many informative results regarding the role of eosinophils in health and disease (see table 10.1, appendix for its use in studying eosinophil function in helminth infection), inconsistencies in methodology, often leading to contradictory outcomes, exposed the need for mice with selective loss of the eosinophil lineage.

1.4.2 ΔdblGATA-1

The first such strain was the ΔdblGATA-1 mouse, which lacks eosinophils due to a deletion in the high affinity GATA-1 binding site within the GATA-1 promoter (Yu et al. 2002). GATA-1, as mentioned above, is essential for eosinophil development, but additionally controls erythroid, megakaryocyte and mast cell development. Mice with a full knock out of the GATA-1 promoter do not survive gestation, but selective deletion of the high affinity GATA-1 binding site leads to targeted deletion of the eosinophil lineage with no reported effects on megakaryocytes or mast cells and with only mild effect on erythrocyte production (Yu et al. 2002). However, in a more recent paper it was additionally demonstrated that ΔdblGATA-1 mice have a mild impairment in basophil levels (Nei et al. 2013).

1.4.3 PHIL

A second mouse strain devoid of eosinophils, the PHIL mouse, was developed two years after the Δ dblGATA-1 mouse and lack eosinophils due to an insertion of the cytocidal diphteria toxin A chain under the EPO promoter, leading to the selective depletion of EPO expressing cells during development (Lee et al. 2004).
Recently inducible PHIL mice (iPHIL) have been developed, in which the human diphteria toxin receptor has been inserted to the EPO promoter, leading to the inducible depletion of eosinophils through injection of diphteria toxin (Jacobsen et al. 2014).

1.5 T-HELPER 2 RESPONSES

1.5.1 The T helper paradigm

Following antigen stimulation via the T-cell receptor (TCR) and depending on the surrounding cytokine milieu, naïve $CD4^+$ T-helper (Th) cells differentiate into distinct subtypes of activated effector cells. To date at least 5 different Th types are described; Th1, Th2, Th17, Th22 and Th9 cells, the latter three of which are named after the defining cytokines they secrete.

The Th1/Th2 dichotomy was formally defined by Mosmann and Coffman in 1987. Th1 cells were described as IFNγ, IL-2 and lymphotoxin (LT) producing cells and Th2 cells as IL-4, 5 and 13 secreting cells. This linked Th2 cells to the development of asthma and allergies and the association of Th2 responses with helminth infections soon followed, (Else et al. 1994; Finkelman et al. 1997; Urban et al. 1995), offering an evolutionary sensible explanation for immune mechanisms, which in the context of atopic asthma or food allergies would appear mal-adaptive.
Thus, the paradigm still in place today, that Th2 immunity evolved in response to extracellular parasites, which are too large to be phagocytosed or to killed via Th1-associated cellular toxicity, took shape.

1.5.2 Th2 induction

Despite the wide range of molecules that can induce Th2 type responses, including helminth and fungal antigens, toxins, insect venoms and other allergens, and notwithstanding the clinical relevance of many Th2 associated conditions, the way(s) in which Th2 immune responses are initially induced and maintained and how they differ between distinct helminth species and allergens is still relatively poorly understood.

1.5.2.1 *Th2 inducing antigens*

In contrast to bacteria, viruses and protozoa, which contain distinct, often well defined molecular patterns that activate specific innate receptors, such molecular patterns are not well characterized for most helminths, or allergens, meaning that the initial events leading to Th2 immune response are less well defined than their Th1 counterparts.
One exception is Omega-1 found in *Schistosoma* egg antigen (SEA)- a glycosylated ribonuclease that is directly responsible for the strong Th2 inducing properties of *Schistosoma mansoni* eggs (Steinfelder et al. 2009). Omega-1 is taken up by dendritic cells via the mannose receptor and inhibits cytosolic protein synthesis by cleavage of rRNA and mRNA (Everts et al. 2012). This means that omega-1 stimulated DCs demonstrate suppressed cytokine secretion and, most importantly, do not secrete IL-12 (see below) (Everts et al. 2012).
Although Omega-1 represents the first (and thus far only) helminth derived molecule in which the molecular mechanism of its Th2 inducing potential has been described, excreted products from other helminth have similarly been shown to directly bestow a Th2 inducing potential on DC via the inhibition of IL-12 production (Semnani et al. 2003; Balic et al. 2004; Massacand et al. 2009; Segura et al. 2007). This includes *H. polygyrus* excretory/secretory products (HES), which not only inhibit IL-12 secretion from bone marrow derived DC (BMDC) stimulated with toll like receptor (TLR) ligands, but also manipulate the expression of a number of other cytokines and chemokines (Segura et al. 2007). This most likely reflects the complex nature of HES, which contains a wide range of molecules with a presumably broad spectrum of functions (Maizels et al. 2012a).

1.5.2.2 *Importance of IL-4*

Ligation of the T-cell receptor in the presence of IL-4 leads to expression of the canonical Th2 transcription factor GATA-3 via STAT-6 signalling (Paul & Zhu 2010). GATA-3 binds to the *IL-4*, *-5* and *-13* loci and promotes their transcription, which, in a positive feedback loop, leads to higher expression of GATA-3. Low strength TCR signalling can induce GATA-3 expressing Th2 cells (Paul & Zhu 2010). Although this can be achieved in an IL-4 independent manner *in vitro*, IL-4$^{-/-}$ mice do not develop stable Th2 cells, suggesting that *in vivo*, IL-4 is essential for Th2 induction (Chu et al. 2014b).
The source of initial IL-4 has been much debated. By now, eosinophils (Voehringer et al. 2006), basophils (Min et al. 2004; Sokol et al. 2008), type 2 innate lymphoid cells (ILC2) (Pelly et al. 2016), natural killer T-cells (NKT) (Yoshimoto & Paul 1994) as well as naive CD4^{+} T-cells (Noben-Trauth et al. 2000) have all been shown competent in providing early IL-4, but

their relative contribution likely differs depending on pathogen and location of inflammation and a considerable level of redundancy most probably exists between them.

1.5.2.3 *Suppression of IL-12*

IL-12 and IFNγ respectively initiate and enhance the expression of T-bet - the canonical Th1 transcription factor (Lazarevic et al. 2013) and IL-12 is thus integral in the induction of Th1 cells. Furthermore, both IL-12 and IFNγ can impair the development of Th2 cells *in vitro* and injection of IL-12 to *Leishmania major* infected mice significantly reduces the level of IL-4 producing cells (Seder & Paul 1994). Thus, the ability of an antigen to block IL-12 secretion in antigen presenting cells (APC) represents a potent pathway in which it could influence Th2 induction. Additionally, degradation of mRNA, as described for the *Schistosome* egg antigen Omega-1 (Everts et al. 2012), could conceivably result in lower expression of MHCII on APC, leading to weaker TCR stimulation, which is known to preferentially induce Th2 cells (Paul & Zhu 2010).
Thus, helminths and allergens may induce Th2 responses in several ways including inhibition of IL-12, weak TCR signalling and induction of IL-4.

1.5.2.4 *Tissue damage and alarmins*

Recently a paradigm has developed in which a second line of early events take place that can lead to initiation or augmentation of Th2 responses and which in some cases can result in helminth expulsion even in the absence of T-cells, due to high levels of Th2 associated cytokines (Voehringer et al. 2006; Fallon et al. 2006).
Helminth or allergen proteases, as well as tissue damage leads to secretion of the alarmins IL-33, TSLP and IL-25 (Saenz et al. 2009; Divekar & Kita 2015). Several cell types can express these mediators, but in delineating the early events of Th2 induction, it is believed that epithelial cells are the most relevant source (Saenz et al. 2008). TSLP, IL-25 and IL-33 have overlapping, but also distinct downstream effects on Th2 induction and Th2 associated events. For example, IL-25, secreted by pulmonary epithelial cells, following infection with the lung migrating nematode *Nippostrongylus brasiliensis*, induces a population of IL-4, IL-13 and IL-5 secreting, non–B/non–T (NBNT), c-kit^{+}, FcεR1^{-} cells, now known as ILC2 and IL-25$^{-/-}$ mice do not expel the parasite (Fallon et al. 2006).
TSLP is constitutively expressed by epithelial cells in the lung, tonsils and intestine, but is up-regulated in response to TLR ligation or stimulation with viral, bacterial and parasitic antigens (Saenz et al. 2008). TSLP influences the ability of DCs to induce Th2 cells by blocking DC derived IL-12 secretion (Taylor et al. 2009a). In line with this, TSLP is essential for Th2 induction and protection during *T. muris* infection (Taylor et al. 2009; Massacand et al. 2009). In contrast, TSLP is dispensable during infection with *H. polygyrus* or *N. brasiliensis* as these nematodes secrete factors directly inhibiting IL-12 secretion by DCs (Massacand et al. 2009).

1.5.3 Eosinophils in Th2 responses

For a long time eosinohilia was described to be a result of Th2 responses, a consequence of the high IL-5 levels produced by Th2 cells. Although this is true, it is since long recognized that eosinophils play a more complex role in Th2 responses, in some cases appearing to contribute to Th2 initiation, in others to inhibit it (Nakagome et al. 2007) and in many cases have no influence at all (Fattouh et al. 2011; Voehringer et al. 2006; Svensson et al. 2011).
Generally is seems that eosinophil involvement in Th2 induction may be more relevant in allergic responses than in responses to helminths, which raises the fascinating possibility of helminths actively modulating eosinophil function. Furthermore, the propensity of eosinophils to influence Th2 induction may be organ specific (e.g Fabre et al. 2009) or vary with the antigen used as well as with the route of antigen application (Chu et al. 2014a).
Finally, an important point is that, although eosinophils can provide early IL-4 during helminth infection both in the lung (Voehringer et al. 2006) and gut draining mesenteric lymph nodes

(mLN) (Svensson et al. 2011), eosinophil derived IL-4, in both these cases, has proved non-essential in inducing Th2 cells.

To summarize, *in vivo* differentiation of Th2 cells can be achieved by a range of mechanisms with overlapping and complementary function, the importance of which will vary with the type of antigen, but include the inhibition of IL-12 directly by antigen (e.g. Omega-1/HES) or by secreted molecules such as TSLP. At the same time IL-4 is made available by a range of cells and epithelial secretion of IL-33, TSLP and IL-25 induces secretion of Th2 cytokines from innate cells and/or influences T-cells directly or modulate the ability of dendritic cells to stimulate Th2 differentiation.

1.6 B-CELLS

Although B-cells have many functions, including cytokine secretion, antigen presentation and immune regulation, their most important function is unarguably the production and secretion of antibodies. Some antigens can stimulate B-cell proliferation and antibody secretion independently of T-cells, either through TLR ligation or by cross-linking several B-cell receptors, through repetitive structures, but these immune responses mainly produce IgM antibodies with low affinity (Janeway et al. 2001). An exception to this is mouse IgG3, which can be induced T-cell independently by LPS stimulation (Deenick et al. 2005). High affinity antibody responses however, require the support of T follicular helper cells (TFH) and the formation of germinal centres in which B-cells proliferate and undergo class switch recombination, affinity maturation through somatic hyper mutation and finally selection by TFH cells and follicular dendritic cells (FDC)- specialized stromal cells with long dendrites, not related to dendritic cells of haematopoetic origin (De Silva & Klein 2015).

1.6.1 Germinal centres

Secondary lymphoid organs such as the spleen, lymph nodes and Peyer's patches (PP) are made up of a central T-cell zone and peripheral B-cell follicles. The formation of germinal centres (GC) takes place within the follicles following B-cell interaction with T-follicular helper cells (TFH). TFH cells are specialized $CD4^+$ cells, recognized via the expression of high levels of CXCR5 and PD-1 (Haynes et al. 2007), which develop in secondary lymphoid organs and provide support for B-cells via CD40:CD40L interaction as well as via the provision of cytokines.
The exact chain of events leading up to the formation of germinal centres have been visualized using two-photon *in vivo* imaging (Kerfoot et al. 2011; Kitano et al. 2011). By transferring fluorescently labelled antigen specific T and B-cells, followed by immunisation of recipient mice, the movement of the transferred cells could be monitored day by day. In this way it was shown that, as early as day 1 post immunisation, antigen activated T and B-cells up-regulate CXCR5 which enables them to migrate to the inter-follicular region (not the B/T boundary), where they interact via CD40-CD40L and MHCII-TCR. On day three, TFH cells migrate into the follicles and B-cells follow on day four at which point they start to proliferate, signalling the start of the germinal centre reaction. From day seven, the germinal centre starts to divide into the dark and light zone, the dark zone consisting of proliferating B-cells and the light zone of TFH, FDC and some B-cells (Keerfoot et al. 2011; Kitano et al. 2011; De Silva & Klein 2015). These B-cells are undergoing selection, in which the affinity of their B-cell receptor (BCR) is tested. B-cells can get "sent back" to the dark zone for several rounds of hyper-mutation until they express highly specific antibodies, at which point they exit the germinal centre either as antigen secreting plasma cells or as memory B-cells (De Silva & Klein 2015).

1.6.2 Class switch recombination

Class switch recombination (CSR) takes place within the GC and is the process by which B-cells switch from expressing IgM and IgD to one of the other immunoglobulin (Ig) isotypes; IgG1, IgG2a, IgG2b, IgG3 (mouse) or IgG4 (only human), IgA and IgE. CSR changes the

immunoglobulin heavy chain constant region (Ch) of the antibody molecule, thereby altering its function. This is in contrast to somatic hypermutation (SHM), in which the variable region is mutated and selected to achieve higher affinity to antigen.
CSR, like SHM, requires expression of the enzyme activation-induced cytidine deaminase (AID) in B-cells, which is up-regulated by CD40 ligation or cross linking of the B-cell receptor and which introduces DNA breaks in specified “switch regions” on the loci containing the Ig Ch genes (Xu et al. 2012). Switch factors such as IL-4 and TGF-β determine which switch region is targeted by AID-mediated DNA breaks. For example, IL-4 induces STAT-6 expression and STAT-6 binds to promoters in the regions immediately preceding the IgG1 and IgE switch regions making them available for AID (Xu et al. 2012).
Thus, IL-4, in addition to working as a survival factors for B-cells, is essential for class switching to IgG1 and IgE.

1.6.3 Eosinophils and B-cell responses

Eosinophils have been shown to support the survival of plasma cells in the bone marrow by secretion of IL-6 and APRIL (Chu et al. 2011). In accordance with this, the high levels of circulating B-cells seen in IL-5 transgenic NJ1638 mice have been shown to be due to eosinophils, not to IL-5 itself, as PHIL/NJ1638 have levels of circulating B-cells comparable to non-transgenic PHIL mice, despite high levels of IL-5 (Wong et al. 2014). Furthermore, eosinophils are found close to the B-cell follicles in human spleen and tonsils and secrete soluble factors *in vitro*, which stimulate antibody secretion from human B-cells (Wong et al. 2014).
Recently, the repertoire of eosinophil-mediated effects on B-cells was expanded by the demonstration that eosinophils additionally support IgA^+ plasma cells in the small intestinal lamina propria (SiLP) and play a role in IgA class switching in the PP in steady state (V.T. Chu et al. 2014; Jung et al. 2015). The role of eosinophils on B-cell responses to infections has not been directly assessed.

1.7 GUT ASSOCIATED LYMPHOID ORGANS

To sufficiently introduce this study, the intestinal tissue compartments in focus of the work and the central features that unite and separate them are important to mention.

1.7.1 Peyer’s patches

The Peyer’s patches (PP) are specialized immune organs of the small intestine with direct access to the intestinal lumen via microfold cells (M-cells). Antigen is transported via the M-cells from the lumen to the subepithelial dome (SED) (Corthésy 2007) and is processed and presented to T and B-cells by dendritic cells in the T-cell zone and B-cell follicles, respectively. As the lumen is inhabited by microbes and contains potentially immune-active food antigens, the PP are perpetually active, visible by the permanent presence of GC (Mowat & Agace 2014). The PP GC are the principal site for IgA production during homeostasis (Macpherson et al. 2008; Fagarasan & Honjo 2003) and infection with intestinal bacteria (Martinoli et al. 2007).
The PP have no afferent lymphatics (Varol et al. 2010), thus leukocyte enter the PP strictly via high endothelial venules (HEV). Once activated, cells leave the PP via the efferent lymphatic and migrate, via the mLN to the thoracic duct where they enter the circulation from which they reach the lamina propria (LP) (Cerutti 2008; Fagarasan & Honjo 2003).

1.7.2 Mesenteric lymph nodes

The mesenteric lymph nodes (mLN) drain the small and large intestine and are thus the main site for immune responses against enteric pathogens. The structure is similar to that of the PP, with the exception of the mLNs being encapsulated by a layer of connective tissue (Mowat & Agace 2014) and the presence of afferent lymphatics. In contrast to the PP, in which antigen

is sampled directly from the lumen, access to intestinal antigen in the mLN is exclusively provided by dendritic cells entering the lymph nodes via the afferent lymph (Milling et al. 2009). Although the mLN are sometimes quoted together with the PP as a site of IgA production and although their size is considerably larger than that of other lymph nodes, suggesting continuous activity (Mowat and Agace 2014), the mLN contain very low levels of GCB-cells and IgA in the absence of infection.

1.7.3 Small intestine lamina propria

The small intestinal lamina propria (siLP) is the layer of tissue situated between the gut epithelia and muscle layer of the small intestine. The epithelium covering the villi contains M-like cells, capable of transcytosing antigen to the underlying siLP (Jang et al. 2004). In addition, dendritic cells (DC) in the siLP sample antigen directly from the lumen by extending dendrites through the epithelial layer or by taking up antibody/antigen complexes present in the siLP (Coombes & Powrie 2008) and transport these to secondary lymphoid organs, in particularly to the mLN. In this way, constitutive migration of DC from the siLP via the lymph to the mLNs is important for maintenance of oral tolerance to food antigens and endosymbionts in the absence of infection (Worbs 2006).
siLP DC also enter the PP (Coombes and Powrie 2008), but what factors determine whether they traffic to the mLN or the PP during the course of an infection and whether they exert different effects in the two compartments remains unknown.

1.8 *HELIGMOSOMOIDES POLYGYRUS*

Heligmosomoides. polygyrus is a trichostrongylid nematode and a commonly used model for studying chronic gastrointestinal nematode infections resembling soil-transmitted nematode infections of humans. As one of the few laboratory models for which the mouse is the natural host, it can offer valuable insight into the evolutionary arms race between parasites and their hosts. The taxonomy of *H. polygyrus* has a complicated history with some long running disagreements as to how the laboratory strain should be properly named in order to distinguish it from strains isolated from wild mice (Behnke & Harris 2010; Maizels et al. 2011). Nevertheless, the vast majority of the literature makes use simply of *H. polygyrus*, although *H. polygyrus bakeri*, as well as the more historical nomenclature of *Nematospiroides dubius* is sometimes seen.

1.8.1 Life cycle

H. polygyrus has a direct life cycle with 2 free living larval stages (L1 and L2) and the infective stage (L3) taken up orally by the host, where it develops via the L4 stage to the mature adult stage found in the small intestine of several mouse species. Eggs are shed in the faeces of infected hosts and L1 larvae hatch into the environment within a few days. They live a short time as feeding L2 larvae before undergoing a semi-molt into the non-feeding, infective L3 stage (Bryant 1973). The molt is incomplete, as L3 larvae retain the L2 cuticle, which enables them to survive for extended periods of time in the environment, adding to their convenience as a laboratory model. L3 larvae enters a new host via the oral route and upon reaching the small intestine, burry into the intestinal wall where they undergo two more molts to reach maturity (Reynolds et al. 2012). After 7-9 days the adults emerge back into the duodenal lumen where they mate and start producing eggs, which can be detected in the faeces from day 9 post infection. Male and female worms can be easily distinguished from one another by the larger size of the females and a characteristic copulatory bursa at the posterior end of the males (Figure 1).

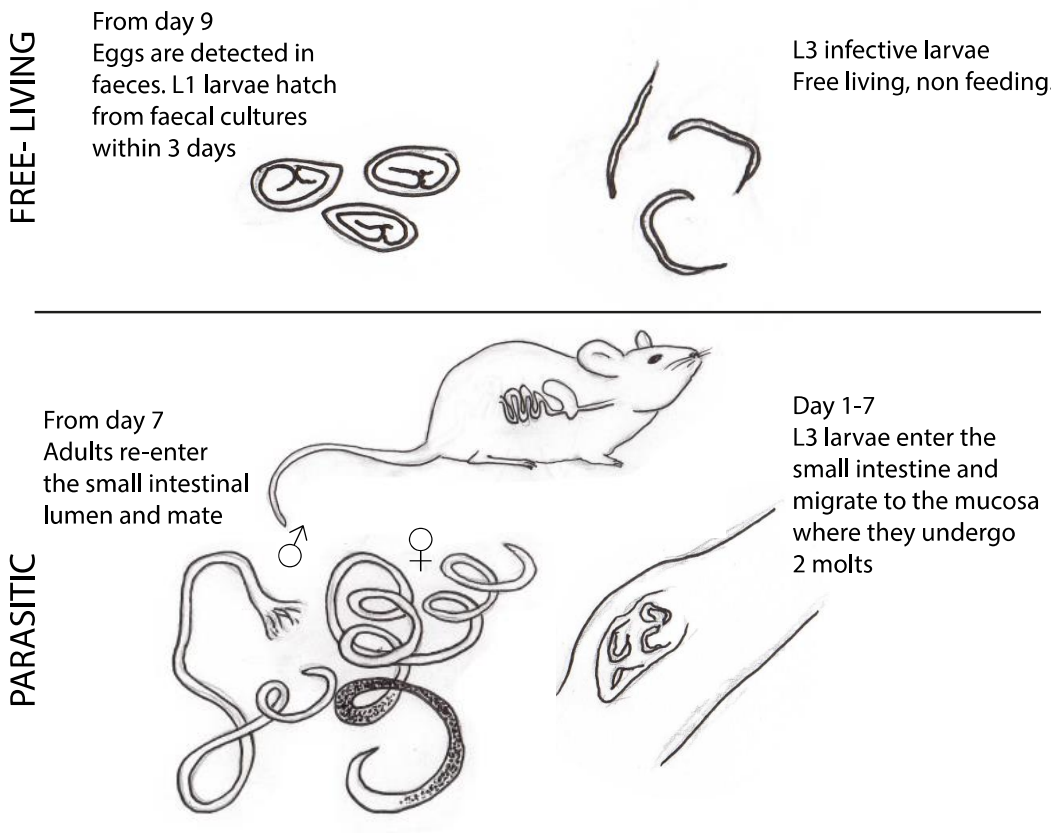

Figure 1.1 Life cycle of *H. polygyrus*
Free-living L3 larvae infect mice via the oral route. Upon reaching the small intestine, larvae burry into the mucosa where they undergo 2 molts. Emerging approximately one week later, adults mate in the lumen of the small intestine after which females start releasing eggs. Male and female worms can be distinguished under the microscope based on size, presence of eggs or copulatory bursa. Eggs are detectable in faeces from day 9 p.i. and increase steadily thereafter, before declining from ca. 4 weeks post infection onwards. L1 larvae hatch from the eggs within 3 days. L1 and L2 larvae feed on bacteria in faeces before undergoing a partial molt into sheathed L3 larvae.

1.8.2 Immune responses to *H. polygyrus*

1.8.2.1 *Primary infection*

During primary infection with *H. polygyrus*, expulsion of adult parasites from the intestine takes place, but is incomplete, with a proportion of worms establishing a chronic infection. The initial immune response is associated with the expansion of IL-4 secreting ILC2 (Pelly et al. 2016), infiltration of eosinophils, neutrophils and macrophage, formation of granulomas, hypergammaglobulinaemia and the induction of Th2 cells, which secrete high levels of IL-4, IL-13 and IL-5, (Reynolds et al. 2012; Maizels et al. 2012b). Although the exact effector mechanisms responsible for expulsion of the majority of worms during the initial phase of primary *H. polygyrus* infection are not well defined, IL-4 and IL-13 have many diverse effects which could be expected to contribute. These include increased muscle contractility (Akiho et al. 2002), enhanced epithelial turnover (Cliffe et al. 2005) and promotion of epithelial cell differentiation into mucus producing goblet cells (Gause et al. 2003; Hasnain et al. 2011). Goblet cells additionally secrete Relm-β, which interferes with parasite feeding (Herbert et al. 2009) and increased mucus production as well as gut permeability, coupled to augmented muscle contractility has been proposed to expulse helminths in a weep (increased intestinal fluids) and sweep (hypercontraction) response (Anthony et al. 2007). Furthermore, IL-4

stimulates alternatively activated macrophages (AAM) which can secrete anti helminthic products such as arginase-1 (Arg-1) leading to larval killing in challenge infections (Anthony et al. 2006), but whether AAM also limit primary *H. polygyrus* infection is unknown.
Interestingly, immunity to other helminths has been shown to be dependent on Th2 associated cytokines, but independent of Th2 cells (Voehringer et al. 2006). In primary *H. polygyrus* infection, $CD4^+$ T-cells appear to be important in limiting worm fecundity, but not necessarily parasite survival, as shown by comparable recovery of adults worms, but a significant increase in faecal egg output in mice treated with an anti-CD4 antibody (Urban et al. 1991a). This effect is most likely driven by T-cell derived IL-4 as suggested by a separate study in which delivery of IL-4 complexes (IL-4C) to severe combined immune-deficient (SCID) mice significantly decreased faecal egg counts (Urban et al. 1995).
Primary infection results in an early burst of IgG and IgE antibodies, but these do not confer protection as they are of low affinity and specific for apparently non relevant antigens (McCoy et al. 2008; Hewitson et al. 2011). Antibodies are however implicated in limiting *H. polygyrus* fecundity during primary infection (McCoy et al. 2008).
Thus, innate cells are likely responsible for the partial expulsion of *H. polygyrus* during a primary infection, with T-cell derived IL-4 and natural antibodies contributing to reduced parasite fitness.

1.8.2.2 *Secondary infection*

H. polygyrus infection, at least at the doses commonly applied in the laboratory, results in sterile immunity, which is wholly dependent on the adaptive immune system, as exemplified by impaired resistance in mice treated with anti-CD4 antibodies (Anthony et al. 2006; Urban et al. 1991a) or mice deficient in mature B-cells (McCoy et al. 2008). IL-4 is imperative in protection, but can be partly compensated for by IL-13, as shown by increased parasite survival in mice treated with anti-IL-4 antibodies, but complete abolishment of protection in mice treated with anti IL-4Rα (Urban et al. 1991b).
In line with this, depletion of macrophages, or Arg-1 had a similar effect on the parasites ability to survive (Anthony et al. 2006), indicating that CD4 mediated alternative activation of macrophages, possibly via IL-4, represent an important pathway for protective immunity.

To summarize, it is likely that partial expulsion of *H. polygyrus* during primary infections is mediated by innate mechanisms such as goblet cell derived mucus and Relm-β production whereas protective immunity to challenge infection is dependent on $CD4^+$ T-cells, antibodies and AAM.

1.8.3 Eosinophils in *H. polygyrus* and other helminth infections

Considering the strong correlation between blood and tissue eosinohilia and parasite infections, it is easy to see why eosinophils have been assumed to play an essential role in host anti-parasite defence, but their role in *H. polygyrus* infection has been surprisingly little studied (Knott et al. 2009; Urban et al. 1991b). To further understand the role of eosinophils in helminth infections, reports from other infection models are informative. An overview of reports in which eosinophils have been studied in the context of anti helminth defence is given in table 10.1 (appendix).
Generally, eosinophils are able to kill larval stages of several helminth species *in vitro* (Butterworth et al. 1975; Kazura and Grove 1978; Shin et al. 2001) and are involved in protection against some tissue migrating helminths *in vivo* (Knott et al. 2007; Knott et al. 2009; Cadman et al. 2014). However, further *in vivo* experiments using other helminth species have shown that the issue of eosinophils role in parasite infections wdoes not let itself be so easily categorized.
For example, although eosinophils have been shown to kill *S. mansoni in vitro* (Butterworth et al. 1975; Ramalho-Pinto et al. 1978), *in vivo* studies using at first anti-IL-5 treated (Sher et al. 1990) and later eosinophil ablated (Swartz et al. 2006) mice, demonstrate no discernible role for eosinophils in protection against this trematode. A further study even suggested that the

parasite benefits from excessive eosinophilia in the liver (Dent et al. 1997). This is similar to experiments with eosinophil ablated mice during a primary infection with *T. spiralis* in which the absence of eosinophils leads to dramatic lethality of muscle stage larvae, due to increased production of nitric oxide in the absence of eosinophils (Fabre et al. 2009; Huang et al. 2014). In many cases however, eosinophils have no or very mild effects on parasite survival (Betts and Else 1999; Hokibara et al. 1997), despite being highly up-regulated and infiltrating the site of infection. This raises the intriguing question of whether some helminth parasites are actively regulating eosinophils, as they have been shown to do with other cell types (Blum et al. 2012; Finney et al. 2007; Rausch et al. 2008; Wilson et al. 2010).

A summary of innate processes known or assumed to take place during primary *H. polygyrus* infection, adaptive mechanisms of Th2 induction and GC formation in the mLN, as well as the role of the PP as sites of homeostatic IgA production is depicted in figure 1.2.

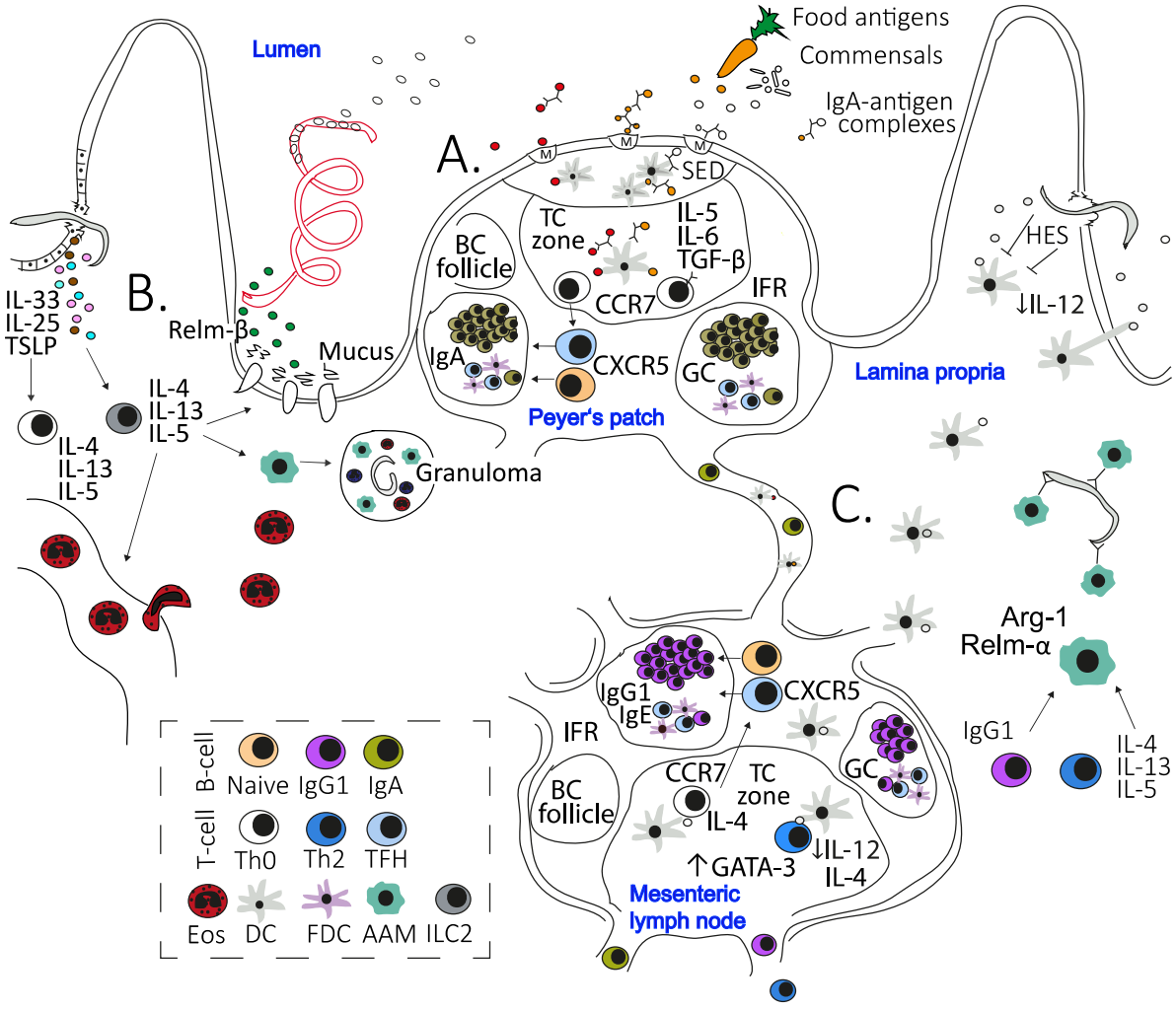

Figure 1.2 Small intestinal immune proceses during homeostasis and infection with *H. polygyrus*
A. Free or IgA bound luminal antigens are transported via M-cells to the underlying SED of the PP. Activated DC travel to the TC zone where they present antigen. Activated T-cells downregulate expression of CCR7 and upregulate CXCR5, facilitating their exit from the TC-zone and entry into the IFR where they initiate interaction with B-cells. This interaction results in T-cell differentiation to TFH cells and the maturation of B-cells. TFH travel into the follicle

and are shortly followed by B-cells, which initiate the GC reaction during which B-cells proliferate, undergo CSR and differentiate to plasma blasts. IgA CSR is facilitated by the presence of IL-6 and TGF-β. B-cells may undergo several rounds of selection by FDC and TFH before leaving the PP via the mLN. **B.** Primary infection with *H. polygyrus* initially results in increased expression of IL-13 and IL-5, which leads to high infiltration of eosinophils to the lamina propria. IL-4 and IL-13 stimulate the differentiation of AAM, which participate both in primary and secondary granulomas. IL-13 induces smooth goblet cell hyperplasia, thereby increasing mucus production and goblet cell derived Relm-β. Relm-β may interfere with parasite feeding and increased muscus, together with IL-13 induced smooth muscle contractility, act in a "weep and sweep" process to expel a proportion of parasites during a primary infection. **C.** DC in the lamina propria may encounter *H. polygyrus* antigen either by direct sampling from lumen or as larvae burrow through the tissue. HES conditioned, antigen loaded DC travel to the mLN where they induce Th2 differentiation in the presence of IL-4 and absence of IL-12. Some DC activated T-cells travel to the IFR where their interaction with B-cells lead to GC formation, as in PP. TFH cells secrete high levels of IL-4 which stimulates CSR to IgG1 and IgE. Th2 cells and $IgG1^+$ plasma cells exit the mLN and home to the LP. Protection against challenge infection is conferred in part by the trapping of larvae in the LP in a mechanism dependent on AAM and parasite specific antibodies. SED Subepithelial dome, M Microfold cells, IFR interfollicular region, TC T-cell zone, BC B-cell follicle, GC Germinal centre, TFH T-follicular helper cell, DC Dendritic cell, FDC Follicular dendritic cell, AAM alternatively activated macrophage, ILC2 Type 2 innate lymphoid cell, Eos Eosinophil, CSR class switch recombination, HES *H. polygyrus* excretory/secretory products.

2 AIMS

It is becoming increasingly clear that eosinophils specifically influence the development of immune events taking place in the PP. However, the contribution of the PP to immune responses against intestinal helminth infections and the influence of eosinophil deficiency on infection induced antibody responses in the PP have not been investigated. Furthermore, although eosinophils are implicated in Th2 induction, their role in this process during helminth infection is contentious. Finally, surprisingly little is known about the role of eosinophils in *H. polygyrus* survival and health.

The primary aims of this thesis therefore are:

1. **To ascertain whether Th2 induction and Th2 associated antibody production takes place in the PP following infection with *H. polygyrus***

2. **To determine whether eosinophil deficiency affects Th2 induction and antibody production following infection with *H. polygyrus***

3. **To revisit the role of eosinophils on the survival and fitness of *H. polygyrus***

These aims are summarized in figure 2.1

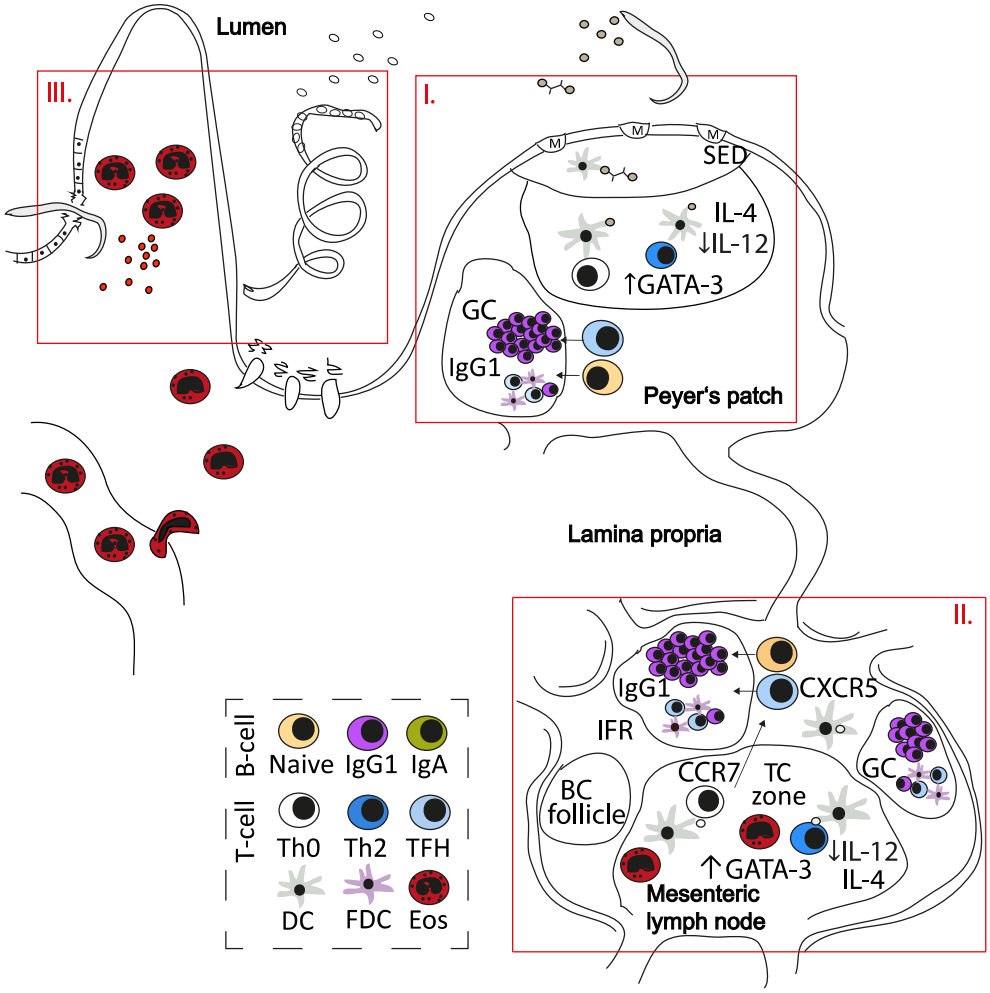

Figure 2.1 Aims of study
Three main aims are defined and highlighted in red boxes. **I.** To assess whether the PP participate in Th2 induction and IgG production in response to *H. polygyrus* infection. **II.** To assess whether eosinophils participate in Th2 induction and antibody production in response to *H. polygyrus*. **III.** To reassess whether eosinophils affect survival and/or fecundity of *H. polygyrus*. SED Subepithelial dome, M Microfold cells, IFR interfollocular region, TC T-cell zone, BC B-cell follicle, GC Germinal centre, TFH T-follicular helper cell, DC Dendritic cell, FDC Follicular dendritic cell, Eos Eosinophil.

3 RESULTS

3.1 ISOLATION OF SMALL INTESTINAL LAMINA PROPRIA CELLS

Isolating lamina propria lymphocytes is associated with lengthily protocols and low cell viability. From uninfected mice, viabilities between 50-70% can be expected. However, during the acute stages of *H. polygyrus* infection, standard protocols yield next to no viable lymphocytes from the siLP. Thus, in order to better study cells at the site of infection, a protocol kindly provided by Dr. W. Gause was repeatedly modified and tested until a procedure was established that yielded acceptable, albeit variable, levels of viable cells. The final protocol is detailed in materials and methods (M&M) and exemplary plots of viable cells, defined as negative for the viability dye Fluor506, are shown in Fig 3.1.

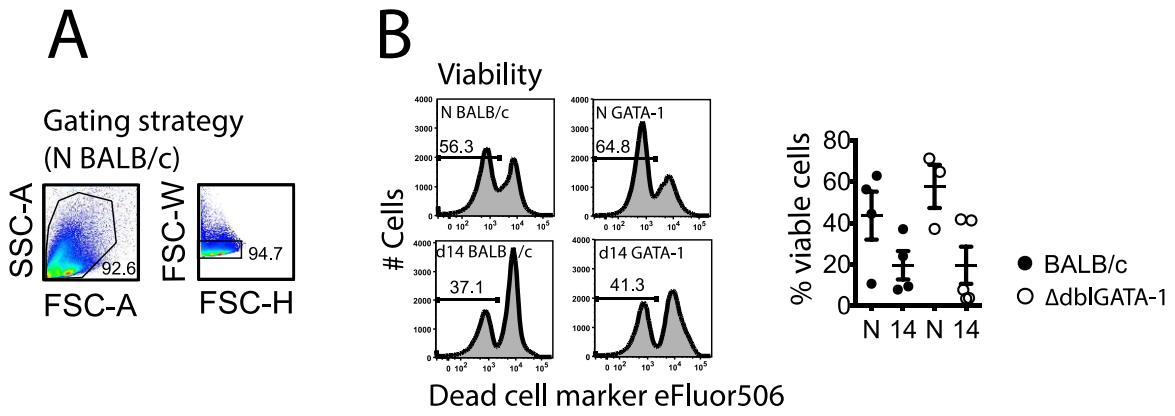

Figure 3.1 Viable cells can be isolated from the siLP of *H. polygyrus* infected mice.
Single cell suspensions from siLP were prepared from naïve and *d14 H. polygyrus* infected BALB/c and ΔdblGATA-1 mice as described in M&M. **A**. Exemplary gating strategy from naïve BALB/c mouse for the removal of duplicate cells. **B.** Exemplary histograms and frequencies of cells from BALB/c and ΔdblGATA-1 mice, negative for viability dye eF506.

Flow cytometry, using a viability dye, was chosen over standard cell-counting methods, to estimate the level of viable intestinal lymphocytes, as lamina propria single cell suspensions inevitably contain contaminating epithelial cells. However, as detailed below, the use of viability dyes may underestimate the levels of live cells from an organ containing high levels of eosinophils.

3.2 DETECTION AND SORTING OF EOSINOPHILS FROM SILP

Having established a protocol of isolating lamina propria cells from *H. polygyrus* infected mice, frequencies of intestinal eosinophils, defined as $CD45^{+}SSC^{hi}$Siglec-$F^{+}CCR3^{+}$, could be determined. By including viability dye eF506 it became apparent that cells qualifying as eosinophils all appeared positive for this marker, suggesting that they were not viable at the time of staining (Fig 3.2A). To confirm whether the entire eosinophil population was in fact dead, as well as to verify their morphology, $CD45^{+}SSC^{hi}CCR3^{+}$Siglec-F^{+} cells were sorted from the small intestine of naïve BALB/c mice and cytospins were performed. Fig 3.2B demonstrates that intact cells with typical eosinophil morphology could be isolated using this gating strategy. To avoid underestimating the frequency of resident and infiltrating eosinophils, viability dye eF506 was thereafter included only to control the quality of samples, but all $CD45^{hi}$ cells were included in the subsequent analysis of eosinophils from the siLP.

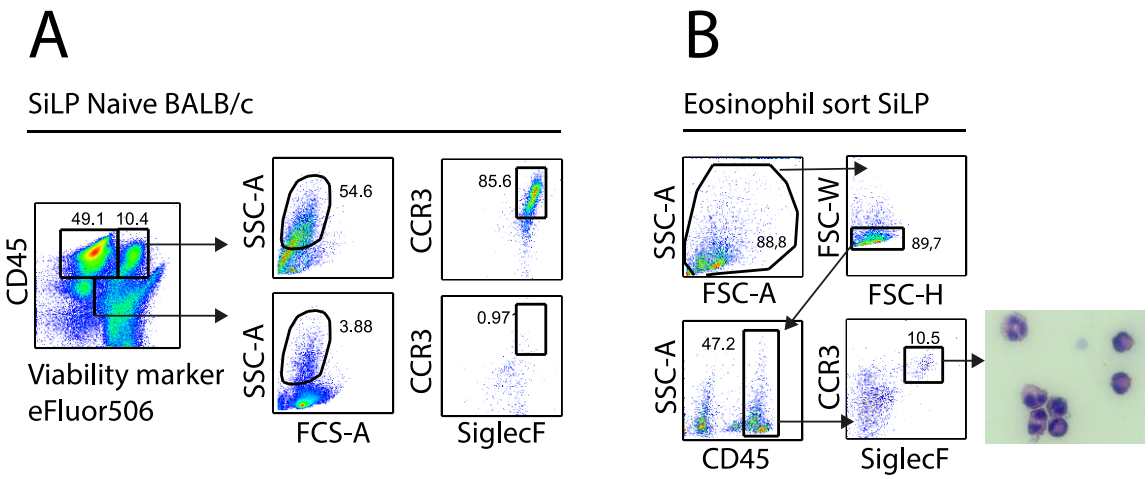

Figure 3.2 Eosinophils appear positive for dead cell marker eF506.
A. siLP single cell suspension from a naïve BALB/c mouse was stained with anti-mouse-CD45 and viability marker eF506. $CD45^{+}eF506^{+}$ (non viable) and $CD45^{+}eF506^{-}$ (viable) cells were analysed for expression of eosinophil markers CCR3 and Siglec-F. **B.** All $CD45^{+}$Siglec-$F^{+}CCR3^{+}$ cells were sorted from the siLP of one naive BALB/c mouse and visualized by cytospin.

3.3 *H. POLYGYRUS* INFECTION IN ΔDBLGATA-1 AND BALB/C MICE

3.3.1 Flow cytometric and histological detection of eosinophils

Being confident that the gating strategy shown in the previous section was appropriate for the detection of eosinophils, it was confirmed that between 12-20% of $CD45^{+}$ isolated siLP cells in uninfected BALB/c mice were eosinophils and that their frequencies increased significantly in mice infected with *H. polygyrus* (Fig 3.3A). As expected, no eosinophils were detected in naive or *H. polygyrus* infected eosinophils deficient ΔdblGATA-1 mice (Fig 3.3A).

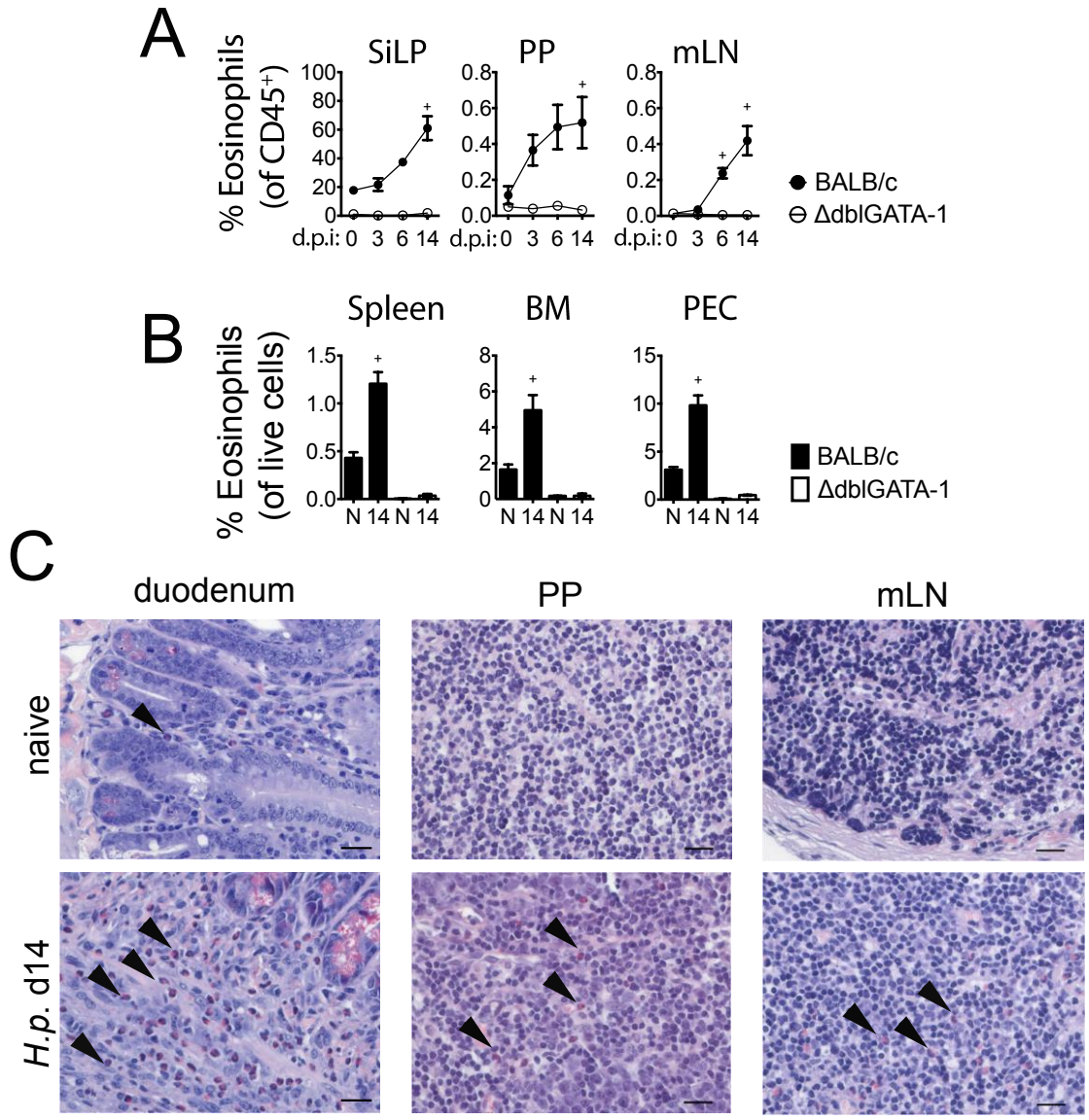

Figure 3.3 Local and systemic eosinophil infiltration following *H. polygyrus* infection. $CD45^{+}SSC^{hi}CCR3^{+}Siglec\text{-}F^{+}$ eosinophils were detected in the SiLP, PP and mLN of BALB/c (filled circles) and ΔdblGATA-1 mice (open circles), on indicated days post infection (d.p.i) with *H. polygyrus*. **B.** $SSC^{hi}Siglec\text{-}F^{+}$ cells were detected in the spleen, bone marrow (BM) and peritoneal exudate cellsy (PEC) of naïve and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars), at day 14 p.i. **C.** Sections of duodenum, PP and mLN from naive and *H. polygyrus* infected BALB/c mice stained with sirius red to detect eosinophils (arrow heads). Scale bar = 20μm. Mean ± SEM. n=3-4. Data are representative of > 2 independent experiments. + depicts a significant difference between naive and infected.

The same gating strategy was then used to detect eosinophils in the mLN and PP. Eosinophils were absent from the mLN of naive BALB/c mice and present only in low frequencies in the PP. Eosinophils were however found to infiltrate both the mLN and PP at day 14 p.i. although frequencies remained low (Fig 3.3A). Additionally, frequencies of eosinophils significantly increased in the bone marrow and infiltrated the spleen and the peritoneal cavity in *H. polygyrus* infected mice (Fig 3.3B). In these organs, eosinophils were detected simply as $SSC^{hi}Siglec\text{-}F$ cells. Histological stains of the duodenum, PP and mLN from BALB/c mice

confirmed that eosinophils are present in the siLP of naive animals and that they infiltrate this site in high numbers during acute *H. polygyrus* infection. Eosinophils were absent from the PP and mLN of naïve mice but were found in some (but not all) sections of PP and mLN from *H. polygyrus* infected animals (Fig 3.3C). Again, histological sections from ΔdblGATA-1 mice confirmed the absence of eosinophils in all compartments (not shown).

3.3.2 Cellularity of gut associated and systemic compartments

In line with previous studies (V.T. Chu et al. 2014; Jung et al. 2015), the PP of ΔdblGATA-1 mice were noticeably smaller than those of BALB/c mice, whereas other lymphoid and non-lymphoid compartments were macroscopically normal. The total numbers of live cells isolated from PP, mLN, siLP, spleen and PEC were therefore compared (Fig 3.4A). The smaller size of PP isolated from ΔdblGATA-1 mice correlated with significantly decreased cellularity compared to BALB/c mice. Considering the low frequencies of eosinophils in the PP of BALB/c mice (Fig 3.3A), the decrease in cell number in the PP of eosinophil deficient mice could not be explained by an absence of eosinophils. Furthermore frequencies of T-cells and B-cells did not differ between BALB/c and dblGATA-1 mice (see Figure 3.5 and 3.11), suggesting that eosinophil deficient mice do not necessarily have a defect in one particular cell population in PP, but rather a general hypo-trophy of the PP. Interestingly, infection with *H. polygyrus* decreased, rather than increased the number of cells in the PP of both mouse strains (Fig 3.4A). This was in contrast to the mLN, in which infection resulted in a major increase in cellularity, indicative of an active immune response as a result of retention of infiltrating and proliferating lymphocytes (Schulz et al. 2014). Eosinophil deficiency did not affect the size or cellularity of the mLN (Fig 3.4B)
Similar to the PP, the number of cells isolated from the siLP was generally lower in ΔdblGATA-1 mice compared to BALB/c (Fig 3.4C). However, considering the pronounced population of eosinophils residing in the siLP of BALB/c mice (Fig 3.3A), this was likely due to the absence of this population in ΔdblGATA-1 mice. The spleen of ΔdblGATA-1 mice were found to be comparable to BALB/c in terms of size and cellularity and the peritoneum contained comparable number of cells, which increased to a similar degree in both strains in response to *H. polygyrus* infection (Fig 3.4D,E).
Thus, eosinophil deficiency specifically affects the cellularity of the PP.

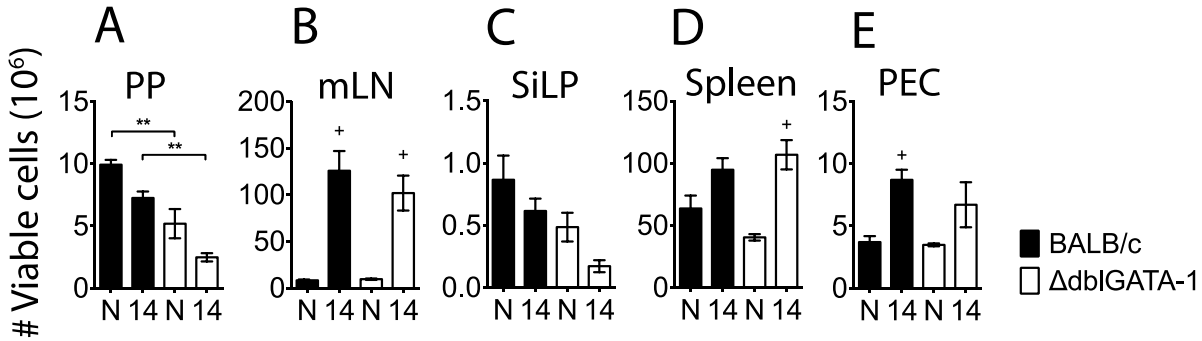

Figure 3.4 Reduced PP cellularity in eosinophil deficient dblGATA-1 mice
Single cell suspensions were obtained from naïve and d14 *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars) and cell numbers were determined using an automated cell counter. ± SEM are shown n=4-9. Data are representative of >2 experiments, except siLP (pooled data from 2 independent experiments). ** $p < 0.01$. + depicts a significant difference between naive and infected.

3.3.3 Th2 induction

Having shown that eosinophils infiltrate local and distal sites following *H. polygyrus* infection

and that their absence results in impaired development of PP, their influence on parasite induced immune responses was investigated in more detail. As helminths induce strong Th2 responses and as the role of eosinophils in such responses is controversial (Spencer & Weller 2010), Th2 responses were first addressed. At this point further analysis focussed on the PP as a gut associated lymphoid organ where eosinophils are known to influence organ development and B-cell responses, the mLN - an important site for intestinal immune responses, where eosinophils have no reported affect in homeostasis or immune responses and, finally, the siLP as the site of infection.

3.3.3.1 *GATA-3 expressing CD4⁺ cells*

To assess whether PP participate in immune responses to enteric helminth infection and whether eosinophils influence Th2 induction during *H. polygyrus* infection the Th2 induction, as measured by the expression of the transcription factor GATA-3, in CD4⁺ helper T-cells was first investigated. Figure 3.5A shows frequencies of CD4⁺ cells in the PP, mLN and siLP of BALB/c and ΔdblGATA-1 mice. Frequencies of CD4⁺ T-cells were not affected by eosinophil deficiency in any of the organs investigated (Fig 3.5A-C), but did decrease in the mLN in response to *H. polygyrus* infection, most likely as a result of increased frequencies of B-cells and other infiltrating cells (Fig 3.5B).

Addressing the first aim - whether the PP participate in the immune response to intestinal helminths, it was determined that GATA-3 expression within CD4⁺ cells was induced both in the PP and mLN of BALB/c mice (Fig 3.5D,E), suggesting that the PP represent an additional site for induction of Th2 cells. Furthermore, GATA-3⁺CD4⁺ cells were present at high frequencies in the siLP (Fig 3.5F).

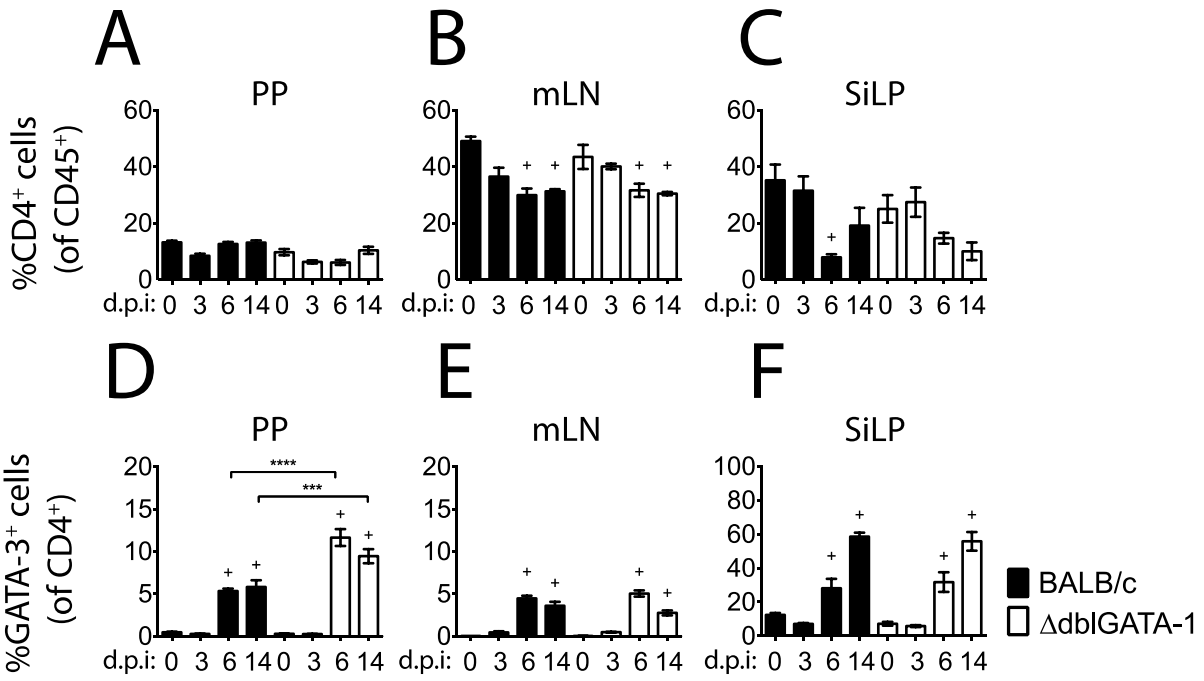

Figure 3.5 Increased CD4⁺ GATA-3 expression in the PP of eosinophil deficient ΔdblGATA-1 mice

A-C. Kinetic of CD4⁺ cells, at indicated days post infecteion (d.p.i.) with *H. polygyrus*, in the PP (A), mLN (B) and siLP (C) of BALB/c (black bars) and ΔdblGATA-1 mice (open bars). **D-F.** Kinetic of GATA-3 expressing CD4⁺ cells in BALB/c and ΔdblGATA-1 mice at indicated time points of *H. polygyrus* infection in the PP (D), mLN (E) and siLP (F). ± SEM. n=4-6. Data are

representative of > 3 independent experiments.. *** p < 0.001, ****p < 0.0001. + depicts a significant difference between naive and infected.

Addressing the second aim - whether eosinophils affect Th2 induction during *H. polygyrus* infection, GATA-3 induction between BALB/c and ΔdblGATA-1 mice was compared. From day 6 post infection (p.i.), frequencies of GATA-3$^+$CD4$^+$ cells had increased in all three compartments and in both mouse strains (Fig 3.5D-F). However, from day 6 p.i., frequencies of GATA-3 expressing CD4$^+$ cells in the PP of ΔdblGATA-1 mice, were significantly higher as compared to the PP of BALB/c mice (Fig 3.5D). High GATA-3 expression was maintained in CD4$^+$ cells from the PP of dblGATA-1 mice on day 14 p.i. In contrast to the PP, eosinophil deficiency had no impact on GATA-3 induction in the mLN, as levels of GATA-3 expression within CD4$^+$ cells was comparable between BALB/c and ΔdblGATA-1 mice on day 6 and 14 p.i. (Fig 3.5.E). In the siLP, GATA-3 expression was generally high amongst CD4$^+$ T-cells of both mouse strains (BALB/c: 14,58%±7,7 SD; ΔdblGATA-1 13,7±6,13 SD) but increase to > 70% of all CD4$^+$ cells in infected BALB/c and ΔdblGATA-1 mice at day 14 post infection (Fig 3.5F). Neither the base-line levels nor their increase as a result of *H. polygyrus* infection was affected by eosinophil deficiency.
Thus, induction of GATA-3 expressing cells takes place in the PP following *H. polygyrus* infection, but in the absence of eosinophils frequencies of Th2 cells in the PP doubles whereas eosinophil deficiency has no effect of Th2 induction elsewhere.

3.3.3.2 *Cytokine expression in CD4$^+$ cells*

Th2 responses are characterized by production of Th2 associated cytokines including IL-4, IL-5, IL-13 and to some extent IL-10 (Paul and Zhu, 2010). Thus, the expression of these cytokines was assessed in CD4$^+$ T-cells from the PP, mLN and siLP in BALB/c and ΔdblGATA-1 mice infected with *H. polygyrus*.
Again, addressing the first aim of this study it was noted that, in contrast to a significant induction of GATA-3 expression, which was demonstrated both in the PP and mLN of BALB/c mice (Fig 3.5), the increase in IL-4 and IL-10 production by CD4$^+$ T cells following *H. polygyrus* infection in PP of BALB/c mice did not reach statistical significance (Fig 3.6A). In contrast, frequencies of IL-4 and IL-10 expressing CD4$^+$ cells in the mLN of BALB/c mice, increased significantly following *H. polygyrus* infection (Fig 3.6B). In a separate experiment IL-13$^+$ CD4$^+$ cells were addressed in the PP and mLN. The presence of IL-13 producing cells in the PP of BALB/c mice had increased at day 14 p.i., but not significantly so, whereas IL-13 producing cells in the mLN had increased significantly (Fig 3.6D). Together, this suggests that GATA-3$^+$Th2 cells are induced in the PP but are restricted in Th2 cytokine production compared to Th2 cells in the mLN.
Comparing the cytokine responses of BALB/c and ΔdblGATA-1 mice it became clear that CD4$^+$ T cells in PP of ΔdblGATA-1 mice substantially increased the production of IL-4 on day 14 post *H. polygyrus* infection (Fig 3.6A). This effect of eosinophil-deficiency was restricted to IL-4, as expression of IL-10, IFN-γ and IL-13 in the PP was not affected (Fig 3.6A,D). Eosinophil deficiency had no effect on cytokine expression in the mLN (Fig 3.6B,D).
In the SiLP, frequencies of cells producing IL-4, IL-5 and IL-13 had all increased in both mouse strains to comparable levels by day 14 post *H. polyygrus* infection (Fig 3.6C).
Together, this suggests that eosinophils specifically regulate both the induction of Th2 cells (GATA-3 expression) and their function (IL-4 production), specifically in the PP.

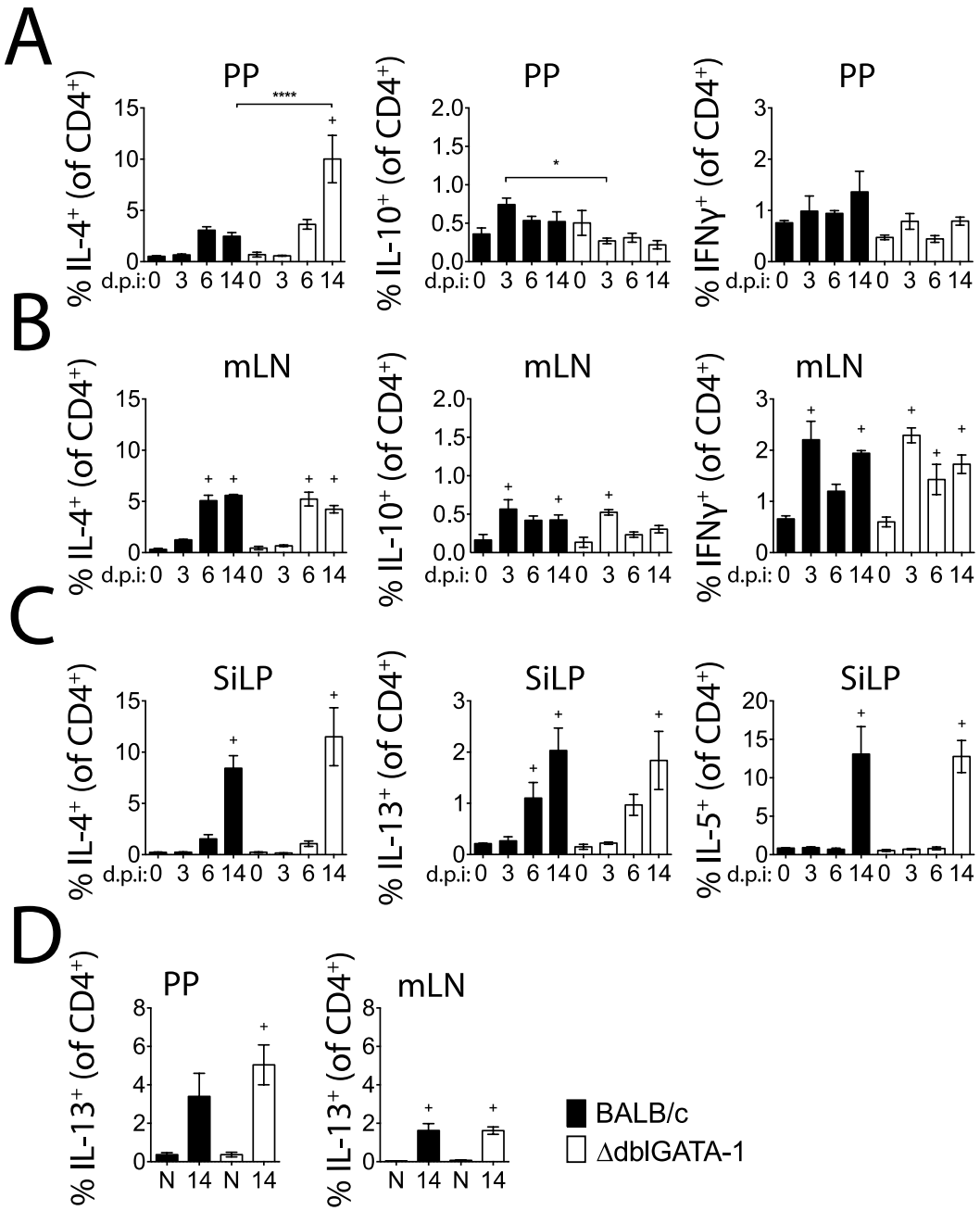

Figure 3.6 IL-4$^+$ CD4$^+$ cells in the PP are specifically regulated by eosinophils
A. Kinetic of IL-4, IL-5 and IL-13 expression within CD4$^+$ cells stimulated with PMA/ionomycin for 3 hours in the siLP of BALB/c (filled bars) and ΔdblGATA-1 (open bars) mice on indicated days post infection (d.p.i.) with *H. polygyrus*. **B, C.** Kinetic of IL-4, IL-10 and IFNγ expressing CD4$^+$ cells in the PP (B) and mLN (C) of naïve and *H. polygyrus* infected mice on indicated d.p.i. **D.** Expression of IL-13 within CD4$^+$ cells in the PP and mLN of naïve and *H. polygyrus* infected mice at d14 post infection. Mean ± SEM. n=4-10 (A,B,C) n=3-5 (D). B and C are representative of > 3 experiments. (A, D) are from 1 experiment. *p < 0.05, ****p < 0.0001. + depicts a significant difference between naive and infected.

3.3.4 FoxP3$^+$ regulatory T-cell frequencies

FoxP3 expressing regulatory T-cells (Treg) regulate Th2 responses during infections with *H.*

polygyrus (Finney et al. 2007) and have recently been suggested as a mechanism by which *H. polygyrus* regulate Th2 responses in the PP (Mosconi et al. 2015). It was thus possible that the regulation of Th2 function in the PP of BALB/c mice and the increased levels of GATA-3 and IL-4 expression in the PP of ΔdblGATA-1 mice were a consequence of diminished Treg numbers. However the levels of FoxP3$^+$ T-cells did not differ significantly between the PP and mLN of BALB/c mice or between ΔdblGATA-1 and BALB/c mice (Fig 3.7A,B). Surprisingly, Foxp3 expressing CD4$^+$ cells were elevated in the SiLP of naive ΔdblGATA-1 mice, but found in similar frequencies in mice infected with *H. polygyrus* (Fig 3.7C).
Thus, the regulation of Th2 function in the PP of BALB/c mice and the lack of regulation in the PP of ΔdblGATA-1 mice is not associated with changes in frequencies of Foxp3$^+$ Treg expression Treg frequencies.

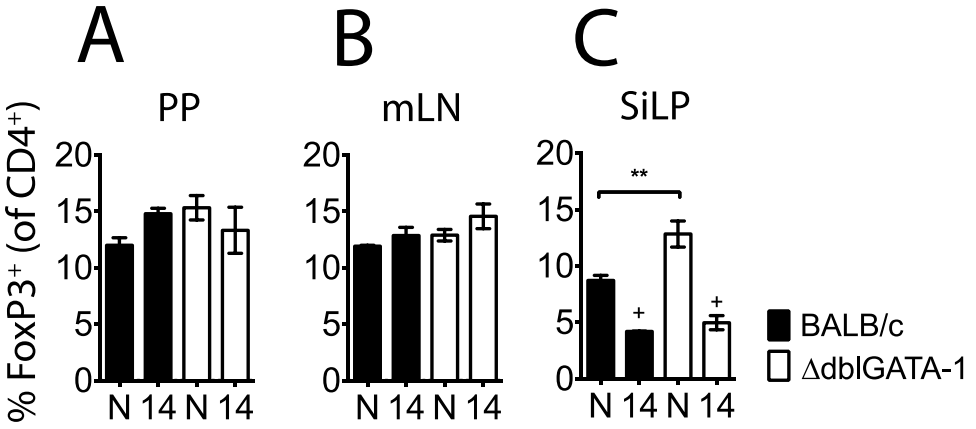

Figure 3.7 Treg cells are not affected by eosinophil deficiency.
A-C. Frequencies of Foxp3$^+$CD4$^+$ cells in the PP (A), mLN (B) and siLP (C) of naive and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars). Mean ± SEM. n=4-5. Data are representative of > 3 experiments. **p < 0.01. + depicts a significant difference between naive and infected.

3.3.5 T-follicular helper cells

It has been demonstrated that most IL-4 producing cells in the mLNs following *H. polygyrus* infection are T follicular helper cells (TFH) (King & Mohrs 2009) marked by the expression of CXCR5 and PD-1 (Haynes et al. 2007). Thus, the frequencies of TFH cells and their expression of IL-4 were assessed in the PP and mLN of BALB/c and ΔdblGATA-1 following infection with *H. polygyrus*.

3.3.5.1 *CXCR5$^+$ PD-1$^+$ TFH cell frequencies*

Firstly, the proportion of TFH cells out of all IL-4 expressing cells from the PP and mLN was analyzed in *H. polygyrus* infected BALB/c and ΔdblGATA-1 mice. 12-15% (mLN) and 19-22% (PP) of all IL-4$^+$CD4$^+$ cells were CXCR5$^+$PD-1$^+$. TFH cells and this did not differ between ΔdblGATA-1 and BALB/c mice (Fig 3.8A).

Next the total frequencies of TFH and non-TFH cells, regardless of their IL-4 status, were analysed. Comparing frequencies of TFH cells in the PP versus the mLN of BALB/c mice it was noted that the PP contained relatively high frequencies of TFH even in the absence of infection (Fig 3.8B), which is in accordance with the continuous IgA class switching at this site (Macpherson et al. 2008). *H. polygyrus* infection resulted in a non-significant increase in TFH in the PP of BALB/c mice (Fig 3.8B). Few TFH cells were detected in the mLN of naive BALB/c

mice, whereas *H. polygyrus* infection resulted in a significant increase in this cell population (Fig 3.8C).
Comparing TFH cell frequencies between BALB/c and ΔdblGATA-1 mice, it was noted that eosinophil deficiency did not affect TFH cells in the PP or the mLN. That is, TFH frequencies were equally elevated in the PP of naive ΔdblGATA-1 mice and were non-significantly up-regulated in response to *H. polygyrus* infection, whereas frequencies of TFH cells in the mLN of naive ΔdblGATA-1 mice were low, but significantly increased in response to *H. polygyrus* infection.
Thus, the PP are a site requiring the continuous presence of TFH cells, whereas the mLN up-regulate this population following enteric infection. Furthermore, eosinophils are not required for the support or regulation of TFH cells in the PP.

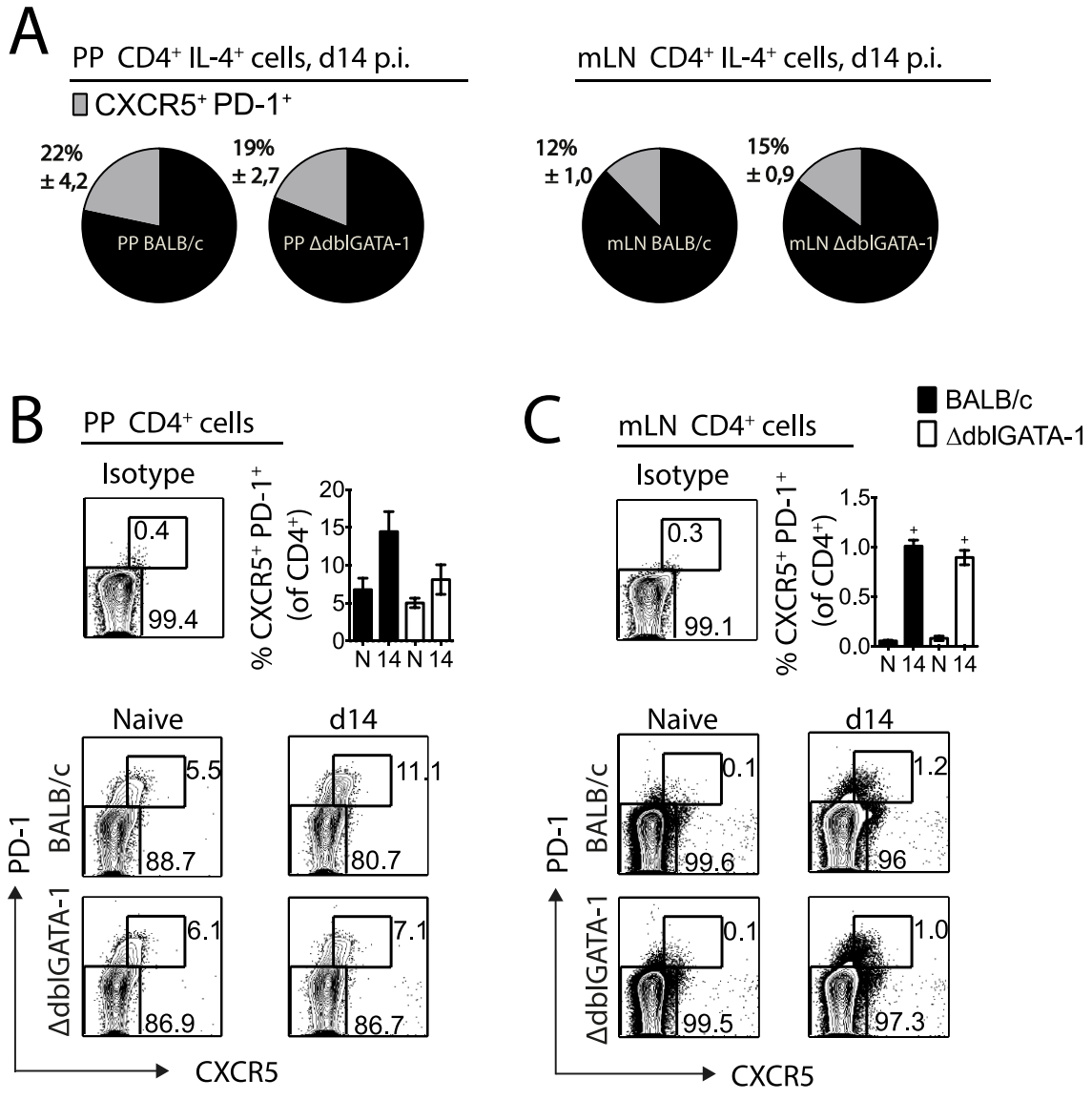

Figure 3.8 Normal frequencies of TFH cells in eosinophil deficient Δ dblGATA-1 mice
A. Proportion of CXCR5+PD-1+ TFH cells within all IL-4 producing cells in the PP and mLN of BALB/c (black bars) and ΔdblGATA-1 (open bars) mice at day 14 post infection with *H. polygyrus*. **B, C** exemplary FACS plots and frequencies of TFH cells in the PPs (B) and mLNs (C) of naïve and *H. polygyrus* infected BALB/c and ΔdblGATA-1 mice. Mean ± SEM. n=4-5.

Data are representative of 4 independent experiments. + depicts a significant difference between naive and infected.

3.3.5.2 *IL-4 expression by CXCR5$^+$ PD-1$^+$ TFH cells*

Having demonstrated that the PP are a site of continuous TFH cell presence and that frequencies of TFH cells were normal in eosinophil deficient mice, IL-4 production by CXCR5$^+$PD-1$^+$ TFH cells and CXCR5$^-$PD-1$^-$ non-TFH cells were next compared.
Comparing the PP and mLN of BALB/c mice it again became apparent that the PP are not a site for significant induction of IL-4 in response to *H. polygyrus* infection (Fig 3.9A). Neither the frequencies of IL-4$^+$ TFH or non-TFH were up-regulated significantly in the PP of BALB/c mice in response to infection. However, IL-4 production per cell basis, as assessed by median florescence intensity (MFI) revealed a slight increase in IL-4 production in non TFH cells present in the PP of BALB/c mice (Fig 3.9C). In contrast to the PP, the mLN of BALB/c mice showed a strong increase in the frequencies of IL-4 producing TFH and non-TFH cells (Fig 3.9B). However, only TFH cells were found to increase IL-4 production on a per-cell basis in the mLN of BALB/c mice (Fig 3.9D).
Comparing the TFH IL-4 response between BALB/c and ΔdblGATA-1 mice, it was confirmed that eosinophils specifically regulate IL-4 responses in the PP. Frequencies of both TFH and non-TFH IL-4$^+$ cells were significantly higher in the PP of ΔdblGATA-1 mice compared to BALB/c animals, this difference being particularly prominent in the TFH cell compartment (Fig 3.9A). Similarly, IL-4 production on a per cell basis was substantially higher in TFH cells from the PP of ΔdblGATA-1 mice compared to WT (Fig 3.9C). In contrast to the PP, eosinophil deficiency had no influence on TFH or non-TFH production of IL-4 in mLN, whether assessed by frequencies of IL-4+ cells or as IL-4 production per cell (Fig 3.9B,D).

Thus, the PP are not a major site for TFH cell IL-4 induction in response to enteric helminth infection, whereas infection in the absence of eosinophils leads to a de-regulated IL-4 response in the PP with a prominent increase in IL-4 production by TFH cells.

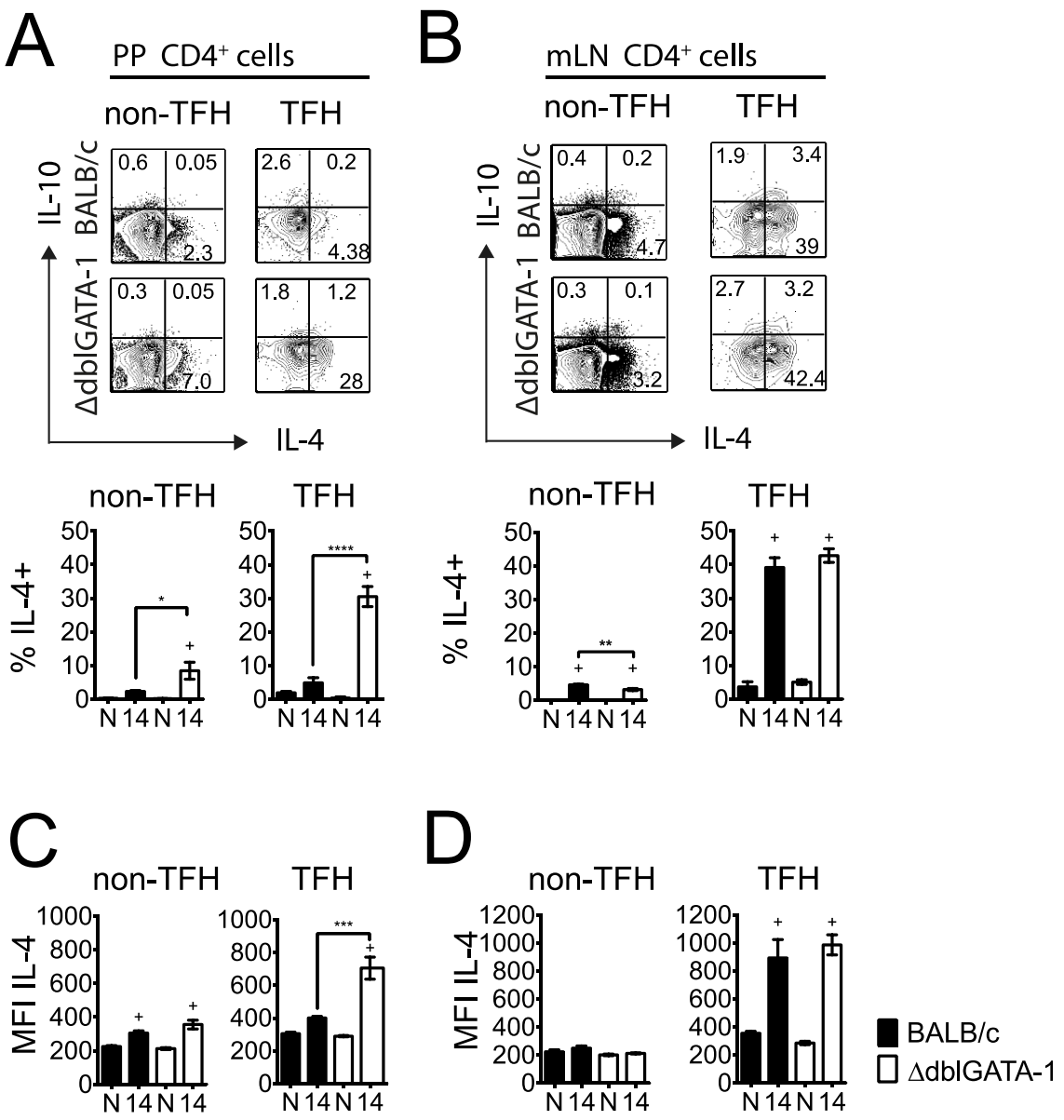

Figure 3.9 Eosinophils regulate TFH cell derived IL-4 in the PP
A, B. Exemplary FACS plots and frequencies of IL-4$^+$ cells within CXCR5$^+$PD-1$^+$ TFH helper cells and CXCR5$^-$PD-1$^-$ non TFH cells in the PP (A) and mLN (B) of BALB/c (black bars) and ΔdblGATA-1 mice (open bars) at day 14 post infection with *H. polygyrus*. **C, D**. Median fluorescence intensity (MFI) of IL-4 within TFH and non-TFH cells in the PP (C) and mLN (D) of naive and *H. polygyrus* infected mice. Mean ± SEM. n=4-5. Data are representative of 4 independent experiments. *p < 0.05, *** < 0.001, ****p < 0.0001. + depicts a significant difference between naive and infected.

3.3.6 B-cell responses

Eosinophils are associated with several aspects of B-cell maintenance (Wong et al. 2014; Chu et al. 2011) and have been demonstrated to support IgA class switching in the PP (V.T. Chu et al. 2014; Jung et al. 2015), but the role of eosinophil in infection induced B-cell responses

was not assessed. Thus, the next step was to investigate the effect of eosinophil deficiency on the induction of *H. polygyrus* induced antibody responses

3.3.6.1 *Total and HES specific serum IgG1 and IgE*

As IL-4 from TFH cells stimulates B-cell class switching to the Th2 associated antibody isotypes IgG1 and IgE (King and Mohrs 2009) and as eosinophil deficiency led to increased levels of TFH derived IL-4 in the PP, serum levels of IgG1 and IgE were determined. In accordance with the increase in IL-4 seen in the PP of infected ΔdblGATA-1 mice, levels of IgG1 and IgE were found to be significantly higher in ΔdblGATA-1 mice at day 14 p.i. as compared to sera from BALB/c mice (Fig 3.10A). At day 14 p.i. antibodies specific for *H. polygyrus* excretory/secretory products (HES) could not be reliably detected. In order to determine whether eosinophil deficiency also influenced the development of parasite specific antibodies, mice were cured form *H. polygyrus* infection with the anti helminthic pyrantel pamoate and re-infected three weeks later. Serum was then taken at day 11 post challenge and tested for HES specific IgG1 and IgE. As seen for total levels of IgG1 and IgE, ΔdblGATA-1 mice had significantly higher levels of circulating HES specific IgG1 and IgE on day 11 post challenge infection (Fig 3.10B).

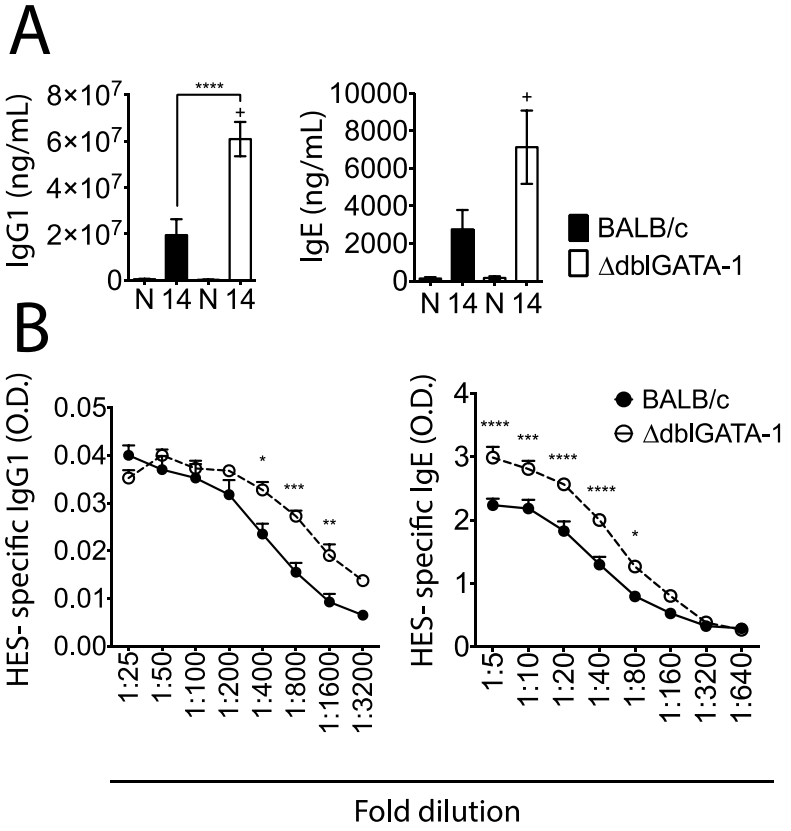

Figure 3.10 Eosinophil deficiency results in increased serum IgG1 and IgE.
A. Total IgG1 and IgE detected in sera of naive and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars). **B.** HES specific serum IgG1 and IgE in sera at day 11 post challenge infection with *H. polygyrus* in BALB/c (filled circles) and ΔdblGATA-1 (open circles). Mean ± SEM. (A) shows pooled data from two independent experiments n=7-11. (B) is from one representative experiment n=4-5. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. + depicts a significant difference between naive and infected.

3.3.6.2 *PNA^{hi}B220^{+} germinal centre B-cell frequencies*

CD40-CD40L interaction between TFH cells and B-cells, together with antigen recognition, results in induction of germinal centres (GC) in which B-cells proliferate, undergo somatic

hypermutation and class switch recombination (De Silva & Klein 2015). To determine whether eosinophils exert a regulatory effect on the formation of GC, cells from the PP and mLN were stained with the B-cell marker B220 and peanut agglutinin (PNA), the latter specifically binding to B-cells that have entered the germinal centre reaction (Shinall et al. 2000).

Firstly, the frequencies of $B220^{+}$ B-cells were compared. The PP of naïve BALB/c mice contained high levels of B-cells, which did not change in response to *H. polygyrus* infection. In contrast, the mLN of naïve BALB/c mice contained lower frequencies of B-cells, but significantly up-regulated this population in response to infection (Fig 3.11A). Eosinophil deficiency did not affect B-cell levels in the PP or in the mLN.
Comparing the levels of $PNA^{hi}B220^{+}$ GC B-cells in the PP and mLN of BALB/c mice, a similar picture was seen concerning GC B-cells as previously demonstrated with TFH cells, illustrating the intimate interplay between these cells types (Crotty 2011). Specifically, the PP of naive BALB/c mice contained relatively high levels of GC B-cells, indicating the continuous class switching to IgA in this compartment (Macpherson et al. 2008). *H. polygyrus* infection did not result in increased frequencies of GC B-cells in the PP of BALB/c mice (Fig 3.11B). In support of this, expression of activation-induced cytidine deaminase (AID) - an enzyme essential for class switch recombination and expressed specifically in the GC (Muramatsu et al. 1999), did not increase in the PP of BALB/c mice infected with *H. polygyrus* (Fig 3.11D). In contrast to the PP, the mLN of naive BALB/c mice contained only low frequencies of GC B-cells, but this population significantly increased in response to *H. polygyrus* infection (Fig 3.11C). Similarly, expression of AID was significantly up-regulated in the mLN of BALB/c mice infected with *H. polygyrus* (Fig 3.11D).
Comparing the frequencies of GC B-cells in BALB/c and ΔdblGATA-1 mice it was determined that eosinophil deficiency did not significantly affect this population in the PP or in the mLN (Fig 3.11B,C). However, GC B-cells tended to be lower in the PP of naive ΔdblGATA-1 mice compared to naive BALB/c mice, although this was not statistically significant (Fig 3.11B). Like in BALB/c mice, frequencies of GC B-cells were low in the mLN of naive ΔdblGATA-1 mice, but increased substantially in response to *H. polygyrus* infection (Fig 3.11C). Eosinophil deficiency did not affect GB B-cell frequencies in the mLN Furthermore, no difference in expression of AID was found between BALB/c and ΔdblGATA-1 mice in the PP or in the mLN (Fig 3.11D).

Thus the PP are a site of continuous GC formation, whereas B-cells in the mLN are induced to enter the GC reaction by *H. polygyrus* infection. Furthermore, eosinophil deficiency does not affect the capacity of B-cells to enter GC reactions in the PP or in the mLN.

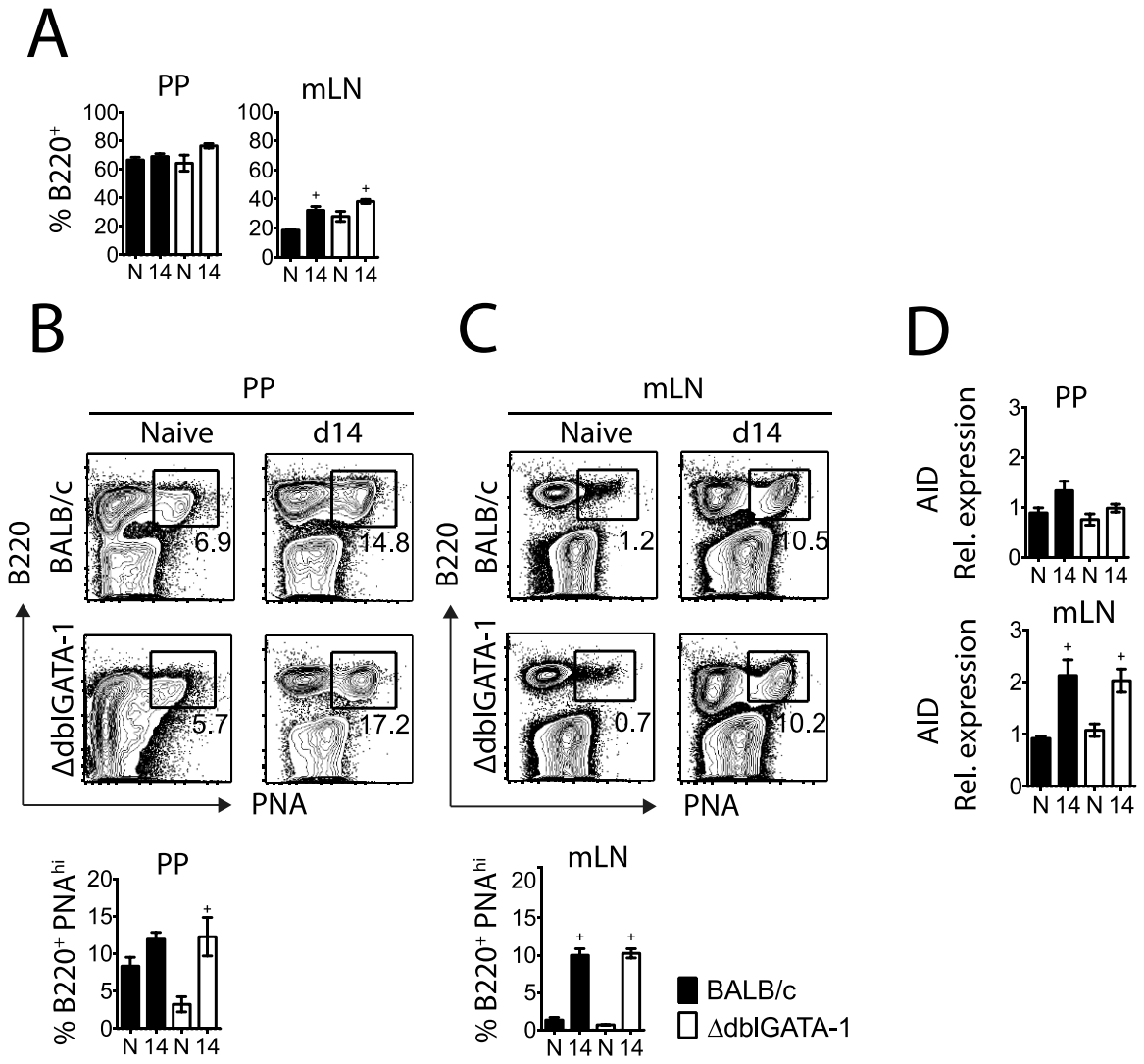

Figure 3.11 Eosinophil deficient Δ dblGATA-1 mice have normal levels of GC B-cells
A. B220$^+$ B-cells in the PP and mLN **B, C.** Representative FACS plots and frequencies of B220$^+$ PNA^{hi} GC B-cells in the PP (B) and mLN (C) of naive and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars). **D.** Relative expression of activation induced cytidine deaminase (AID), normalized to housekeeping gene GUSB and expressed relative to naive BALB/c, in the PP and mLN of naive or *H. polygyrus* infected BALB/c and ΔdblGATA-1 mice. Mean ± SEM. n=3-5. (A) Pooled data from two experiments. (B-D) Data representative of > 3 independent experiments. + depicts a significant difference between naive and infected.

3.3.6.3 *Germinal centre IgA and IgG1 isotype class switching*

As differences in GC formation between BALB/c and ΔdblGATA-1 mice infected with *H. polygyrus* could not explain the differences seen in serum IgG1 and IgE, it was likely that the flavour of the B-cell response was affected by eosinophil deficiency. Thus, the expression of IgG1, IgM and IgA were co-examined in B220$^+$ PNA^{hi} GC B-cells.
Comparing the PP and mLN of BALB/c mice, it was noted that both organs contained only low levels of IgG1$^+$ B cells in the absence of infection (Fig 3.12A). Furthermore, *H. polygyrus* infection did not result in a significant increase in IgG1$^+$ GC B-cells in the PP of BALB/c mice (Fig 3.12A). This was in contrast to the mLN, in which *H. polygyrus* infection resulted in substantial IgG1 class switiching (Fig 3.12B), which again indicates a dichotomy between the immune responses taking place in these two gut associated lymphoid organs.

Analysis of IgA$^+$ GC B-cells confirmed the PP as the main site for IgA class switching, as up to 30% of GC B-cells from naive BALB/c mice were IgA$^+$. *H. polygyrus* infection resulted in an additional small increase in IgA$^+$ GC B-cells in the PP of BALB/c mice. In contrast to the PP, the mLN of naive BALB/c mice contained only ca. 1% of IgA$^+$ GC B-cells and *H. polygyrus* infection resulted in almost complete abolishment of this population (Fig 3.12B).
Both the PP and mLN of naive BALB/c mice contained high levels of IgM$^+$ GC B-cells, which decreased as a result of *H. polygyrus* infection, as class switching to IgA (PP) and IgG1 (mLN) increased (Fig 3.12A, B).
Comparing the antibody response in BALB/c and ΔdblGATA-1 mice, it was confirmed that eosinophils specifically regulate immune events in the PP. Specifically, *H. polygyrus* infection resulted in a considerable increase in frequencies of IgG1$^+$ GC B-cells in the PP of ΔdblGATA-1 mice compared to *H. polygyrus* infected BALB/c mice. In contrast, frequencies of IgA$^+$ GC B-cells decreased substantially in the PP of ΔdblGATA-1 mice (Fig 3.12A).
B-cell class switching in the mLN was not affected by eosinophil deficiency, as ΔdblGATA-1 mice significantly increased IgG1$^+$ GC B-cells in response to *H. polygyrus*, similar to BALB/c mice and had comparable frequencies of IgA$^+$ cells (Fig 3.12B).
To summarize, the main IgG1 response against *H. polygyrus* takes place in the mLN, and is not affected by eosinophil deficiency. Eosinophils however regulate the B-cell response in the PP ensuring maintenance of IgA antibodies during the course of enteric infections. In the absence of eosinophils, *H. polygyrus* infection leads to an immune response in the PP comparable to the mLN, that is; increased B-cell class switching to IgG1 and significant reduction in IgA production.

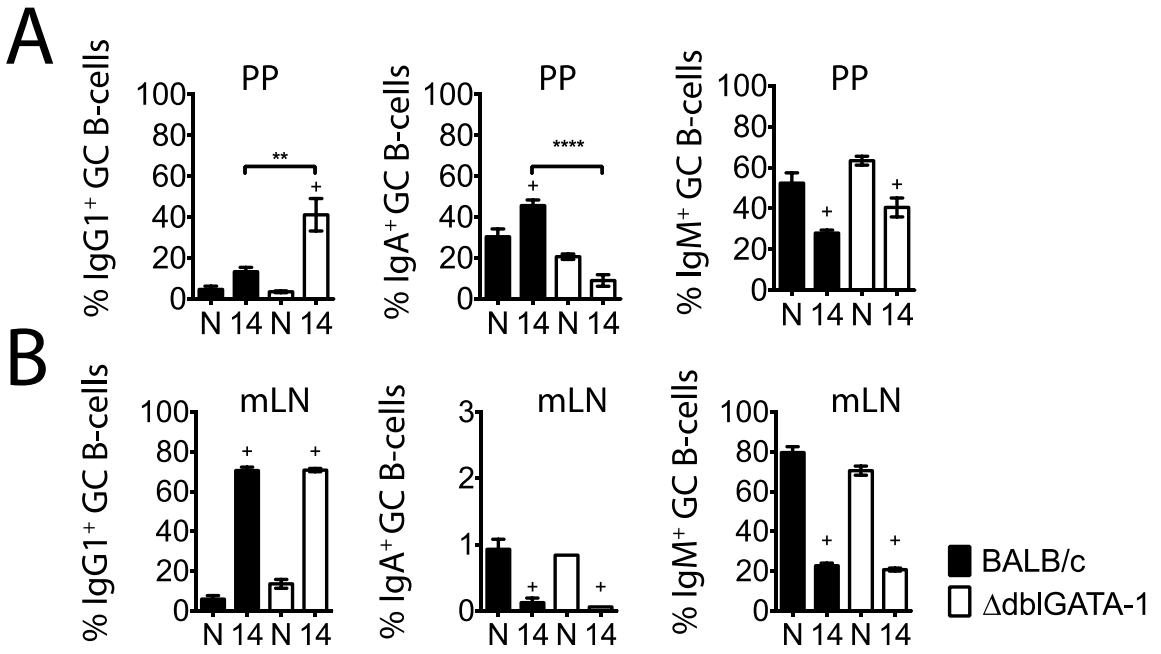

Figure 3 12 High levels of IgG1 class switching in the PP of eosinophil deficient Δ dblGATA-1 mice
A, B. Frequencies of IgG1$^+$, IgA$^+$ and IgM$^+$ GC B-cells in the PP (A) and mLN (B) of naive and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars). Mean ± SEM. n=3-5. Data are representative of > 3 independent experiments. $^{**}p < 0.01$ and $^{****}p < 0.0001$. + depicts a significant difference between naive and infected.

3.4 SUMMARY AIM 1 AND 2

The two initial aims of this thesis were to investigate the role of the PP (irrespective of eosinophils) and the role of eosinophils, in Th2 induction and antibody responses to infection with an enteric nematode. Towards this, the data presented so far clearly demonstrate a dichotomy existing between the functions of PP and mLN. Specifically; the PP are an important site for IgA production and do not significantly contribute to Th2 and IgG1 responses following *H. polygyrus* infection. Rather, the mLN represent the main site for the induction of immune responses against the parasite. These data further demonstrate that the "division of labor" between the PP and mLN seen in BALB/c mice is disturbed in the absence of eosinophils, as the PP of eosinophil deficient mice respond to nematode infection in a way comparable to the mLN.
The next task was to assess processes known to be important in Th2 induction in an attempt to explain how the presence of eosinophils may regulate Th2 responses in the PP.

3.5 TISSUE DERIVED CYTOKINE EXPRESSION IN THE SILP

The tissue derived cytokines IL-33, TSLP and IL-25, also called 'alarmins', have been implicated in early events leading to the induction of Th2 responses (Fallon et al. 2006). It was thus hypothesized that eosinophils present in the small intestine in high numbers might regulate the expression of alarmins and hence restrict overt local Th2 induction in the PP embedded in the mucosal tissue. Real time quantitative PCR (RT-qPCR) was therefore performed to investigate whether eosinophil deficiency resulted in impaired expression of one or more of these cytokines. Expression of IL-33 did not significantly increase in the siLP of BALB/c mice infected with *H. polygyrus* and only slightly increase in ΔdblGATA-1 mice (Fig3.13A). Furthermore, expression of TSLP did not increase in the siLP of infected BALB/c mice. However, TSLP expression was up-regulated in the intestine of ΔdblGATA-1 mice in response to *H. polygyrus* infection and was significantly higher compared to BALB/c mice on day 3 and 6 p.i. (Fig3.13B). On the contrary, whereas *H. polygyrus* infection potently boosted expression of IL-25 in the siLP of BALB/c mice, ΔdblGATA-1 mice did not significantly up-regulate expression of this cytokine (Fig 3.13C). As this coincided temporally with significant eosinophil infiltration to the lamina propria (Fig 3.3) and as eosinophils have been shown to express high levels of IL-25 mRNA (Wang et al. 2007), it is possible that the increase in IL-25 expression in BALB/c mice infected with *H. polygyrus* was due to infiltrating eosinophils.
Thus, eosinophils regulate expression of TSLP and may contribute to increased IL-25 production in the siLP during *H. polygyrus* infection.

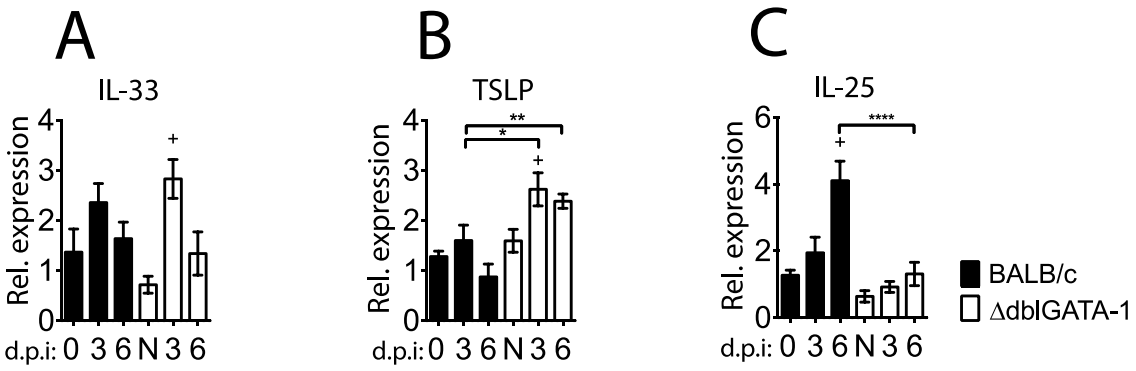

Figure 3.13 Small intestinal expression of tissue derived cytokines
A-C IL-33 (A), TSLP (B) and IL-25 (C) mRNA expression in the siLP of BALB/c (black bars) and ΔdblGATA-1 mice (open bars) at indicated days post infection (d.p.i.). Expression is normalized to housekeeping gene GUSB and calculated relative to naive BALB/c. Mean ± SEM. n = 6-8. Data are representative of 3 independent experiments. * $p < 0,05$ ** $p < 0.01$ and **** $p < 0.0001$. + depicts a significant difference between naive and infected.

3.6 CD103+ DENDRITIC CELLS

One task of TSLP is to support Th2 responses via the suppression of DC derived IL-12 (Massacand et al. 2009). More [SR1]recently and in contrast, $CD103^{+}CD11b^{-}$ dendritic cells have been implicated in inhibition of Th2 responses through the production of IL-12 (Everts et al. 2015) Thus, DC cell subsets were next analysed in the PP and mLN of BALB/c and ΔdblGATA-1 mice.

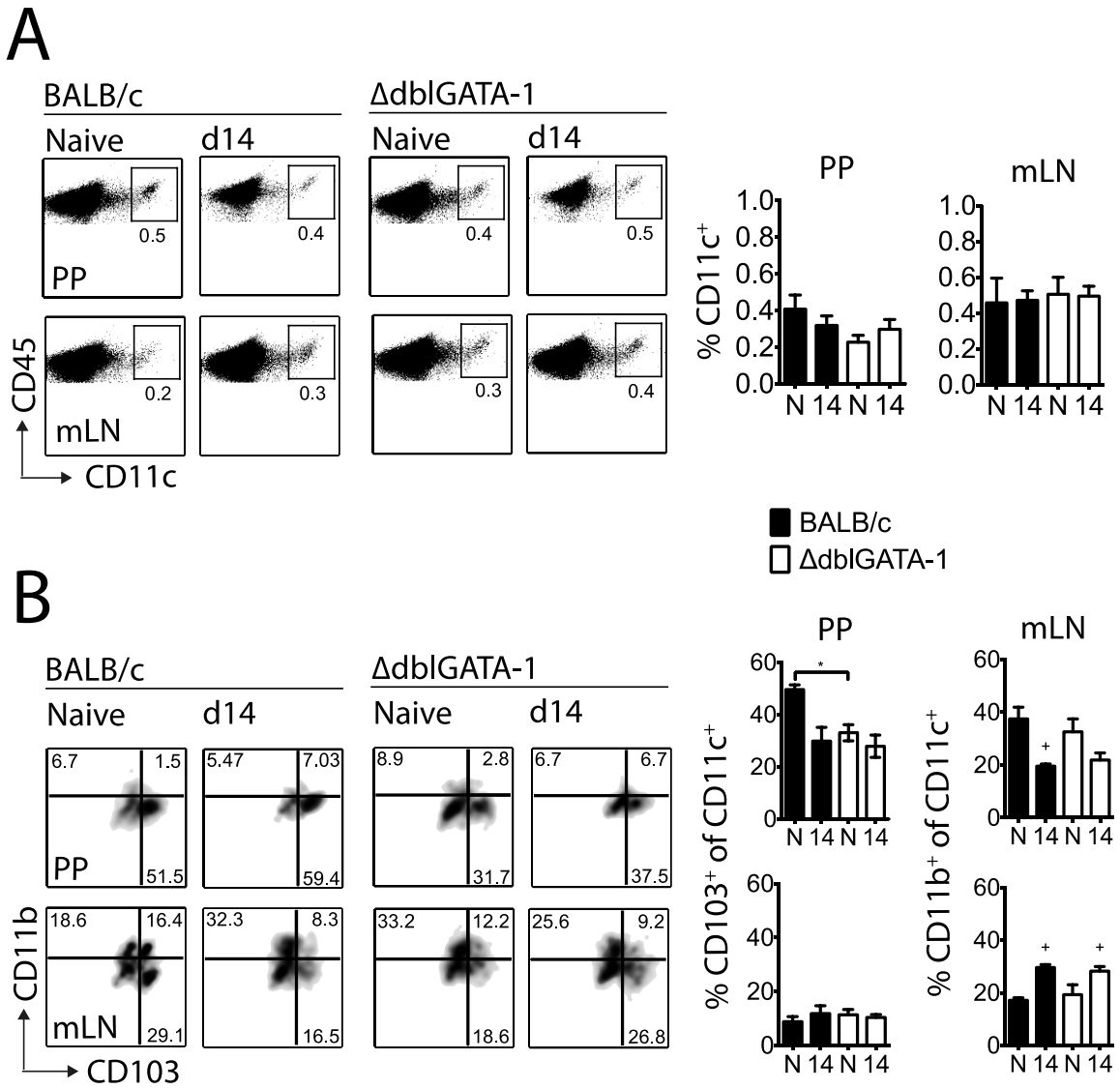

Figure 3.14 Base line levels of $CD103^{+}$ DCs are decreased in PP in the absence of eosinophils
A. Exemplary FACS plots and frequencies of $CD11c^{hi}$ cells in the PP and mLN of naive and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars). Cells are pre-gated on live, $CD45^{+}$ cells **B.** Exemplary FACS plots depicting expression of CD103 and CD11b by $CD11c^{hi}$ DC in the PP and mLN of BALB/c and ΔdblGATA-1 mice. Mean ± SEM. Plots are representative from one experiments, bar graphs represent pooled data from two experiments. Data are representative of two independents experiments, n = 5-10. ** $p < 0,01$. + depicts a significant difference between naive and infected.

The frequencies of $CD11c^{hi}$ cells were comparable between the PP and mLN as well as between BALB/c and ΔdblGATA-1 mice (Fig 3.14A). When analysing DC subsets based on CD103 and CD11b expression, it was noted that, although frequencies of $CD11b^{+}$ DC were comparable between BALB/c and ΔdblGATA-1 mice, both in the PP and mLN, levels of

CD103^{+} DC were significantly higher in PP of naïve BALB/c mice compared to the PP of ΔdblGATA-1 mice (Fig 3.14B). As these cells are known to produce IL-12 (Everts et al. 2015), it will be important in the future to analyse the cytokine expression of these cells in the PP of BALB/c and ΔdblGATA-1 mice, as a decreased availability of IL-12 could contribute to an increase in Th2 responses in the absence of eosinophils. Furthermore, it will
be important to assess this population early following infection, as the current analysis was restricted to day 14p.i., at which point all DC subsets were comparable between BALB/c and ΔdblGATA-1 mice.

3.7 EOSINOPHIL DEPLETION

In an attempt to confirm that eosinophils were responsible for increases in Th2 induction and B-cell class switching to IgG1 in the PP of *H. polygyrus* infected ΔdblGATA-1 mice, an eosinophil depletion regime using anti-Siglec-F monoclonal antibodies (mAb) was applied in BALB/c mice and Th2 and B-cell responses were evaluated in the PP and mLN.

3.7.1 Treatment with anti-Siglec-F

BALB/c mice were injected intra-peritoneally with 20µg anti-Siglec-F mAb, starting 2 days prior to *H. polygyrus* infection, followed by every second day until 14 days p.i. Fig 3.15A shows a schematic overview of the injection/infection regime. Blood was taken before commencement of mAb injection and at regular intervals thereafter in order to monitor circulating eosinophil levels. In mice injected with control RatIg2a, circulating eosinophils increased steadily starting 6 days post *H. polygyrus* infection, but were effectively depleted from the circulation of naïve and *H. polygyrus* infected mice treated with anti-Siglec-F antibodies (Fig 3.15B).
At day 14 p.i. mice were sacrificed and eosinophils detected in the siLP by flow cytometry and histology. Although eosinophils were completely depleted from the blood, a portion of eosinophils remained in the siLP of anti-Siglec-F treated mice (Fig 3.16A, B). Both in naïve and *H. polygyrus* infected mice anti-Siglec-F treatment resulted in a 50% reduction in frequencies of SSC^{hi}CCR3^{+}Siglec-F^{+} cells detected in the siLP, compared to RatIgG2a treated mice (Fig 3.16C, D). Histological duodenum sections confirmed that anti-Siglec-F treatment incompletely depleted siLP eosinophils with a reduction of about 66% (Fig 3.16E, F).
Thus, treatment with anti-Siglec-F effectively depleted peripheral, but not intestinal eosinophils.

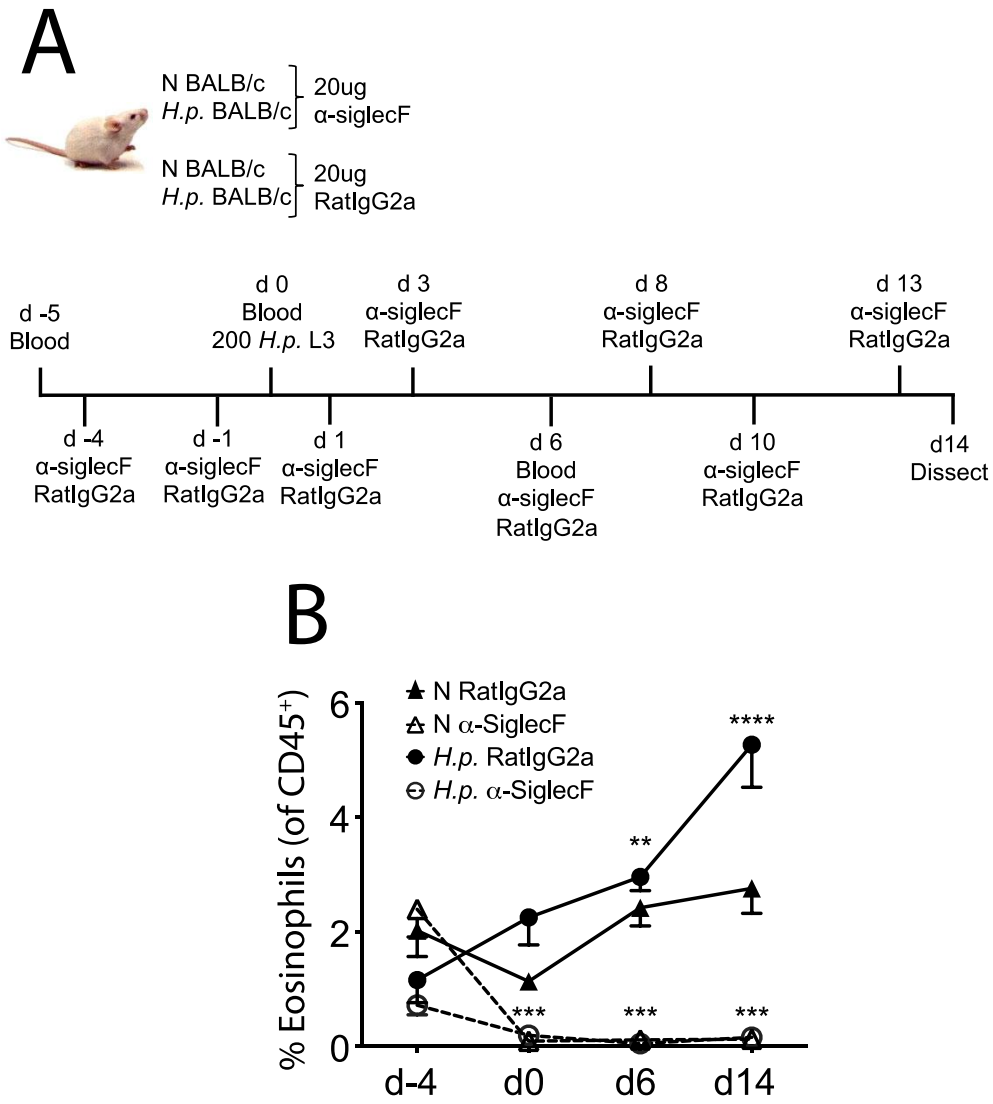

Figure 3.15 Treatment with anti-Siglec-F antibodies depletes circulating eosinophils in BALB/c mice

A. Schematic showing regime for injection of control RatIgG2a or anti-Siglec-F mAb, *H. polygyrus* infection and blood acquisition. **B.** $CD45^{+}SSC^{hi}Siglec\text{-}F^{+}CCR3^{+}$ eosinophils were detected in blood on indicated days, of naïve and *H. polygyrus* infected BALB/c mice treated with RatIgG2a (filled symbols) or anti-Siglec-F mAb (open symbols). Mean ± SEM. n=3-5. Data are representative of 2 independent experiments. Significance was tested using a two-way ANOVA and indicates differences in eosinophil levels compared to pre-treatment with mAb (d-4). ** $p < 0{,}01$ *** $p < 0{,}001$ **** $p < 0{,}0001$. αSiglec-F, anti-Siglec-F.

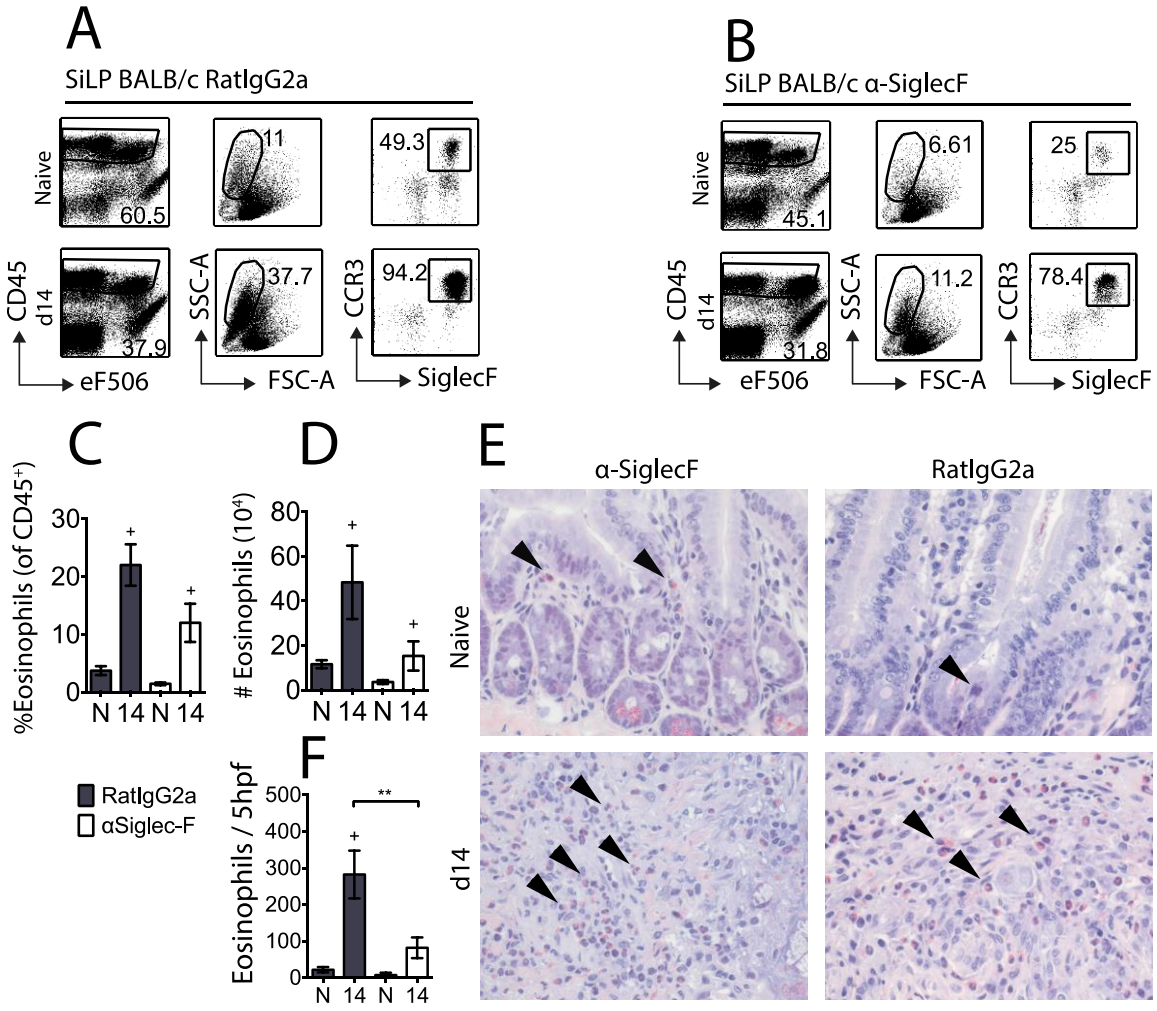

Figure 3.16 Treatment with anti-Siglec-F incompletely depletes siLP eosinophils
A, B. Exemplary FACS plots for the detection of $CD45^+SSC^{hi}Siglec\text{-}F^+CCR3^+$ eosinophils in the siLP of naïve or *H. polygyrus* infected BALB/c mice treated with RatIgG2a (A) or anti-Siglec-F (B). **C.** Frequencies of eosinophils within all $CD45^+$ siLP cells in naïve and *H. polygyrus* infected BALB/c mice treated with RatIgG2a (grey bars) or anti-Siglec-F (open bars). **D.** Total number of eosinophils calculated by multiplying frequencies with number of cells isolated. **E, F.** Exemplary pictures (E) and quantification of (F) of duodenum stained with Sirius red to visualize eosinophils, magnification x400. Hpf: high power field. Mean ± SEM. n=3-5. Data are representative of 2 independent experiments. ** $p < 0{,}01$. + depicts a significant difference between naive and infected. αSiglec-F, anti-Siglec-F.

3.7.2 T and B-cell responses

Although eosinophils were incompletely depleted in the SiLP, the possibility remained that the partial decrease would suffice for an increase in PP Th2 responses as seen in eosinophil deficient ΔdblGATA-1 mice. Thus, PP and mLN cellularity and the Th2 and B-cell response were analysed in BALB/c mice receiving RatIgG2a or anti-Siglec-F treatment.
Firstly, PP size in control and anti-Siglec-F treated BALB/c mice were comparable. As seen

previously (Fig 3.4), the cellularity of PP decreased significantly in mice infected with *H. polygyrus*, but there was no difference between controls and anti-Siglec-F treated mice (Fig 3. 17A). Expectedly, *H. polygyrus* infection resulted in a significant increase in mLN size and cellularity in controls and anti-Siglec-F treated mice (Fig 3.17A).

Next, GATA-3 expression within $CD4^+$ cells was assessed in the PP and mLN and was found to be comparably increased in both the PP and mLN of infected controls and anti-Siglec-F treated mice (Fig 3.17B). Finally, levels of IgA^+ and $IgG1^+$ B-cells were assessed in the PP and mLN. In accordance with previous results, the main IgG1 response took place in the mLN, whereas the PP only mildly up-regulated levels of $IgG1^+$ B-cells but maintained IgA class switching during *H. polygyrus* infection (Fig. 3.17C, D). The partial depletion of intestinal eosinophils by anti-Siglec-F treatment had no impact on the levels of IgA or IgG1in the PP or the mLN.

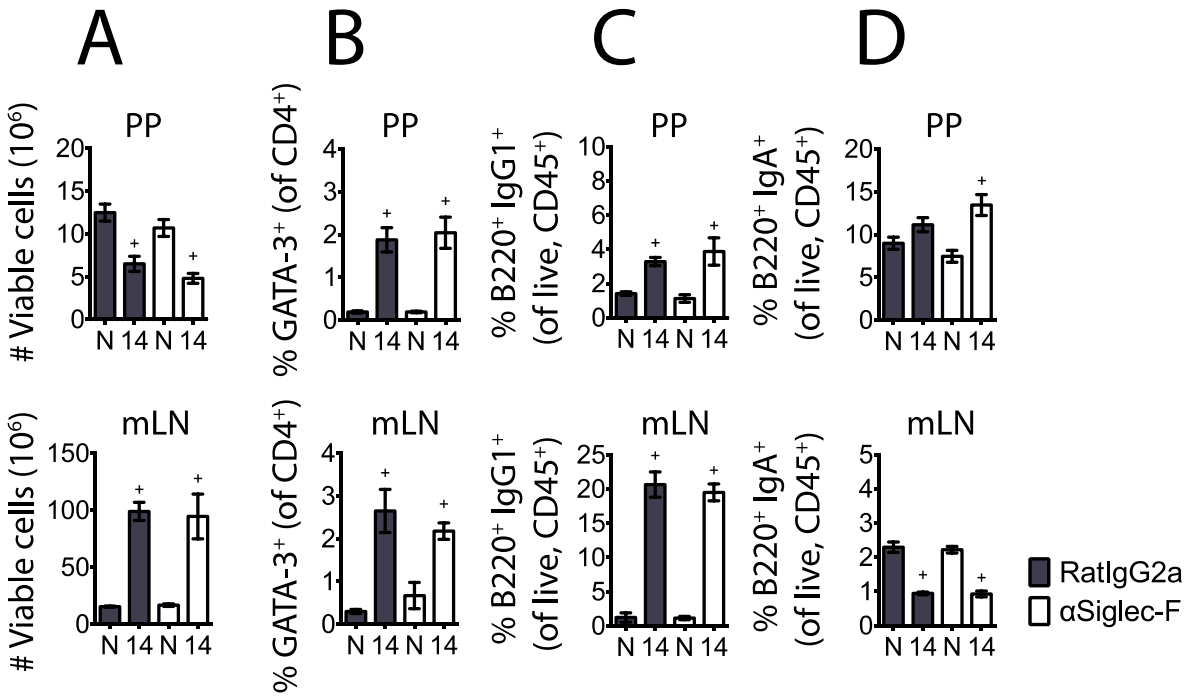

Figure 3.17 Treatment with anti-Siglec-F does not affect the immune response to *H. polygyrus*
A. Single cell suspensions were obtained from the PP and mLN of naïve and d14 *H. polygyrus* infected BALB/c mice treated with RatIgG2a (grey bars) or anti-Siglec-F mice (open bars) and cell numbers were counted. **B.** Frequencies of GATA-3 expressing $CD4^+$ cells in the PP and mLN of BALB/c mice treated with RatIgG2a or anti-Siglec-F. **C,D.** Frequencies of $IgG1^+$ (C) and IgA^+ (D) GC B-cells in the PP and mLN of BALB/c mice treated with RatIgG2a or anti-Siglec-F. Mean ± SEM. n=3-5. Data from mLN are representative of 2 independent experiements. Data from PP represent one experiment. + depicts a significant difference between naive and infected. αSiglec-F, anti-Siglec-F.

Of note, GC B-cells were not stained in this experiment, therefore the frequencies of IgA^+ and $IgG1^+$ cells in Fig 3.17 are a function of B-cells. This explains the low frequencies of $IgG1^+$ and IgA^+ cells relative to those shown in Figure 3.12, where frequencies of $IgG1^+$ and IgA^+ cells within GC B-cells are given. However, almost all non-GC B-cells in both the PP and mLN were found to be IgM^+, thus the approach applied here should not influence the observed pattern.
Summing up, it is likely that the insufficient depletion of intestinal eosinophils by anti-Siglec-F antibody-treatment resulted in T and B cell responses indistinguishable from controls harbouring normal eosinophil levels in the gut.

3.8 *H. POLYGYRUS* INFECTION IN PHIL AND C57BL/6 MICE

As intestinal eosinophil depletion by anti-Siglec-F antibody treatment was incomplete and did not result in a phenotype comparable to ΔdblGATA-1 mice, the importance of eosinophil in the observed scenario was assessed in a second eosinophil-deficient mouse strain. PHIL mice, in which genetic background and the mechanism leading to eosinophil deficiency are distinct to that of ΔdblGATA-1 mice (Lee et al. 2004), were acquired and infected with *H. polygyrus*.

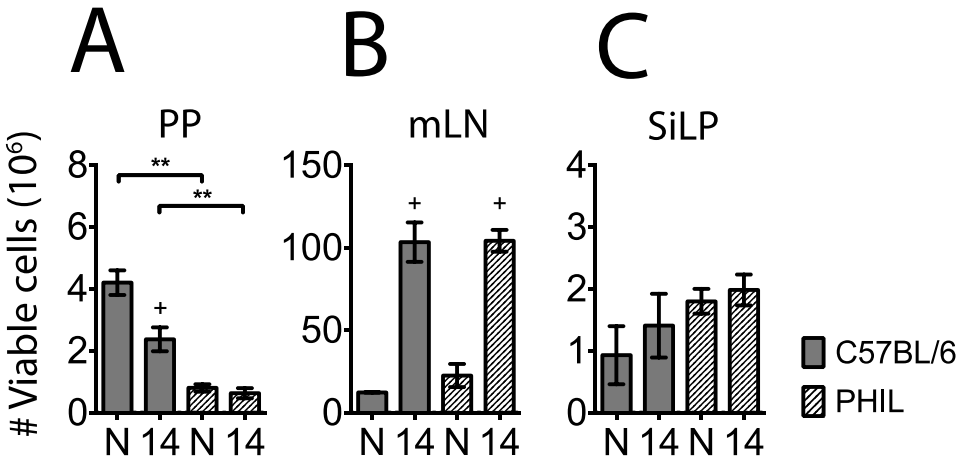

Figure 3.18 Reduced PP cellularity in eosinophil deficient PHIL mice
A-C. Single cell suspensions were obtained from the siLP (A), PP (B) and mLN (C) of naïve and d14 *H. polygyrus* infected C57BL/6 (grey bars) and PHIL mice (hatched bars) and cell numbers were counted using an automated cell counter. Mean ± SEM. Performed once with 2 mice per naïve group and 5-6 mice per infected group. ** $p < 0.01$. + depicts a significant difference between naive and infected.

3.8.1 Cellularity of gut associated compartments

Examining the PP of C57BL/6 mice it was noted that these organs were not as well developed as those of BALB/c mice and contained only about half as many cells (Fig 3.18A), suggesting that the genetic background alone can influence the activity of the PP. This is in agreement with previously published findings (Jung et al. 2015).
Comparing C57BL/c and PHIL mice PP cellularity was significantly reduced in eosinophil deficient PHIL mice, with only approximately 0,5 x 10^6 cells isolated per mouse. Furthermore, *H. polygyrus* infection resulted in a decrease in PP size in C57BL/6 mice, while
PP cellularity of PHIL did not change significantly after infection (Fig 3.18A). Eosinophil deficiency did not affect the cellularity of the mLN either in naïve or *H polygyrus* infected mice (Fig 3.18B).
Hence, PP size under steady state conditions is impaired in two strains of eosinophil deficient mice, confirming previous studies (V.T. Chu et al. 2014; Jung et al. 2015).

3.8.2 Th2 induction

Next the induction of Th2 cells as assessed by GATA-3 expression was assessed in the PP, mLN and siLP of C57BL/6 and PHIL mice.

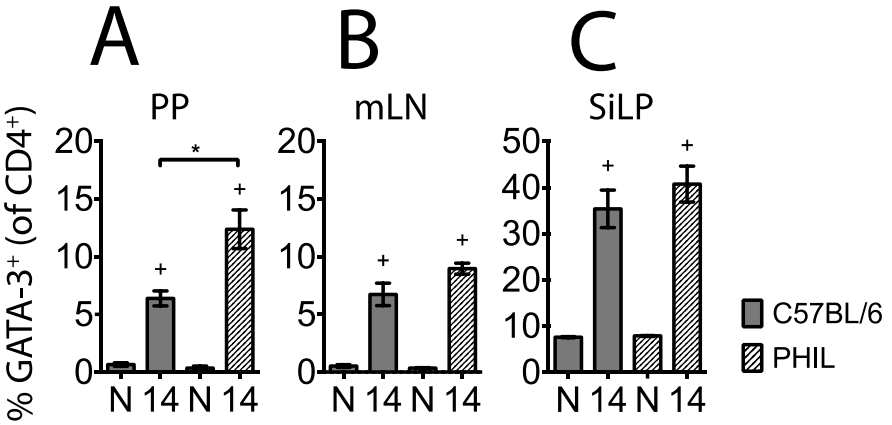

Figure 3.19 Increased GATA-3 expression by $CD4^+$ T cells in the PP of eosinophil deficient PHIL mice
A-C. GATA-3 expression within $CD4^+$ cells in the PP (A), mLN (B) and siLP (C) of naïve and d14 *H. polygyrus* infected C57BL/6 (grey bars) and PHIL mice (hatched bars). Mean ± SEM. Performed once with 2 mice per naïve group and 5-6 mice per infected group. * $p < 0.05$. + depicts a significant difference between naive and infected.

In agreement with findings in BALB/c and ΔdblGATA-1 mice it was noted that significantly more $GATA\text{-}3^+CD4^+$ cells were induced in PP of PHIL mice compared to the PP of C57BL/6 mice (Fig 3.19A). Eosinophil deficiency in PHIL mice did not influence induction of GATA-3 expression by $CD4^+$ cells in the mLN (Fig 3.19B). Furthermore, highly elevated levels of GATA-3 expressing $CD4^+$ cells were detected in the siLP at day 14 p.i. but were comparable between C57BL/6 and PHIL mice (Fig 3.19C).
Thus, Th2 induction is significantly enhanced in PP of two strains of eosinophil deficient mice following infection with *H. polygyrus*.

3.8.3 Germinal centre reaction and isotype class switching

To complete the analysis of PHIL mice, TFH cells, GC formation and isotype class switching was analysed in the PP and mLN of C57BL/6 and PHIL mice following infection with *H. polygyrus*.

3.8.4 $CXCR5^+PD\text{-}1^+$ TFH and $PNA^{hi}B220^+$ GC B-cells

Corresponding to findings from ΔdblGATA-1 mice, frequencies of $CXCR5^+PD\text{-}1^+$ TFH were relatively high in the PP of naïve C57BL/6 and PHIL mice and did not change significantly in response to *H. polygyrus* infection (Fig 3.20A). In the mLN, TFH cells were present at lower levels but did significantly increase in response to infection (Fig 3.20B). There was no influence of eosinophil deficiency on the frequencies of TFH cells in either PP or mLN, as levels of $CXCR5^+PD\text{-}1^+$ cells were comparable between C57BL/6 and PHIL mice in both compartments (Fig 3.20A, B).

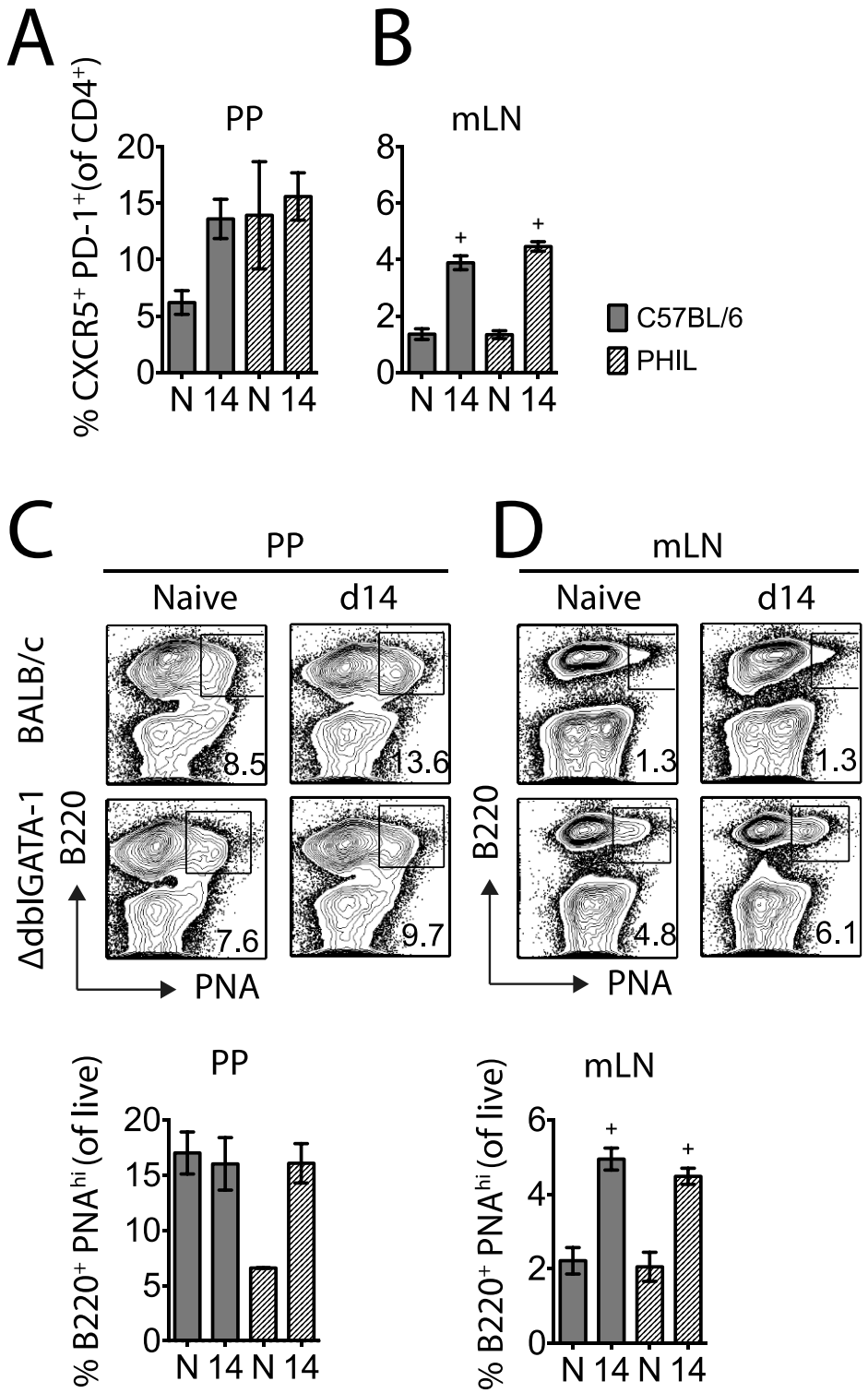

Figure 3.20 Eosinophil deficient PHIL mice have normal levels of TFH cell and GC B-cells **A, B.** Frequencies of CXCR5$^+$PD-1$^+$ TFH cells in the PP (A) and mLNs (B) of naïve and d14 *H. polygyrus* infected C57BL/6 (grey bars) and PHIL mice (hatched bars). **C, D.** Exemplary FACS plots and frequencies of B220$^+$PNA^{hi} GC B-cells in the PP (C) and mLN (D) of naïve and d14 *H. polygyrus* infected C57BL/6 and PHIL mice. Mean ± SEM. Performed once with 2 mice per naïve group and 5-6 mice per infected group. + depicts a significant difference between naive and infected.

A similar picture was seen when assessing $B220^{+}PNA^{hi}$ GC B-cells. It was noted that the PP of naïve C57BL/c mice contained relatively high frequencies of GC B-cells, which did not change in mice infected with *H. polygyrus*. Once again, naïve eosinophil deficient mice had lower levels of GC B-cells. Nevertheless, following *H. polygyrus* infection, GC B-cell frequencies were enhanced in PHIL mice to similar levels as was seen in WT animals (Fig 3.20C). Compared to the PP, the mLN of naïve C57BL/6 and PHIL mice contained lower levels of GC B-cells, but this population was expanded significantly following *H. polygyrus* infection. Eosinophil deficiency in PHIL mcie did not affect GC B-cells in the mLN (Fig 3.20D).

3.8.5 $PNA^{hi}B220^{+}$ GC B-cells and IgA, IgG1 class switching

Next, the expression of IgG1 and IgA within GC B-cells was assessed. Confirming findings from BALB/c mice, it was apparent that the PP of C57BL/c mice did not significantly participate in *H. polygyrus* induced IgG1 class switching, but were rather involved in continuous IgA production, as evident by the relatively high frequencies of IgA^{+} GC B-cells in the PP of naïve C57BL/6 and a maintenance of IgA class switching during *H. polygyrus* infection (Fig 3.21A). In agreement with data from ΔdblGATA-1 mice, GC B-cells isolated from infected PHIL mice contained significantly higher frequencies of $IgG1^{+}$ cells. Levels of IgA^{+} GC B-cells were slightly decrease in the PP of PHIL mice, but not significantly so and did not change in response to infection (Fig 21A).

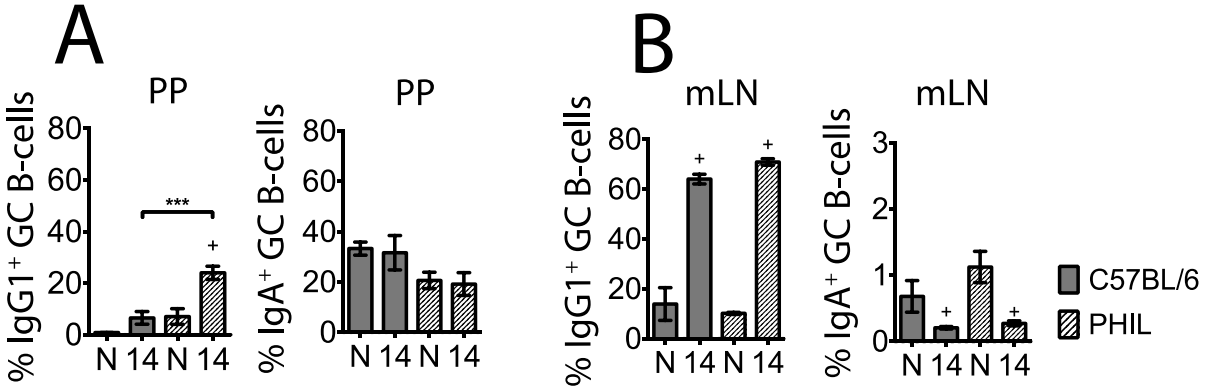

Figure 3.21 High levels of IgG1 class switching in the PP of eosinophil deficient PHIL mice
A, B. Frequencies of $IgG1^{+}$ and IgA^{+} GC B-cells in the PP (A) and mLN (B) of naive and d14 *H. polygyrus* infected C57BL/6 (grey bars) and PHIL mice (hatched bars). Mean ± SEM. Performed once with 2 mice per naïve group and 5-6 mice per infected group. *** $p < 0.001$. + depicts a significant difference between naive and infected.

It was confirmed that the mLN represent the main site for induction of IgG1 following *H. polygyrus* infection and that this site is not affected by eosinophil deficiency (Fig 3.20B). Furthermore, levels of IgA were low in the mLN of both C57BL/6 and PHIL mice, but decreased even further following infection (Fig 3.20B).
In conclusion the induction of IgG1 in the PP following *H. polygyrus* infection is regulated in presence of eosinophils and mice deficient in this innate cell population react with overt IgG1 class switching in these local lymphoid structures.

3.9 ROLE OF EOSINOPHILS IN *H. POLYGYRUS* SURVIVAL AND FITNESS

The third aim of this thesis was to address the affect of eosinophils on *H. polygyrus* survival and fecundity. Based on studies where eosinophil depletion was achieved by administration of anti-IL-5 mAb, eosinophils are thought not to play a major part in the defence against *H. polygyrus* (Urban et al. 1991a; Knott et al. 2009). However, with the exception of one recent study (Hewitson et al. 2015), the consequence of eosinophil deficiency on *H. polygyrus* has not been investigated. Furthermore, measures of fitness affecting propagation can be more

informative than survival when considering host effects on pathogens, but the fecundity of *H. polygyrus* in eosinophil ablated mice has not been assessed. In the next section, the effect of eosinophil deficiency was analysed and host protective innate responses compared in BALB/c and ΔdblGATA-1 mice.

3.9.1 Adult worm burden and fecundity

On day 14 post infection, ΔdblGATA-1 and PHIL mice were found to have comparable numbers of adult worms in the duodenum to their respective WT strains (Fig 3.22A, B). In addition, ΔdblGATA-1 mice were equally protected against challenge *H. polygyrus* infection as BALB/c mice (Fig 3.22A). When faecal egg shedding was assessed, both ΔdblGATA-1 and PHIL mice tended to shed fewer eggs compared to BALB/c and C57BL/6 mice, respectively, but this was not statistically significant (Fig 3.22C, D). Faecal egg counts can be problematic, due to variation in the number of adult females present in the intestine. A more reliable measure of fecundity is thus to isolate female worms and assess their individual egg output. Using this method it was demonstrated that the fecundity of parasites developing in ΔdblGATA-1 and PHIL mice was significantly impaired compared to those isolated from the respective WT strains (Fig 3.22E, F).

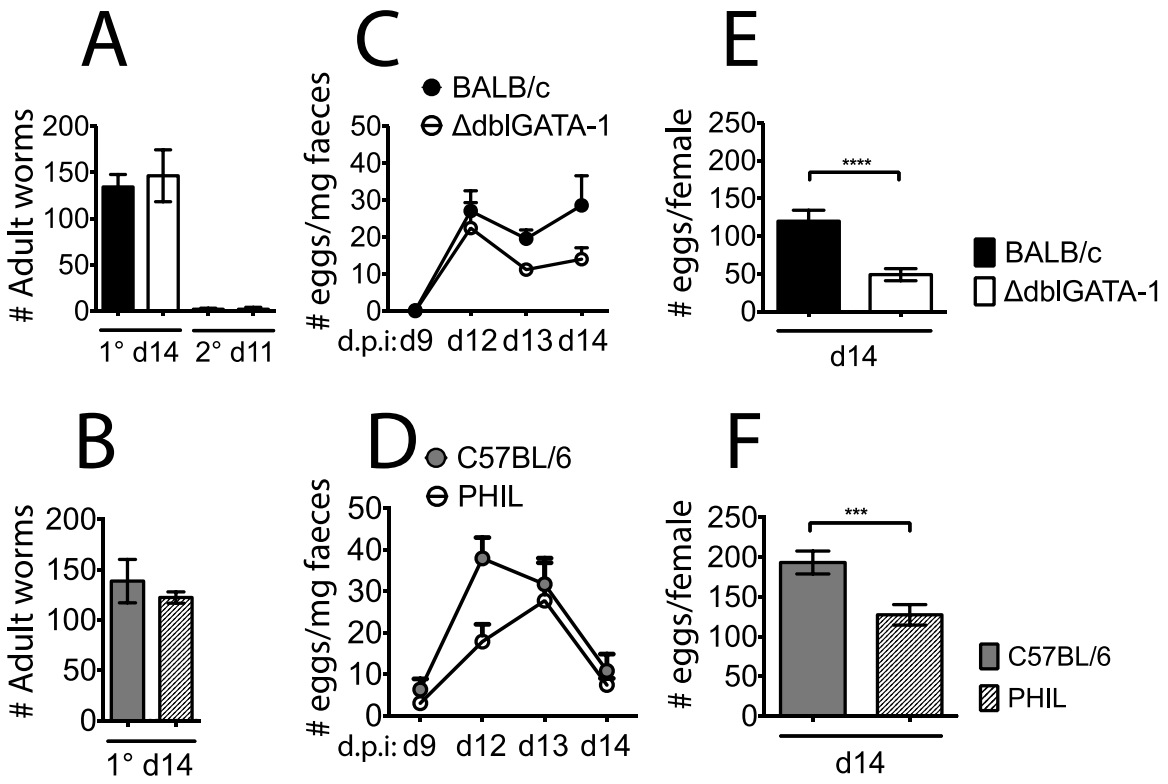

Figure 3.22 Eosinophil deficiency impairs parasite fecundity
A, B. Adult worm burden in BALB/c (black bars) and ΔdblGATA-1 (open bars) (A) or C57BL/6 (grey bars) and PHIL (hatched bars) (B) at day 14 post primary infection or day 11 post challenge with *H. polygyrus* (A). **C, D.** Faecal egg output by BALB/c and ΔdblGATA-1 (C) or C57BL/6 and PHIL (D) mice on indicated days post *H. polygyrus* infection (d.p.i.). **E, F.** Average egg output in 24 hours, per *H. polygyrus* female isolated from indicated mouse strain on day 14 post infection. Mean ± SEM. Data from BALB/c and ΔdblGATA-1 mice are representative of > 3 independent experiments, n=3-5. Data from C57BL/6 and PHIL mice

represent one experiment n=5-6. Significance tested with Mann Whitney U test. *** $p < 0.001$ *** $p < 0.0001$.

3.9.2 Anti *H. polygyrus* effector mechanisms

The unexpected finding that eosinophil deficiency resulted in decreased parasite fecundity prompted further analysis of innate responses that may be detrimental to helminth health. As mechanisms by which the immune system may harm *H. polygyrus* during a primary infection are not well described, two processes known to participate in worm expulsion were assessed.

3.9.2.1 *Alternative activation of macrophages*

In response to IL-4Rα signalling, macrophages acquire an alternatively activated phenotype marked by the expression of resistin-like molecule alpha (RELM-α) and arginase-1 (Arg-1), the latter being central to the control of challenge infection with *H. polygyrus* (Esser-von Bieren et al. 2013; Anthony et al. 2006). Although the role of AAM on the fecundity of *H. polygyrus* has not been investigated, they have been shown to target L3 *H. polygyrus* larvae *in vitro* (Esser-von Bieren et al. 2013). Thus, expression of Relm-α and Arg-1 mRNA was assessed in the siLP of BALB/c and ΔdblGATA-1 mice to determine whether differences in alternative macrophage activation could explain the decreased fecundity of worms developing in eosinophil deficient mice.

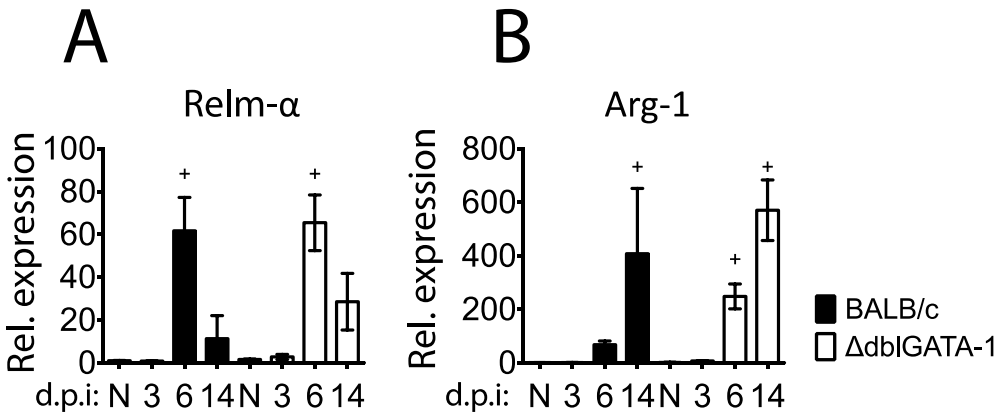

Figure 3.23 Eosinophils do not affect the alternative activation of macrophages in the SiLP
A, B. Relm-α (A) and Arginase-1 (Arg-1) (B) mRNA expression in the siLP of BALB/c (black bars) and ΔdblGATA-1 mice (open bars) at indicated days post infection (d.p.i.). Gene expression was normalized to housekeeping gene GUSB and expressed relative to naive BALB/c. Mean ± SEM. n=3-5. Data are representative of 3 independent experiments. + depicts a significant difference between naive and infected.

Both Relm-α and Arg-1 mRNA increased significantly in the small intestine in response to infection (Fig 3.23A, B). Interestingly there was a temporal difference in the expression of these two markers with Relm-α reaching the highest levels at day 6 p.i., whereas Arg-1 expression was highly increased at day 14 p.i. Importantly however, expression of both these AAM markers did not differ between BALB/c and ΔdblGATA-1 mice.
Thus, a difference in alternative activation of macrophages is unlikely to underlie the decreased parasite fecundity observed in eosinophil deficient mice.

3.9.2.2 *Goblet cell responses*

Infection with intestinal helminths results in IL-13 production, which stimulates the differentiation of epithelial cells into mucus secreting goblet cells and induces smooth muscle contraction, both of which are thought to aid in helminth expulsion (Gause et al. 2003; Hasnain

et al. 2011). Furthermore, goblet cells secrete Relm-β which can interfere with *H. polygyrus* feeding (Herbert et al. 2009).
Thus, in order to determine whether differences in goblet cells or their products could be responsible for the decreased fecundity of parasites developing in eosinophil deficient mice, goblet cells were quantified via periodic acid Schiff (PAS) staining of the duodenum.

In accordance with comparable adult worm counts in the intestine of eosinophil deficient and WT mice (Fig 3.22A, B), number of PAS^+ goblet cells did not differ between BALB/c and ΔdblGATA-1 mice (Fig 3.24A, B). Furthermore, levels of Relm-β mRNA was comparable between BALB/c and ΔdblGATA-1 mice infected with *H. polygyrus* (Fig 24B, C), indicating that this goblet cell derived product is not responsible for the reduced fitness of worms developing in eosinophil deficient mice. Interestingly, it was noted that, although goblet cell numbers were comparable between mouse strains, the mucus layer present on the luminal side of the intestine from BALB/c mice was mostly absent from the corresponding region of ΔdblGATA-1 derived duodenal sections (Fig 3.24A). This is in accordance with previous studies that have demonstrated an impaired ability of eosinophil deficient mice to retain a mucus layer in the duodenum (V.T. Chu et al. 2014; Jung et al. 2015). Finally, although ΔdblGATA-1 mice showed impaired retention of a protective mucus layer and although they demonstrated higher Th2 and B-cell responses in the PPs, intestinal inflammatory scores in infected ΔdblGATA-1 mice were comparable to control animals (Fig 3.24D).

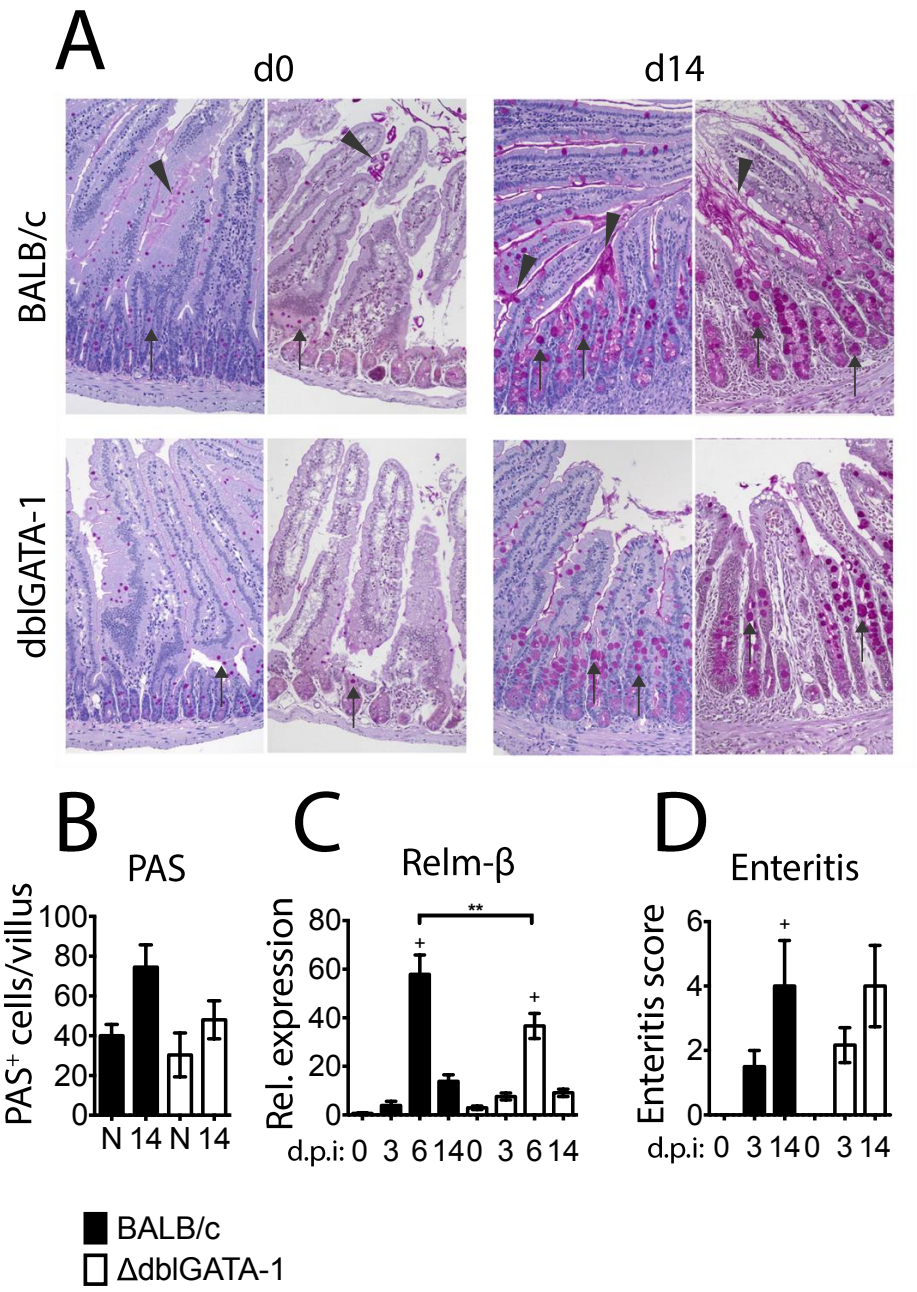

Figure 3.24 Impaired retention of mucus layer in eosinophil deficient Δ dblGATA mice
A. Duodenal cross sections, stained with periodic acid/Schiff (PAS), from naive and d14 *H. polygyrus* infected BALB/c and ΔdblGATA-1 mice showing goblet cells (arrows) and mucus layer (arrow heads). Exemplary pictures from two experiments are shown, magnification x 100. **B.** Number of PAS$^+$ goblet cells/villi in the duodenum of naive and *H. polygyrus* infected BALB/c (black bars) and ΔdblGATA-1 mice (open bars). **C.** Relative expression of Relm-β mRNA in the siLP of BALB/c and ΔdblGATA-1 mice at indicated days post infection (d.p.i.). Gene expression was normalized to housekeeping gene GUSB and expressed relative to naive BALB/c. **D.** Inflammatory score of the duodenum at indicated d.p.i. of BALB/c and ΔdblGATA-1 mice. Mean ± SEM. n=3-5. Data are representative of 3 independent experiments. ** *p* 0,001. + depicts a significant difference between naive and infected.

3.10 SUMMARY AIM 3

To summarize data addressing the third aim stated in this thesis, it was demonstrated that eosinophils do not affect *H. polygyrus* survival but, surprisingly, their presence appeared to benefit parasite fecundity. This did however not correlate with differences in innate molecules reported as detrimental to helminth health. Retention of luminal mucus appeared to be impaired in the absence of eosinophils, indicating that eosinophils may play an important role in the maintenance of intestinal health.

3.11 FUTURE WORK TOWARDS UNCOVERING THE MECHANISM OF IMMUNE REGULATION BY EOSINOPHILS

As eosinophils are rare in peripheral blood and as their isolation from the gastrointestinal tract is associated with cumbersome protocols giving low yields, investigations of eosinophil functions are hampered by the difficulty in obtaining sufficient cell numbers for performing cell transfer experiments or *in vitro* assays. This is often overcome by isolating eosinophils from IL-5Tg eosinophilic mice or repeated administration of rIL-5. However, Dyer et al. (2008) describe a method by which high levels of pure eosinophils can be differentiated from mouse bone marrow (BmEos).
To facilitate the continuation of this work, this method was established and initial *in vitro* assays of BmEos function were carried out.

3.11.1 Establishement of bone marrow eosinophils cultures

To generate eosinophils from mouse bone marrow, cells were isolated from BALB/c mice and cultured for an initial 4 days with stem cell factor (SCF) and fms-related tyrosine 3 ligand (FLT-3L). Thereafter, cells were cultured with IL-5, which was replenished every second day.
The final protocol describing the necessary steps for successful differentiation of eosinophils from mouse bone marrow is detailed in M&M.

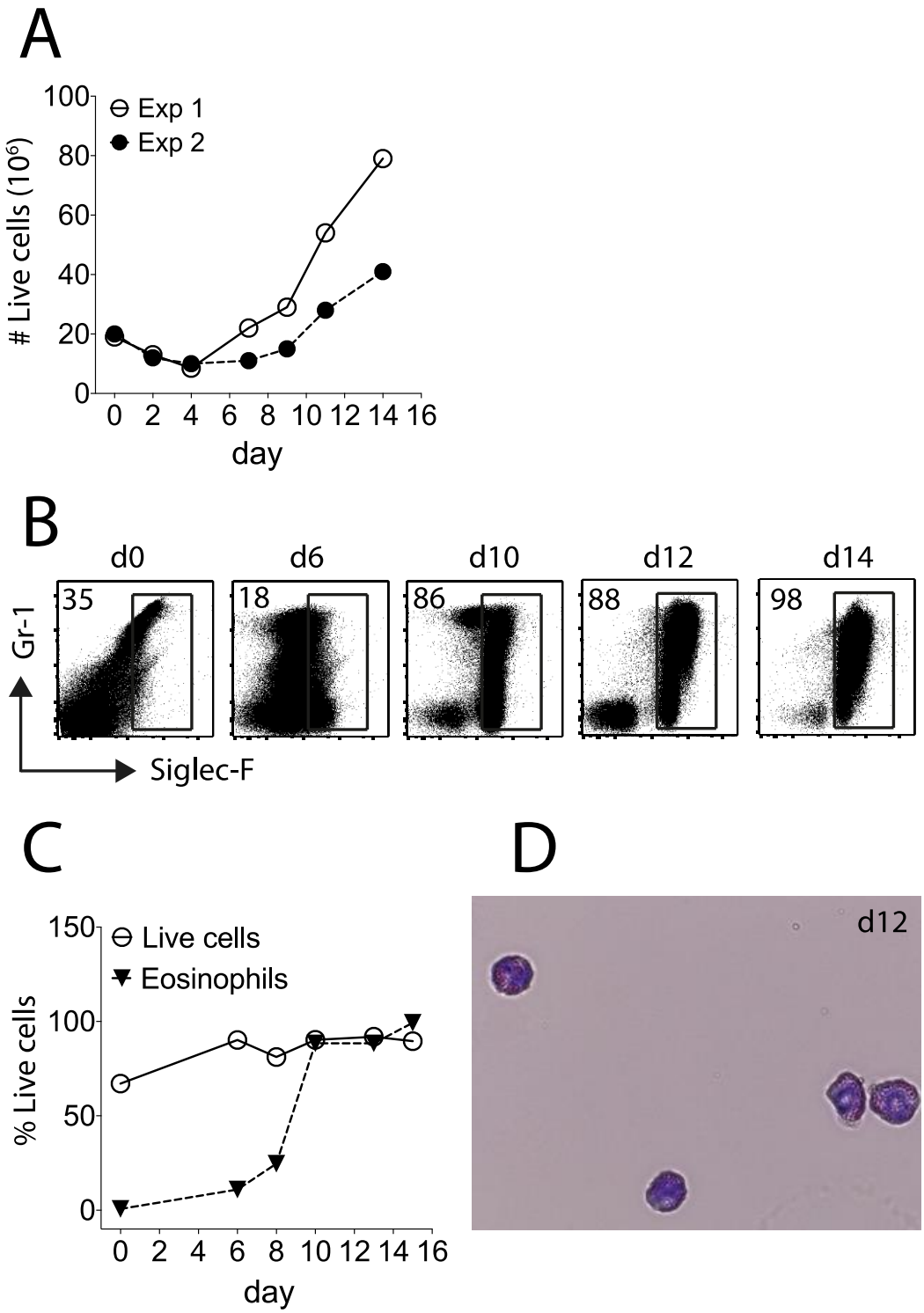

Figure 3.25 Differentiation of eosinophils from female BALB/c bone marrow
A. Number of live cells on indicated days, from two independent experiments,. **B.** Exemplary FACS plots of BmEos between day 0 and 14 of culture, showing the gradual differentiation of Siglec-F$^+$ cells. **C.** Exemplary plot showing % live cells as determined by viability marker eF780 and % Siglec-F$^+$ cells within live cells, in BmEos culture between day 0 and 16. **D.** Cytospin of BmEos harvested at day 12.

Cells start to proliferate between day 6-8 and adopt a granular appearance under the phase contrast microscope. By day 12-14, cells have proliferated substantially and a high proportion

of cells express Siglec-F (Fig 3.25B, C) and show an eosinophil cell morphology, as assessed by cytospin (Fig 3.25D).

3.11.2 *In vitro* stimulation of BmEos

Eosinophils express IL-25 and the IL-25 receptor (Tang et al. 2014) and IL-25 enhances expression of adhesion molecules on human eosinophils (Cheung et al. 2006). Similarly, eosinophils express the IL-33 receptor ST2 and IL-33 enhances eosinophil infiltration to the lung during inflammation, up-regulates eosinophil expression of CCR3 and stimulates the secretion of several cytokines from eosinophils *in vitro*, including IL-6 (Stolarski et al. 2010). IL-6 is important for B-cell maintenance (Chu et al. 2011), IgA class switching (Sato et al. 2003) and regulation of Th2 responses (Smith & Maizels 2014). Furthermore, eosinophil derived IL-1β has been suggested to underlie their support of IgA in the intestine (Jung et al. 2015) and eosinophil derived IL-10 is implicated in the support of chronic helminth infection (Huang et al. 2014). Thus in order to test the activity of BmEos, cells were stimulated with IL-25, IL-33 and PMA/Ionomycin *in vitro* and their secretion of IL-6, IL-1β and IL-10 was assessed. IL-33 in combination with PMA/ionomycin (PMA/iono), but not either alone, induced high amounts of IL-6 and low amounts of IL-1β (Fig 3.26A, B). In addition, BmEos secreted IL-10 in response to IL-33 or PMA/iono alone or in combination (Fig 3.26C). In contrast, stimulation with IL-25 did not result in secretion of IL-6, IL-1β or IL-10 from BmEos whether this was present alone or in combination with PMA/iono or IL-33. IL-25 did not further enhance cytokine secretion induced by IL-33, but inhibited production of IL-10 in response to IL-33 (Fig 3.26C).

Together these initial experiments demonstrate that high levels of functional eosinophils can be differentiated *in vitro* from mouse bone marrow. These cells are competent in the secretion of cytokines to selective stimuli and will prove useful in continuing the research on the role of eosinophils in T and B-cell responses during *H. polygyrys* infection.

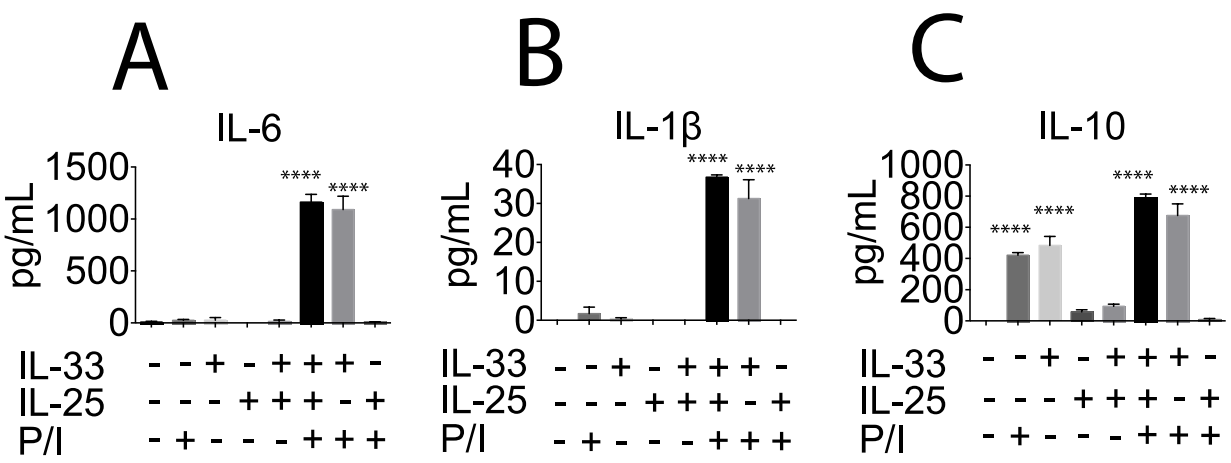

Figure 3.26 BmEos secrete IL-6, IL-1 β and IL-10 in response to IL-33
50x10^4 BmEos (harvested on day 14 of culture) were stimulated for 5 days with indicated stimuli. **A-C.** IL-6 (A), IL-1β (B) and IL-10 (C) were measured in the supernatants by ELISA. Mean ± SEM. Representative of two independent experiments with 2-3 replicate wells/condition. **** *p* 0,0001 indicates difference to unstimulated.

4 DISCUSSION

Findings presented here demonstrate an exciting, novel role for eosinophils in maintaining a dichotomous response between the PP and mLN during enteric infection. Specifically, the main T and B-cell response to an enteric nematode infection was found to take place in the mLN of BALB/c mice whereas the frequencies of IL-4 producing T-cells and $IgG1^+$ B-cells in the PP did not increase significantly. In two strains of eosinophil deficient mice, this dichotomy was found to be disturbed, as a strong increase of Th2 cells and $IgG1^+$ B-cells was seen in the PP of both ΔdblGATA-1 and PHIL mice infected with *H. polygyrus*. IL-4 producing TFH cells were found to be particularly affected by eosinophil deficiency, as both their frequencies and cytokine secretion per cell increased substantially in the absence of eosinophils. As immune responses to enteric pathogens are most commonly studied in the context of the mLN with the processes taking place in the PP only rarely considered (Mosconi et al. 2015; Boullier et al. 2009), this study contributes to the field by demonstrating that the PP maintain an immune regulated environment during intestinal helminth infection and by indicating the involvement of eosinophils, a cell most commonly associated with anti parasite defences (Meeusen & Balic 2000), in such regulation.
Further, this study confirms that eosinophils have no direct influence on the survival of *H. polygyrus*, however, that their presence support parasite fecundity, opening interesting questions about parasite solicitation of eosinophils, of life history choices and eosinophil mediated support of parasite niches.
I will now discuss each of the presented results as they appear in this thesis, starting with the interesting finding that eosinophils infiltrate the PP and mLN during infection with *H. polygyrus*.

4.1 EOSINOPHIL INFILTRATION TO THE PP AND MLN FOLLOWING *H. POLYGYRUS* INFECTION

That eosinophilpoesis and circulating eosinophils increase following helminth infection and that these cells traffic to the site of invasion is well established. However, whether eosinophils infiltrate the mLNs and PP during *H. polygyrus* infection was unknown.
In this study cells with an eosinophilic phenotype (CCS^{hi}Siglec-F^+CCR3^+) were found to make up less than 0,2% of all $CD45^+$ cells isolated from the PP of naive BALB/c mice. However, when assessed by immunohistochemistry, no eosinophils were found in the PP of naive animals. This discrepancy could be explained by a number of inherent differences to the two approaches. Firstly, the chances of “encountering” a cell that is present in such low frequencies is higher by flow cytometry, as many more cells can be analysed than what is practical via visual inspection of tissue sections. Secondly, single cell suspensions contain cells from all areas of the specific organ, whereas immunohistochemistry requires the selection of, in this case 5, areas in which cells are counted. This means that cells restricted to distinct architectural sites risk being missed. Thirdly, the possibility exist that single cell suspensions prepared for flow cytometry contain some cells from the proximal lamina propria that get included as the PP are excised. Deciphering which analysis provides the right answer is important as the question of whether eosinophils are present in the PP of naive mice will indicate whether the effects of eosinophil mediated immune regulation is likely to be direct or indirect. Few studies have investigated the presence of eosinophils in the PP, but one previous report suggests that eosinophils do reside in the PP of naive mice in low numbers, but that their presence is confined to the peripheral regions of the outer cortex and the interfollicular regions (Mishra et al. 2000). This would place them in a prime position to influence B and T-cell interactions taking place in the interfollicular region prior to the commencement of the GC formation (Kerfoot et al. 2011). Mishra et al. (2000) additionally report that eosinophils infiltrate the PP of IL-5Tg and *A. fumigatus* challenged mice. This is in agreement with results presented here: eosinophils were found to infiltrate the PP in response to an enteric nematode infection, detectable both by flow cytometry and immunohistochemistry.
To further understand the mechanism by which eosinophils influence events in the PP, it will be imperative to perform more detailed histological analysis of distinct architectural sites in

naive and *H. polygyrus* infected mice. Furthermore, if eosinophils are indeed present in the interfollicular regions, fluorescent co-stainings may reveal whether they are in direct contact with interacting B and T-cells.
The mLN of naive mice contained no eosinophils as assessed both by flow cytometry or by immunohistochemistry. Nevertheless, *H. polygyrus* infection resulted in their infiltration also to this organ. This is in agreement with a previous study demonstrating that eosinophils infiltrate the mLN during infection with the large intestinal nematode *T. muris* and that they in that case produce IL-4 (Svensson et al. 2011). Importantly however, the absence of eosinophils and their early provision of IL-4 had no influence on *T. muris* stimulated Th2 induction in the mLN (Svensson et al. 2011). The role of eosinophils infiltrating the mLN during such intestinal nematode infections therefore remains elusive.

4.2 THE INVOLVEMENT OF PP IN THE IMMUNE RESPONSE TO *H. POLYGYRUS*

The first aim of this study was to assess the involvement of PP in the immune response to *H. polygyrus*. Studies on intestinal immunity to helminths most commonly address events taking place in the mLN (King & Mohrs 2009; Svensson et al. 2011), even though the PP are in direct contact with the site of infection and in some cases are invaded by *H. polygyrus* larvae (Mosconi et al. 2015). Here it was found that, whereas strong Th2 induction and IgG1 production was induced in the mLN, these processes were suppressed in the PP of BALB/c mice. To understand why this might be, it is important to outline any known differences between these organs.

4.2.1 Distinctions between PP and mLN during homeostasis

There is widespread agreement that the mucosal and systemic immune systems are in many ways distinct and that cells from the PP and mLN often have divergent functions compared to cells in the spleen or peripheral lymph nodes (Mowat 2003; Iwasaki & Kelsall 1999). For example DC from the PP produce high levels of IL-10 in response to stimulation with CD40 (Iwasaki & Kensall 1999) or receptor activator of NF-kappa B (RANKL) (Williamson et al. 2002), whereas DC from the spleen produce IL-12 in response to the same stimulus. Furthermore, the composition of DC subsets is almost identical between the PP and mLN, but distinct from that of the spleen (Mowat 2003). Fewer studies have contrasted the function of the PP and mLN (Shiokawa et al. 2009; Fink & Frøkiaer 2008). Yet some data indicate that the PP may represent an inherently immune regulated environment. For example, $CD4^+$ cells from the PP are less responsive to chemokines and polyclonal stimulation compared to $CD4^+$ cells from other organs, including the mLN (Kellermann & McEvoy 2001). Interestingly, $CD4^+$ cells isolated from the PP and kept in culture gradually regain their responsiveness, indicating that the PP environment is decisive in maintaining leukocyte hypo-responsiveness (Kellermann and McEvoy 2001). Further studies indicate that PP DC may behave differently to both mLN and spleen DC. For example, in mice fed OVA in their drinking water, PP DC cells were found to secrete higher levels of IL-10 than DCs from both mLN and spleen (Shiokawa et al. 2009). Furthermore, when stimulated with three different commensal bacteria, PP DC produce high levels of IL-6, but no IFN-γ and no TNF-α, in contrast to mLN and spleen DCs which produced both cytokines, but lower levels of IL-6. Moreover, PP DC produced the highest levels of TGF-β (Fink & Frøkiaer 2008). This is in agreement with the central role for PP in the induction of IgA^+ B-cells (Sato et al. 2003; Macpherson et al. 2008), as both TGF-β (Coffman et al. 1989) and IL-6 (Sato et al. 2003; Ramsay et al. 1994) are implicated in this process.
The mLN are also competent in IgA induction and seem to, in some, but not all cases, overtake this function in mice lacking PP (Yamamoto et al. 2000; Hashizume et al. 2008), but levels of IgA^+ B-cells are low in the mLN when PP are functioning normally, as reported here and elsewhere (Macpherson et al. 2008). Furthermore, DC from the PP are inherently superior to DC from other organs in inducing IgA class switching *in vitro* (Mora et al. 2006). In contrast, the mLN are the main site for induction of oral tolerance, in which immune responses to orally applied antigens are suppressed (Pabst & Mowat 2012; Mowat 2003). This is shown by the

maintenance of tolerance to oral antigens in mice lacking PP as a result of lymphotoxin-β inhibition (Spahn et al. 2002; Yamamoto et al. 2000) and functioning oral tolerance in rats in which the PP have been surgically removed (Enders et al. 1986).
Thus, one separation of the PP and mLN under homeostatic conditions may be that the PP are responsible for IgA production in response to commensal bacteria whereas the mLN maintain oral tolerance to food antigens (Pabs and Mowat 2012). However, it is likely that the PP additionally contribute to tolerance both directly, as the presence of retinoic acid, TGF-β and IL-10 - all molecules associated with oral tolerance induction, are present in high levels in the PP (Cerutti & Rescigno 2008) and indirectly via the provision of the mLN with antigen (Huang et al. 2000). Thus, the PP and mLN are likely to have overlapping functions, with the mLN capable of compensating for the loss of PP.

4.2.2 Distinctions between PP and mLN during infection

With the exception of one recent study, the division of immune responses between the PP and mLN during intestinal helminth infection has not been investigated. Mosconi et al. (2015) demonstrated that the main Th2 response to *H. polygyrus* takes place in the mLN. In agreement with the current study, Th2 cytokine expression increased significantly in the mLN from day 6 p.i., whereas up-regulation of Th2 cytokines was moderate in the PP. The authors argue that *H. polygyrus* actively inhibits immune responses in the PP, as shown by the higher levels of activated ($CD25^+FoxP3^+$) Treg cells in PP where *H. polygyrus* larvae could be proximally found, as compared to PP without proximal larvae. *H. polygyrus*-secreted products contain a TGF-β homologue that potently induces Treg cells (Grainger et al. 2010). It is thus reasonable to speculate that the low induction of Th2 cytokine expression in the PP of *H. polygyrus* infected mice could be a result of active modulation by *H. polygyrus*.
In the current study, only overall frequencies of $FoxP3^+$ Treg cells were assessed and found to be approximately equal between the PP and mLN, but further analysis of Treg activation status and possibly additional phenotyping of Treg subsets, may reveal results in agreement with Mosconi et al. (2015). Nevertheless, if *H. polygyrus* is actively regulating the induction of Th2 responses in the PP (Mosconi et al. 2015), the work presented here imply that such a regulation is mediated via eosinophils, as the PP of eosinophil deficient mice were highly reactive to *H. polygyrus* infection.
Several pathogenic bacteria and viruses infiltrate the PP and mLN and can thus provide further clues to possible differences in response to infection within these organs. For example, the PP are the main route of entry for *Salmonella typhimurium* and only after initial colonisation of the PP do bacteria disseminate to the mLN and later to systemic sites such as spleen and liver (McSorley et al. 2002). The PP are responsive to *S. typhimurium* invasion as shown by significant hypertrophy (Schulz et al. 2014), induction of iNOS and TNFα (Rydström & Wick 2007) and the accumulation of *Salmonella* specific T-cells frequencies comparable to those in the mLN (McSorley et al. 2002; Salazar-Gonzalez et al. 2006). Similarly, infection with murine rota virus (MRV) results in infiltration of virus to the PP and mLN (Dharakul et al. 1988), hypertrophy of both organs (Blutt et al. 2002), migration of DC to the subepithelial dome and increase of IL-12, TNF-α, IFN-β and IL-10 expression of PP DC (Lopez-Guerrero et al. 2010). In contrast to the examples given above, in which infection result in increased cellularity of the PP, intraperitoneal infection with vesicular stromatitis virus (VSV) or treatment with the TLR3 ligant PolyI:C, leads to a major and specific decrease in PP cellularity, explained by type I interferon mediated reduction in B-cell recruitment to the PP, but not mLN (Heidegger et al. 2013).
In the current study, *H. polygyrus* infection resulted in decreased PP cellularity, whereas the mLN increased dramatically in size and cellularity. The reason for this was not further analysed but, as a decreased expression of B-cell α4β7 was demonstrated to underlie the impaired infiltration of B-cells specifically to the PP in PolyI:C treated mice (Heidegger et al. 2013), it will be important to investigate the expression of homing receptors on PP lymphocytes as well as the expression of integrins and selectins in the PP following *H. polygyrus* infection.

These examples demonstrate that the PP are not inherently a site of exclusive immune regulation, but appear to be selectively participating in some infections, but not others. It is interesting to note, that IgA has been implicated in protection to both *Salmonella* (Michetti et al. 1992; Mantis et al. 2011) and MRV (Blutt et al. 2002), whereas IgG is of higher importance in VSV (Kalinke et al. 1996) and *H. polygyrus* infections with only a minor role for IgA in the latter (McCoy et al. 2008). Considering the importance of the PP as a site for IgA induction it can therefore be speculated that the PP participate in immune responses to pathogens against which significant levels of IgA are induced, but do not substantially contribute to pathogens against which IgG is of importance, which could explain the differences in PP hypertrophy (*Salmonella*, MRV) versus atrophy (VSV, *H. polygyrus*) (Schultz et al. 2014; Blutt et al. 2002; Heidegger et al. 2013).
The idea that IgA versus IgG responses are divided between the PP and mLN respectively was underscored by the current study. The PP of BALB/c mice contained relatively higher frequencies of TFH cells and IgA^+ GC B-cells compared to the mLN, and their frequencies were maintained or slightly expanded during infection with *H. polygyrus*. However, infection did not significantly increase the level of IL-4 expressing TFH cells and, in line with this, did not significantly increase class switching to IgG1 in the PP of BALB/c mice. In the mLN however, *H. polygyrus* infection resulted in a sharp increase in IL-4 expressing TFH cells and a concomitant increase in IgG1 class switching.

To summarize, although the PP and mLN are often compared to peripheral sites, intra-comparisons between these organs can provide much valuable information about the organisation of the mucosal immune system. Under homeostatic conditions, the PP are the main site for IgA production with possible contribution to oral tolerance, whereas the mLN are the main site for the latter with little contribute to IgA induction. During infection with intestinal pathogens, the mLN are critical for efficient immune responses. This includes *H. polygyrus* infection, during which the main T and B-cell response takes place in the mLN, as shown in the current study. In contrast, the PP do not contribute significantly to responses again *H. polygyrus*, although their involvement in immunity to enteric pathogens is not categorically excluded. Rather their participation appears to depend on the type of pathogen investigated, potentially determined by the requirements for IgA production.

4.3 EOSINOPHIL MEDIATED REGULATION OF TH2 AND IGG1 RESPONSES TO *H. POLYGYRUS*

The second aim of this study was to investigate the role of eosinophils in Th2 induction during *H. polygyrus* infection. The presented findings illustrate the exciting point that the differences between the PP and mLN immune responsiveness, as discussed in the previous section, is in part driven by eosinophils. This was shown by a significant increase in IL-4 and IgG1 antibody production in the PP of eosinophils deficient mice, whereas T and B cell responses were unaffected in the mLN.

4.3.1 Eosinophils in Th2 resopnses

The finding that the frequencies of IL-4 competent Th2 cells were significantly increased in the PP of eosinophil deficient mice was at first surprising. Eosinophils are routinely cited as an inherent part of Th2 responses, whether it is as initiators (Padigel et al. 2007), or responders (Li et al. 1999), thus an increase in Th2 cells and IL-4 production in their absence seemed counterintuitive. However, when consolidating data on the role of eosinophils in Th2 responses using eosinophil deficient mice, it becomes apparent that, far from being a generalized feature of the functions of eosinophils, their propensity to support Th2 immune responses varies with the antigen (Fulkerson et al. 2006; Fattouh et al. 2011), genetic pre-disposition (Walsh et al. 2008) and route of antigen exposure (Chu et al. 2014a) and the success in demonstrating an involvement of eosinophils will depend on the compartment analyzed (Fabre et al. 2009; Huang et al. 2015). Table 4.1 summarizes studies in which eosinophil deficient mice have been used to investigate the role of eosinophils in Th2 induction. It can be seen that most of

the evidence for eosinophils supporting Th2 immunity comes from studies in which responses to allergens have been investigated. However, even here it is not straight forward, as illustrated by the following: Th2 responses are impaired in the absence of eosinophils in response to OVA on a C57BL/6 background (Lee et al. 2004), but not on a BALB/c background (Humbles et al. 2004), whereas at the same time being impaired on a BALB/c background in response to *A. fumigates* (Fulkerson et al. 2006), but not in response to house dust mites (Fattouh et al. 2011). This indicates that both the genetic background as well as the antigen used influences the involvement of eosinophils in Th2 immune responses to pulmonary allergens.

With regards to intestinal allergens, a recent study demonstrates that eosinophil deficiency results in impairment of Th2 induction in the mLN and spleen in response to oral challenge with peanut allergen co-applied with the mucosal adjuvant cholera toxin, whereas the lack of eosinophils has no effect on Th2 responses if the same antigen is applied parentally (Chu et al. 2014a). This is an interesting study, as it is one of the few addressing Th2 induction to an orally applied allergen, although sadly PP responses were not included. Nevertheless, it reports that levels of antigen specific serum IgG and IgE, as well as splenic Th2 cells are severely decreased in ΔdblGATA-1 mice and that this is due to an impairment in up-regulation of CCR7 on intestinal dendritic and thereby a decrease in the migration of Th2 inducing DC to the mLN (Chu et al. 2014a). In contrast to this, the current study showed no effect of eosinophil deficiency on Th2 induction in the mLN during *H. polygyrus* infection and no difference in DC frequencies in the mLN. This exemplifies the plasticity of eosinophil function with regard to Th2 induction and reiterates their potential role of initiators of Th2 responses following long sensitisation regimes with allergens, but an absence of such function during infection with naturally occurring intestinal helmniths.

With the exception of *T. spiralis*, in which levels of IL-4 producing $CD4^+$ cells are impaired in the muscle of eosinophil deficient mice, an involvement of eosinophils in helminth induced Th2 responses has not been demonstrated, whether tissue migrating (Voehringer et al. 2006; Cadman et al. 2014), liver dwelling (Swartz et al. 2006) or intestinally residing (Svensson et al. 2011) helminths were investigated. The current study adds to this field by demonstrating that eosinophil deficiency has no influence on Th2 induction to *H. polygyrus* in the mLN but additionally demonstrates the novel finding that the role of eosinophils may be to maintain a non-responsive state in the PP - an organ not previously investigated during helminth infection

Table 4.1 Summary of studies investigating Th2 induction in eosinophil deficient mice
NB. few studies (marked by *) have directly quantified cells based on GATA-3 or cytokine expression by $CD4^+$ cells. Rather, many studies deal with Th2 associated responses. + indicates and involvement of eosinophils in Th2 or Th2 associated responses, ~ indicates no involvement and – indicates negative regulation of eosinophils on Th2 associated responses.

Organ	Type of model	Antigen/pathogen	Mouse strain and background		Effect on Th2 responses	Reference
LUNG	**Allergens**	OVA	ΔdblGATA-1 (BALB/c)	~	* Th2 cells and IL-4,5,13 levels in the lung comparable both in acute and chronic OVA induced inflammation	Humbles et al. 2004, Science
			PHIL (BL/6)	+	Decreased lung pathology (proxy of Th2) in PHIL mice	Lee et al. 2004, Science
			ΔdblGATA-1 (BL/6)	+	* Decreased Th2 cell recruitment and IL-4,5 and 13 secretion in lung of PHIL mice	Walsh et al., 2008, JEM
			PHIL (BL/6)	+	Lower levels of IL-4,5,13 in BAL fluid of PHIL mice. Only rescued when OTII Th2 cells are transferred together with eosinophils	Jacobsen et al. 2008, JEM
			PHIL (BL/6)	+	Lower levels of IL-13 in BAL fluid and milder lung pathology in PHIL mice. Only transfer of blood eosinophils competent of IL-13 and pre-treated with cocktail of IL-4, GM-CSF and IL-33 restored lung IL-13 production and pathology.	Jacobsen et al. 2015, Allergy
		A. fumigatus	ΔdblGATA-1, $CCR3^{-/-}$ (BALB/c)	+ ~	Decreased IL-4 and IL-13 levels and mucus production in lung of eosinophil deficient mice. No effect on Th2 associated Antibody production.	Fulkerson et al. 2006, PNAS
		House dust mite antigen	PHIL , ΔdblGATA-1 (BALB/c)	~	* Comparable levels of lung Th2 cells and serum IgE in PHIL and ΔdblGATA-1 and similar splenocyte derived IL-5 and IL-13 in ΔdblGATA-1	Fattouh et al. 2011. Am J Respir Crit Care Med
	Helminths	*N. brasiliensis*	ΔdblGATA-1 (BALB/c)	~	* Normal Th2 induction and serum IgE levels in ΔdblGATA-1 mice.	Voehringer et al. 2006, JEM

		B. malayi	PHIL	_ ~	Increased serum IgE in PHIL mice during secondary infection. Levels of IL-5 and IL-13 from splenocytes comparable to WT.	Cadman et al. 2014, PLoS Path
INSTESTINE	**Allergen**	Peanut allergen + cholera toxin	ΔdblGATA-1 (BALB/c)	+ ~	IL-4 and IL-13 production from PN stimulated splenocytes abolished in ΔdblGATA-1 mice orally fed PN+CT. Not the case when PN administered parenterally. IgG1 IgE not affected in either case.	Chu et al. 2014a, JEM
	Helminths	*S. mansoni*	ΔdblGATA-1, PHIL (BALB/c)	- ~	Increased serum IL-5, but comparable IL-4 in eosinophil deficient mice.	Swartz et al. 2006, Blood
		T. muris	ΔdblGATA-1 (BALB/c)	~	* Eosinophils accumulate and produce IL-4 in mLNs, but no effect of eosinophil deficiency on Th2 induction.	Svensson et al. 2011, Parasite Immunol
		T. spiralis	PHIL (BL/6)	~ +	Antigen stimulated mLN cells produce similar levels of IL-4 and IL-13. Less IL-4 production in muscle draining LN.	Fabre et al. 2009, JI
			PHIL (BL/6)	+	* Fewer IL-4$^+$ CD4 and CD8 in diaphragm of PHIL mice	Gebreselassie et al. 2012, JI
			ΔdblGATA-1 (BL/6)	+	* Fewer IL-4$^+$ CD4 in diaphragm. Only transfer of IL-4$^+$ eos restore IL-4$^+$CD4$^+$ cells.	Huang et al. 2014, JI
			ΔdblGATA-1 (BL/6)	~ +	ΔdblGATA-1 mice have similar levels of Th2 cytokines in the mLNs, but specific impairment of IL-4 in cervical lymph nodes during secondary infection. Comparable serum levels of IgE and IgG1	Huang et al. 2015, JI

4.3.2 Eosinophils in B-cell responses

Several observations have associate eosinophils with B-cell survival and antibody production during homeostasis. Firstly, IL-5Tg mice experience high B-cell lymphocytosis in addition to eosinophilia (Wong et al. 2014). Crucially, higher levels of circulating B-cells is not due to the increased availability of IL-5, but rather to increases of eosinophils, as shown by normal levels of circulating B-cells in IL-5Tg PHIL mice, which have high serum IL-5 but are devoid of eosinophils. Secondly, eosinophils co-localize to B-cell areas of the human spleen and thymus (Wong et al. 2014) and are found in close proximity to plasma cells in the murine bone marrow (Chu et al. 2011). Thirdly, human eosinophils cultured with CpG stimulated B-cells increase B-cell proliferation and significantly enhances their secretion of IgM, IgG and IgA (Wong et al. 2014). Similarly, mouse intestinal eosinophils cultured with IgM^+ lamina propria B-cells significantly enhances their class switching to IgA, although the mechanism by which eosinophils support IgA class switching was not directly addressed (V.T. Chu et al. 2014). Thus several lines of evidence indicate that eosinophils can directly influence B-cell function during homeostasis. However, the influence of eosinophils on infection induced B-cell class switching has not previously been directly addressed. Cadman et al. (2014) report that serum IgE levels are significantly increased in PHIL mice infected with the filarial nematode *B. malayi*, although B-cells were not analysed. This is in agreement with the current study, in which it was demonstrated that ΔdblGATA-1mice had higher levels of circulating IgE and IgG1 following infection with *H. polygyrus*. Furthermore, this study is the first to report on the effect of eosinophil deficiency on GC formation and B-cell class switching during the course of an infection. It was shown that during *H. polygyrus* infection, eosinophils limit IgG1 class switching in the PP, without affecting the formation of GC, as assessed by normal levels of TFH cells and GC B-cells, but significantly increased levels of $IgG1^+$ GC B-cells in the PP of ΔdblGATA-1 mice. This correlated with a major increase in TFH cell derived IL-4.

The substantial increase in TFH cell derived IL-4, suggests that eosinophil mediated regulation of B-cell responses is likely an effect of eosinophil interaction with cell types upstream from B-cell class switching, rather than a direct interaction between eosinophils and B-cells.

As mentioned above, previous studies have demonstrated a severe impairment in the maintenance of intestinal IgA and IgA class switching in eosinophil deficient mice (V.T. Chu et al. 2014; Jung et al. 2015). In accordance with this, the results of the current study demonstrate a severe impairment in the maintenance of IgA^+ GC B-cells in the PP of ΔdblGATA-1 mice infected with *H. polygyrus*. However, although frequencies of IgA^+ GC B-cells were consistently lower in the PP of naive ΔdblGATA-1 mice compared to BALB/c mice, this difference did not reach statistical significance and thereby contradict previous reports from uninfected mice (V.T. Chu et al. 2014; Jung et al. 2015). Although the reason for this is unknown, the most likely explanation may be differences in intestinal microbiota between mice housed in different animal facilities. The development and maintenance of IgA is intimately linked with intestinal bacteria as demonstrated by the decreased levels of IgA in germ free mice (Round & Mazmanian 2009). Conversely, intestinal bacterial composition is regulated by IgA (Suzuki et al. 2004). Some data point to a difference in bacterial composition between BALB/c and ΔdblGATA-1 mice (V.T. Chu et al. 2014; Jung et al. 2015), which is in agreement with a discrepancy in the levels of intestinal IgA. However, as intestinal bacteria not only regulate IgA, but also the opposite is true, it is hard to determine what comes first. Do eosinophil deficient mice have an altered microbiota due to impaired IgA production, or do they have an impaired IgA production due to an altered microbiota? To address this, it would be necessary to co-house ΔdblGATA-1 and BALB/c mice for extended times prior to assessing whether a deficiency in IgA class switching is retained in eosinophil deficient animals.

4.4 HOW MAY EOSINOPHILS REGULATE PP IMMUNE RESPONSIVENESS TO *H. POLYGYRUS*?

In an effort to explain the increase in Th2 responses in the PP of eosinophil deficient mice, several mechanisms with known involvement in Th2 immunity were assessed. Here I will discuss the results dealing with expression of the alarmins IL-33, IL-25 and TSLP as well as

siLP, PP and mLN DC subsets. I will further discuss the possibility that eosinophils regulate Th2 responses in the PP via their support of IgA class switching.

4.4.1 TSLP, IL-25, IL-33

The alarmins TSLP, IL-25 and IL-33 are produced during helminth infection and allergic reactions following epithelial damage and recognition of a variety of molecules including proteases, chitins and β-glucans (Hammad & Lambrecht 2015). They have all been implicated in several aspects of Th2 immunity and protection to intestinal helminths (Fallon et al. 2006; Humphreys et al. 2008; Taylor et al. 2009) and have both overlapping (Taylor et al. 2009; Owyang et al. 2006; Humphreys et al. 2008) and distinct (Chu et al. 2013) functions. In addition, the expression of one alarmin can influence the responsiveness to another as demonstrated by increased expression of IL-25 receptor (IL-25R) on Th2 cells cultured with TSLP stimulated DC (Wang et al. 2007).

4.4.1.1 *IL-33*

IL-33 is expressed under basal conditions in a wide range of organs including the intestine, lungs and central nervous system (Schmitz et al. 2005) and is significantly up-regulated during infection with *T. muris* (Humphreys et al. 2008) or exposure to allergens such as the fungus *Alternaria alternata* (Bartemes et al. 2012). IL-33 can activate a range of cells including eosinophils, basophils, mast cells and T-cells (Saenz et al. 2008) and is a strong inducer of IL-5 and IL-13 from type 2 innate lymphoid cells (Bartemes et al. 2012; Hung et al. 2013). In accordance with this, IL-33$^{-/-}$ mice fail to significantly increase ILC2 derived IL-13 during *N. brasiliensis* infection and are unable to expel parasites during a primary infection or mount protective responses against re-infection (Hung et al. 2013). IL-33 additionally enhances IL-5 and IL-13 (but not IL-4) secretion from differentiated Th2 cells stimulated with CD3/28 and exogenous treatment with IL-33 leads to the development of Th2 cells *in vivo* (Schmitz et al. 2005). Finally, exogenous IL-33 confers protection against *T. muris* in otherwise susceptible mice via inhibition of IL-12 (Humphrey et al. 2008).
In this study IL-33 was not significantly up-regulated in the siLP of BALB/c mice infected with *H. polygyrus* and expression was only slightly increased in ΔdblGATA-1 mice. This is in accordance with a previous study in which IL-33 was not up-regulated in the siLP of C57BL/c mice infected with *H. polygyrus* (Zaiss et al. 2013). Interestingly, administration of HES has been shown to inhibit IL-33 expression in an OVA/*Alternaria* allergy model (McSorley et al. 2014). Thus it could be that *H. polygyrus* is actively inhibiting up-regulation of IL-33 in the intestine in order to facilitate its prolonged retention.
As IL-33 expression did not significantly differ between BALB/c and ΔdblGATA-1 mice, it is concluded that IL-33 is not implicated in increased Th2 responses to *H. polygyrus* in the PP of eosinophil deficient mice.

4.4.1.2 *IL-25*

IL-25 is up-regulated in the lungs of mice infected with *N. brasiliensis* and in response to pulmonary allergens (Zhao et al. 2010; Angkasekwinai et al. 2007). Administration of IL-25 switches the immune response of AKR mice following *T. muris* infection from a Th1 response to a protective Th2 response, which is mediated via the inhibition of IL-12 (Owyang et al. 2006). Nevertheless, the role of IL-25 in Th2 induction is contentious. Although IL-25$^{-/-}$ mice fail to develop protective Th2 immunity during *T. muris* infection (Owyang et al. 2006), *N. brasiliensis* infection of IL-25$^{-/-}$ results in significantly higher levels of IL-4 and IL-13 in the mLN following stimulation with antigen, although the secretion of IL-13 (and IL-5) is delayed (Fallon et al. 2006). Interestingly, despite the high levels of IL-4, IL-25$^{-/-}$ mice experienced

delayed worm expulsion. (Fallon et al. 2006). These data indicate the importance of IL-25 in the stimulation of early production of protective cytokines, but also makes the important point that IL-25 is not needed for Th2 differentiation, as antigen specific secretion of Th2 cytokines was increased in IL-25$^{-/-}$ mice (Fallon et al. 2006).

In the current study, IL-25 expression was significantly enhanced in the siLP of BALB/c mice following *H. polygyrus* infection, whereas ΔdblGATA-1 mice did not up-regulate IL-25 expression. The increased expression of IL-25 mRNA in BALB/c mice coincided with significant infiltration of eosinophils at day 6 p.i. Human esoinophils isolated both from normal and allergic subjects express the highest levels of IL-25 out of a wide range of cells isolated from blood (Wang et al. 2007). It is therefore possible that the increase in IL-25 expression in BALB/c mice was a direct effect of infiltrating eosinophils. Nevertheless, given that IL-25 is implicated in Th2 induction at least in some settings and considering that eosinophil derived IL-25 enhances the secretion of Th2 cytokines from TSLP-DC activated Th2 memory cells (Wang et al. 2007), the increased expression of IL-25 in BALB/c mice was surprising. It may be that infiltrating eosinophils express IL-25 mRNA but do not secrete the protein, but rather store it in their granules, as is the case with many cytokines (Rothenberg & Hogan 2006). If so, then the high levels of IL-25 mRNA detected in the siLP of BALB/c mice would not necessarily translate into an increase in Th2 associated processes. Cell sorting and *in vitro* stimulation of siLP eosinophils will provide information as to whether mouse eosinophils are equally potent in secretion of IL-25 as their human counterparts. This, as well as co-culture of mouse eosinophils with TSLP-DC activated Th2 cells will also provide valuable information regarding the similarities between mouse and human eosinophils (Lee et al. 2012).
In conclusion, increased Th2 responses in the PP of ΔdblGATA-1 mice did not correlate with increased expression of IL-25 in the gut of eosinophil deficient mice. Whether murine eosinophils, similar to their human counterparts, express IL-25 deserves further investigation.

4.4.1.3 *TSLP*

TSLP is constitutively expressed in epithelial cells of the gastrointestinal tract and skin and increases during infection with viral, bacterial and parasitic infections as well as in response to Th2 cytokines (Saenz et al. 2008). TSLP has been repeatedly shown to inhibit IL-12 production (Soumelis et al. 2002; Taylor et al. 2009) and enhance OX40L expression (Ito et al. 2005; Seshasayee et al. 2007) in both mouse and human DC, thereby rendering them potent inducers of Th2 differentiation. Furthermore, TSLP can directly act on $CD4^+$ T-cells and induce expression of GATA-3 as well as secretion of IL-4, IL-5 and IL-13 in polyclonally stimulated naive T-cells (Omori & Ziegler 2007).
TSLP is implicated in the support of a protective Th2 response and worm expulsion during infection with *T. muris* and $TSLP^{-/-}$ mice are completely unable to control infection (Taylor et al. 2009). Importantly, blocking of IFN-γ restores a protective Th2 response in $TSLP^{-/-}$ mice, suggesting that TSLP is not directly instructing Th2 induction, but rather facilitates Th2 immunity by inhibiting Th1 responses (Taylor et al. 2009). Interestingly, during *H. polygyrus* and *N. brasiliensis* infection, TSLP is redundant in Th2 induction and parasite expulsion, as both these helminths secrete products that directly inhibit IL-12 production by DC (Massacand et al. 2009).
In the current study, TSLP expression was significantly higher in the siLP of *H. polygyrus* infected ΔdblGATA-1 mice compared to WT, both at day 3 and 6 p.i. This might affect the ability of gut DC to express IL-12 and thus be involved in facilitating Th2 induction in the PP (see next paragraph). In addition, Eosinophils in the siLP express IL-1β (Jung et al. 2014), which has been demonstrated to regulate the expression of IL-25 and IL-33 in C57BL/67 mice infected with *H. polygyrus* and by doing so to promote helminth chronicity (Zaiss et al. 2013). As it is not known whether IL-1β additionally regulate the expression of TSLP, it remains to be assessed whether the increased expression of TSLP in the absence of eosinophils is a consequence of a lack of eosinophil-derived IL-1β.
Although TSLP is redundant in Th2 induction in the mLN during *H. polygyrus* infection, its effect on responses in the PP has not previously been investigated. Thus, it is possible that an increase in TSLP in the absence of eosinophils may influence the phenotype of DC migrating to the PP during *H. polygyrus* infection. As mentioned above, TSLP can directly stimulate secretion of Th2 cytokines from naive $CD4^+$ T-cells (Omori & Ziegler 2007). Thus, although levels of IL-4, IL-5 and IL-13 producing T-cells were comparable in the siLP of BALB/c and dblGATA-1 mice, increased expression of TSLP in the absence of eosinophils may extend to

the epithelia covering the PP, in which case it is possible that TSLP directly contributes to enhanced Th2 responses in ΔdblGATA-1 mice. *In vivo* blocking of TSLP in ΔdblGATA-1 mice will determine whether TSLP is involved in enhancing Th2 immunity in the PP of eosinophil deficient mice.

4.4.2 Intestinal dendritic cells

Intestinal DC are commonly categorized based on expression of CD103, CD11b and CD8α. $CD103^+$ DC are the main migratory subset from the lamina propria to the mLN, but $CD103^-$ cells are also found in the efferent lymph (Cerovic et al. 2013). $CD11b^+$ cells are of myeloid origin and, in the PP, reside in the sub-epithelial dome. In contrast, $CD8\alpha^+$ DC are of lymphoid origin and reside in the interfollicular region. In addition, CD11b CD8α double negative DC reside in both areas of the PP (Iwasaki & Kelsall 2001). To aid the discussion of intestinal DC subsets, table 4.2 outlines some of the described functions of $CD11b^+$ ($CD103^{+/-}$) and $CD8^+$ ($CD103^{+/-}$) DC.

An important point underscored by the summary in table 4.2 is that surface expression of CD11b or CD8α is often not sufficient to discern distinct subsets of DC in orchestrating immune responses. For example, although both $CD103^+CD11b^+$ and $CD103^+CD8a^+$ DC secrete IL-6 and IL-12, albeit in response to distinct stimuli, and thereby support Th1 and TH17 cell differentiation (Uematsu et al. 2008; Fujimoto et al. 2011), $CD103^+CD11b^+$ DC additionally express high levels of the enzyme Aldh1a2, which catalyses retinal into retinoic acid (Uematsu et al. 2008). In this way, $CD103^+CD11b^+$ DC additionally support B-cell class switching to IgA, which is not a feature of $CD103^+CD8a^+$ DC (Fujimoto et al. 2011; Uematsu et al. 2008). Furthermore, $CD103^-CD11b^+$ DC have been implicated in Th2 induction *in vivo*, (Plantinga et al. 2013), whereas the same phenotype promotes Th1 cells *in vitro* (Cerovic et al. 2013), underscoring the importance of environmental cues in determining DC function.

In the current study, frequencies of $CD11b^+$ DC did not significantly differ between BALB/c and ΔdblGATA-1 mice. However, given the above, it will be important to analyze cytokine expression within $CD11b^+$ DC, as this group is likely heterogeneous. In contrast, frequencies of $CD103^+CD8\alpha^+$ DC were found to be higher in the PP of naive BALB/c mice as compared to naive ΔdblGATA-1 animals. This subset has been implicated in regulation of Th2 responses via the production of IL-12 (Everts et al. 2015). Although, the difference in $CD103^+CD8\alpha^+$ DC was no longer apparent in the PP of *H. polygyrus* infected mice two weeks post infection, it is nevertheless possible that eosinophil mediated support of $CD103^+CD8\alpha^+$ DC during homeostasis and, possibly, early after infection, provide conditions in the PP unsuitable for or at least limiting Th2 differentiation. In order to further understand whether difference in $CD103^+CD8a^+$ DC may contribute to the disparity in PP Th2 immune responses between BALB/c and ΔdblGATA-1 mice, it will be important to additionally assess their kinetics at early time points post infection. Furthermore, as with $CD11b^+$ DC, an important next step will be to analyze the cytokine expression of $CD103^+CD8\alpha^+$ intestinal DC subsets, to confirm whether a difference in IL-12 secretion can be detected between BALB/c and ΔdblGATA-1 DC in the PP.

Table 4.2 Overview of intestinal dendritic cell subsets and their described functions

CD103 expression	Phenotype	Function	Reference
$CD103^+$	$CD11b^+$ ($CD8\alpha^-$)	Express TLR5. Produce IL-6, IL-12 and retinoic acid. Promote Th1 and Th17 differentiation as well as IgA class switching, in response to flagellin	Uematsu et al., 2008, Nat Immunol
		Produce IL-6. Support Th17 cell differentiation	Persson et al. 2013, Immunity
		Support Th1 induction	Milling et al. 2009 Mucosal Immunol
	$CD8\alpha^+$ ($CD11b^-$)	Express TLR3, TLR7, TLR9. Produce IL-6 and IL-12 and promote Th1 and Th17 differentiation, but not IgA class switching	Fujimoto et al. 2011, JI
		Cross present antigen to $CD8\alpha^+$ T-cells	Cerovic et al. 2015, Mucosal Immunol
		Produce IL-12. Prime $CD8^+$ T-cells for gut homing	Moretto et al. 2015, Infect Immun
		Produce IL-12. Suppress Th2 responses during *S. mansoni* and *H. polygyrus* infection	Everts et al. 2015, J Ex Med
$CD103^-$	$CD11b^+$ ($CD8\alpha^-$)	Express IL-12. Support Th1 and Th17 cell differentiation	Cerovic et al. 2013, Mucosal Immunol
		Induce Th2 responses to house dust mite antigen	Plantinga et al. 2013, Immunity
	$CD8\alpha^+$ ($CD11b^-$)	Support Th17 cells	Cerovic et al. 2013, Mucosal Immunol
CD103?	$CD11b^+$ ($CD8\alpha^-$)	Produce IL-10 in the PP	Shiokawa et al. 2009, Immunol lett
		Produce IL-10 in the PP. Prime IL-4 and IL-10 production in $CD4^+$ T-cells	Iwasaki & Kelsall 2001, JI
		Produce IL-6 in the PP and support IgA class switching	Sato et al. 2003, JI
		Cross present intragastric OVA in the mLN	Chung et al. 2005, Blood
		Support IgA class switching via iNOS mediated enhancement of TGF-β receptor on B-cells	Tezuka et al. 2007, Nature
	CD8α+ (CD11b-)	Cross present intravenous OVA in the spleen	Chung et al. 2005, Blood
		Produce IL-12. Prime IFNγ production in $CD4^+$ T-cells	Iwasaki & Kelsall 2001, JI
	$CD11b^-$ $CD8\alpha^-$	Produce IL-12. Prime IFNγ production in $CD4^+$ T-cells	Iwasaki & Kelsall 2001, JI

4.4.3 Potential role of IgA

As mentioned at the beginning of this chapter, some reports suggest that the PP represent an inherently “hypo-immune” environment. That is, $CD4^+$ T-cells isolated from the PP show diminished actin polymerisation and chemotaxis towards several chemokines, compared to T-cells isolated from mLN (Kellermann & McEvoy 2001). Furthermore, PP DC tend to secrete high levels of IL-10 in response to stimuli that induces IL-12 secretion from DC isolated from the periphery (Shiokawa et al. 2009; Iwasaki & Kelsall 1999) and are particularly good at supporting IgA class switching from B-cells (Sato et al. 2003). Some data indicate that the maintenance of IgA may be key in inhibiting other types of immune responses in the PP (Favre et al. 2005; Diana et al. 2013).

Mouse and human M cells express a receptor of unknown identity that specifically binds IgA (but not IgM or IgG1) (Mantis et al. 2002) and transports it, with or without bound antigen, from the lumen into the PP interior (Rey et al. 2004). Translocated IgA is internalized by DCs residing in the SED and is bound by B and T-cells on their surface via an equally unidentified receptor (Rey et al. 2004). Following oral application of soluble IgA (SIgA), IgA/*Shigella flexneri* immune complexes or *S. flexneri* alone, immune complexes were found to be taken up by rabbit PP, inhibit expression of pro-inflammatory mediators and prevent excessive inflammation and pathology caused by *S. flexneri* treatment alone (Boullier et al. 2009).

A more recent study investigated the effect of SIgA on bone marrow derived DC (BMDC) *in vitro.* The authors found that BMDC bind SIgA via ICAM-3 grabbing non-integrin receptor 1-SIGNR1- the murine homologue of DC-SIGN (Diana et al. 2013) and that this leads to the development of tolerogenic DC, as assessed by high expression of IL-10 and impaired maturation in response to several TLR ligands. In accordance with this, IL-12 secretion in response to LPS, zymosan or CpG, was fully inhibited in SIgA-DC, whereas they were potent inducers of Treg. Unfortunately the ability of SIgA-DC to inhibit secretion of Th2 cytokines was not assessed (Diana et al. 2013).

Considering the role of eosinophils in maintaining IgA class switching in the PP (V.T. Chu et al. 2014; Jung et al. 2014) it may be that eosinophil mediated support of IgA class switching underlies their regulation of Th2 responses in the PP. Oral application of SIgA to ΔdblGATA-1 mice followed by *H. polygyrus* infection may offer a way in which to investigate this possibility further. However, although assessing the Th2 and IgG1 response in ΔdblGATA-1 mice treated with SIgA would determine whether eosinophil associated regulation of PP immune responses is mediated via IgA, it will not answer the question of how eosinophils support IgA maintenance, an issue that is still unresolved.

Jung et al. (2015) suggest that IL-1β, secreted by intestinal eosinophils, may underlie their support of IgA, as $IL\text{-}1\beta^{-/-}$ mice have low levels of intestinal IgA, comparable to eosinophil deficient mice. The authors further suggest that eosinophil derived IL-1β supports IgA maintenance via iNOS expressing $ROR\gamma t^+$ ILC, although the evidence for this is rather indirect (Jung et al. 2015). Another potential function of IL-1β may be to maintain IL-12 producing DC, as has been shown in BMDC *in vitro* (Wesa & Galy 2001). Therefore, in addition to the postulated role of IL-1β in IgA maintenance (Jung et al. 2015), IL-1β may further underlie the ability of eosinophils to inhibit Th2 responses in the PP via the support of IL-12 producing DC. The actions of IL-6 may have a similar dual role in IgA maintenance and Th2 regulation, as DC derived IL-6 is an important cytokine in IgA class switching (Sato et al. 2003), but has also been implicated in the suppression of Th2 responses (Mayer et al. 2014; Smith & Maizels 2014).

Thus, a possible model of eosinophil mediated regulation of Th2 and IgG1 responses in the PP can be put together in which eosinophils in the SiLP, possibly in response to IL-33, produce IL-1β and/or IL-6 thereby maintaining populations of DC producing IL-12 and/or IL-6. As these cells enter the PP, they may already be prone to Th2 inhibition and IgA stimulation, respectively. The establishment of such a base line environment and the resulting presence of high levels of IgA, may be sufficient to maintain a regulated state in the PP following infection with intestinal helminths. If IgA regulates Th2 responses in the PP, it would offer a rationalization for the lack of eosinophil influence on immune responses in the mLN, as the

mLN is not continuously exposed to IgA and IgA immune complexes from the lumen. These ideas are summarized in figure 4.1.

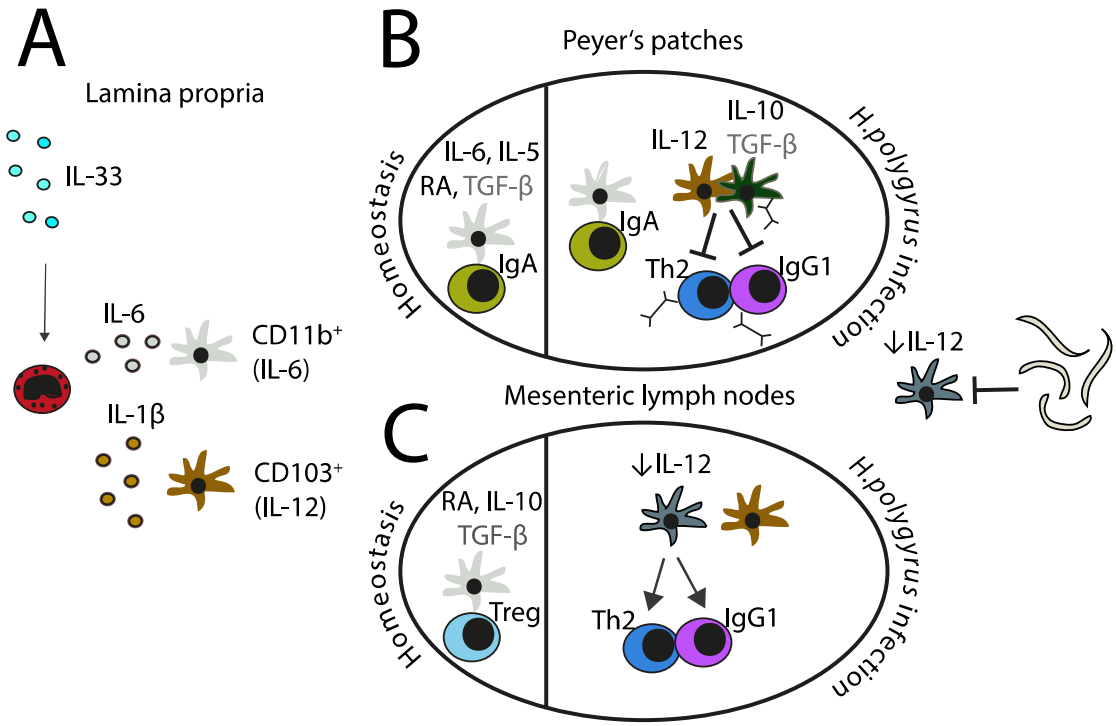

Figure 4.1 Potential role of IgA in the regulation of Th2 responses
A. Constitutively secreted IL-33 may maintain IL-6 and IL-1β secretion from siLP eosinophils, which in turn may contribute to the support of IL-6 and IL-12 secreting DC. **B.** In the PP, IL-6 secreting DC support IgA class switching during homeostasis (Sato et al. 2003) together with IL-5, retinoic acid (RA) and TFG-β. The availability of TGF-β is essential for IgA class switching, but is not derived from DC, but from Treg cells. During *H. polygyrus* infection, IL-12 producing DC may contribute to the inhibition of Th2 responses following *H. polygyrus* infection (Everts et al. 2015). In addition, the presence of IgA-immune complexes, transported into the PP via M-cells, promote a tolerogenic DC phenotype that secrete high levels of IL-10 and induce TGF-β secretion from Treg (Diana et al. 2013). T and B-cells also bind IgA on their surface (Rey et al. 2004), which could be responsible for the regulated T-cell phenotype demonstrated from PP T-cells (Kellermann & McEvoy 2001). **C.** In the mLN, DC induce T-reg cells and tolerance to commensal bacteria, food and self antigens during homeostasis. During *H. polygyrus* infection, incomplete maturation of DC (low levels of co-stimulatory molecules) and low secretion of IL-12 results in the induction of Th2 cells, followed by Th2 associated antibody responses. Although the mLN and PP are exposed to the same DC from the siLP, availability of IgA-immune complexes in the PP may underlie differences in immune reactivity to infection. Furthermore, it is likely that differences in stromal cell mediators provide additional environmental differences.

4.4.4 Future studies on eosinophil regulation of PP Th2 and IgG1 responses

Although an increase in Th2 responses and IgG1 induction in the PP was demonstrated in two strains of eosinophil deficient mice, depletion of eosinophils via administration of anti-Siglec-F antibodies did not result in an increase in Th2 or IgG1 responses in the PP. This correlated with an incomplete depletion of eosinophils in the lamina propria following anti-

Siglec-F treatment. This was unexpected, as a previous study, using the same amount of mAb and the same route of administration, demonstrated an almost complete absence of eosinophils in the siLP of BALB/c mice treated with anti-Siglec-F (V.T. Chu et al. 2014). Nevertheless, a considerable level of eosinophils remaining in the siLP of mAb treated mice may explain why no enhancement of Th2 and IgG1 antibody responses, similar to that seen in eosinophil deficient mice, could be detected in mice treated with anti-Siglec-F mAb.
An alternative approach for demonstrating that eosinophils are responsible for the observed phenotype of ΔdblGATA-1 and PHIL mice is to reconstitute eosinophil deficient mice with eosinophils prior to *H. polygyrus* infection. A major hindrance to this approach is the relatively low numbers of eosinophils that can be isolated from a WT mouse. The generation of high numbers of pure eosinophils from bone marrow may provide a tool for circumventing this problem. This approach has been previously used to study eosinophil trafficking to the lung following intra pulmonary application of eotaxin (Sturm et al. 2013). However, whether BmEos traffic to the small intestine in the absence of additional stimuli is not known. In the future, this approach should be applied to the *H. polygyrus* infection model in order to find additional support for the role of eosinophils as regulators of immune events in the PP. Furthermore, this approach can address the important question of whether the observed phenotype in the PP of eosinophil deficient mice is a result of a long term absence of eosinophils. That is, whether the results presented in this thesis stem from eosinophil maintenance of homeostasis or eosinophil mediated functions in response to helminth infection.

4.5 ROLE OF EOSINOPHILS IN *H. POLYGYRUS* SURVIVAL AND FECUNDITY

The third aim of this study was to investigate the influence of eosinophils on *H. polygyrus* survival and fecundity. Although no influence of eosinophil deficiency was seen on the survival of *H. polygyrus*, the presence of eosinophils in BALB/c mice appeared to benefit *H. polygyrus* fitness, as assessed by decreased fecundity of individual females isolated from ΔdblGATA-1 mice. This was despite comparable intestinal levels of mediators known to impair helminth health and motility (Esser-von Bieren et al. 2014; Herbert et al. 2009). These somewhat surprising findings are discussed in the following section.

4.5.1 *H. polygyrus* survival and fecundity

Despite the wide use of this parasite infection model and notwithstanding the high levels of eosinophilia it induces, reports on the effects of eosinophil deficiency on *H. polygyrus* survival are rare (Knott et al. 2009; Urban et al. 1991a) and, until very recently (Hewitson et al. 2015) effects of eosinophil deficiency have not been assessed in this model. The few reports on the role of eosinophils in *H. polygyrus* infection, as investigated with the use of IL-5$^{-/-}$ or anti-IL-5 treated mice, have demonstrated no role for eosinophils in *H. polygyrus* survival, establishment of chronicity (Knott et al. 2009) or in protection against re-infection (Urban et al. 1991a). However, a recent study investigating antibody responses to HES vaccination reported that ΔdblGATA-1 mice have more adult worms in the small intestine on day 28 post infection compared to BALB/c mice (Hewitson et al. 2015). Both mouse strains were however protected against *H. polygyrus* infection following vaccination with HES. In agreement with this last point, ΔdblGATA-1 and BALB/c mice were found to be equally protected against re-infection with *H. polygyrus* in the current study. However, adult parasites recovered from the lumen of ΔdblGATA-1 and BALB/c mice were comparable at day 14 post primary infection, which is in disagreement with results obtained from day 28 p.i. by Hewitson et al. (2015). This apparent discrepancy is intriguing and may indicate that *H. polygyrus* is able to survive for extended times in the absence of eosinophils. This is particularly interesting if one interprets the differences in female worm fecundity differently: it might be that the decreased egg production of parasites derived from eosinophil-deficient mice reflects an adaptation by *H. polygyrus* to a decreased immunological pressure. Conversely, increased egg production by worms developing in wild type mice may represent an appropriate adaptation to the presence of eosinophils, during which a rapid reproductive success may be advantageous. This idea has

been previously suggested in the context of the filarial nematode *Litosomoides sigmodontis. L. sigmodontis* develop faster and produce more eggs in the presence of high eosinophilia in IL-5Tg mice (Martin et al. 2000). Interestingly however, IL-5Tg mice expel worms quicker than WT mice (Martin et al. 2000). The authors argue that, in the presence of eosinophils, *L. Sigmodontis* changes its strategy to fast, but brief development in order to maximise its reproductive success. Thus, conversely, in the absence of such a pressure, it can be speculated that *H.* polygyrus adopts a slow, but prolonged strategy, as would be suggested by the combined results of Hewitson et al. (2015) and the present investigation. Further studies on the kinetics of *H. polygyrus* expulsion in ΔdblGATA-1 and BALB/c mice may gain additional support for this theory, although an "intentional" adaptation of *H. polygyrus* to the external pressure of eosinophils may be difficult to conclusively prove, as many other factors likely contribute to the impaired fecundity of parasites developing in eosinophil deficient hosts. Furthermore, the absence of antibodies (McCoy et al. 2008) and $CD4^+$ cells (Urban et al. 1991b)- factors which may also be viewed as sources of stress for *H. polygyrus*, have both been shown to increase parasite fecundity, as discussed below.

4.5.2 Anti-parasite immune responses

The most obvious explanation for the decrease in *H. polygyrus* fecundity in the absence of eosinophils at first seemed to be the enhancement of Th2 induction and IgG1 class switching demonstrated in the PP of ΔdblGATA-1 mice. Indeed, both $CD4^+$ cells and IgG antibodies have been shown to control fecundity in primary *H. polygyrus* infection (Urban et al. 1991b; McCoy et al. 2008). However, a closer look at these processes gave no indication that a connection exist between enhanced immune responses in the PP of ΔdblGATA-1 mice and the decreased fecundity of parasites developing in this host.

4.5.2.1 *IgG1*

IgG is associated with control of *H. polygyrus* fecundity as shown by decreased egg shedding in $JH^{-/-}$ mice, which lack mature B-cells, following transfer of purified IgG (McCoy et al. 2008). It therefore seemed plausible that the increased levels of circulating IgG1 seen in ΔdblGATA-1 mice infected with *H. polygyrus* could be a contributing factor to the decreased parasite fecundity in these mice. However, although the mechanism by which IgG limits parasite fecundity is not known, it may be expected that it involves FcγR mediated binding of IgG1 to effector cells present at the site of infection. Yet, at day 14 p.i. IgG1 was not detected in intestinal tissues of either BALB/c or ΔdblGATA-1 mice (data not shown). Although it is possible that IgG is present in very low amounts, not detected here, the effect of IgG on *H. polygyrus* fecundity is conferred even when transferred IgG is isolated from naive mice (McCoy et al. 2008), suggesting that the mechanism of IgG mediated impairment of *H. polygyrus* fecundity is unspecific. This indicates that the increased circulating levels of IgG1 seen in ΔdblGATA-1 mice is not responsible for the decrease in fecundity of *H. polygyrus* developing in the absence of eosinophils. Nevertheless a pressing question is whether the $IgG1^+$ cells, induced to excess in the PP of eosinophil deficient mice, execute a function in the SiLP. Looking closer at the presence of cell bound IgG1 in the lamina propria and on the effect this may have on worm fitness will be informative. A further possibility could be that IgA antibodies bind to the surface of *H. polygyrus* in the lamina propria and thereby protects it from antibody or cell mediated damage and that the decrease of available IgA in dblGATA-1 mice could thereby expose the parasite to enhanced stress.

4.5.2.2 *Th2 cells, AAM and goblet cells*

It has been shown that depleting antibodies against $CD4^+$ cells, though not affecting survival of *H. polygyrus*, results in significantly increased egg production (Urban et al. 1991b). Furthermore, IL-4 and IL-13 induce the development of AAM and the differentiation of mucus producing goblet cell (Anthony et al. 2007). The role of AAM during primary *H. polygyrus* infection is not known, but they are present in the granulomas that form along the duodenum (Reynolds et al. 2012) and could therefore have detrimental effects on *H. polygyrus* health.

Furthermore, goblet cells produce Relm-β, which has been shown to interfere with *H. polygyrus* feeding. These processes could therefore potentially contribute to the impairment of parasite fecundity in eosinophil deficient mice. However, in the lamina propria, comparable levels of GATA-3$^+$, IL-4$^+$, IL-5$^+$ and IL-13$^+$ CD4$^+$ cells were present in *H. polygyrus* infected BALB/c and ΔdblGATA-1 mice. In accordance with comparable levels of IL-4 and IL-13 in the siLP, the expression of the AAM markers Relm-α and Arg-1 was similar in BALB/c and ΔdblGATA-1 mice, as was the number of goblet cells and the expression of Relm-β. Thus, none of these processes are likely to explain the impaired parasite fecundity seen in *H. polygyrus* developing in eosinophil deficient mice.

4.5.3 Eosinophils as maintainers of *H. polygyrus* niche

Another possibility as to why the eosinophils are of apparent benefit to the fitness of *H. polygyrus* may be that eosinophils are involved in maintaining the general health of the intestine and that this is essential for optimal development of *H. polygyrus*. Two studies have previously indicated that eosinophil deficient mice have a disrupted mucus layer in the duodenum (V.T. Chu et al. 2014; Jung et al. 2015) and this study confirms these findings. Although excessive mucus production is thought to be implicated in expulsion of *H. polygyrus*, it is possible that a healthy mucus layer is important for *H. polygyrus* development. Only one study has investigated the feeding habits of *H. polygyrus* and concludes that its main diet consists of host epithelial cells (Bansemir & Sukhdeo 1994). If the mucus constitutes an additional source of nutrients, then resource limitation could contribute to the reduced fitness in worms developing in a sub-optimal mucus layer. This is not an easy idea to test, as a specific depletion of the mucus layer is not possible. However, mucus is made up of a complex matrix of molecules including the gel-forming Muc2 (Johansson et al. 2011). Thus, infection with *H. polygyrus* in Muc2$^{-/-}$ mice may provide clues as to whether a normal mucus layer is supportive of *H. polygyrus* health and fecundity.

4.5.4 The potential of helminths in regulating eosinophil function

This thesis and many other reports where eosinophils, despite being highly upregulated during helminth infection, appear to have little effect on parasite survival (see table 10.1, appendix), raises the interesting possibility of parasite mediated modulation of eosinophil function. From an evolutionary point of view it makes sense- if we assume, as many do, that eosinophils evolved to participate in host defence against helminths, then helminths probably evolved to counter regulate their function.

Some studies hint at a role for helminth mediated immune modulation on eosinophil function. Firstly the observation that eosinophils from patients infected with *S. mansoni* are less potent in the killing of schistosomula *in vitro* than are eosinophils from healthy people (Butterworth et al. 1975) suggests that *S. mansoni* may have evolved processes by which it can reduce eosinophil function. Secondly, E/S products from *Toxocara canis* added to cultures of eosinophils and L3 *N. brasiliensis* larvae, inhibits eosinophil attachment to *N. brasiliensis* and thereby eosinophil mediated killing (Giacomin et al. 2008). Thirdly- *H. polygyrus* infection leads to down-regulation of eotaxin as well as CCR3 expression on eosinophils during murine pulmonary inflammation (Rzepecka et al. 2007). Fourthly, levels of small intestinal eosinophils in BALB/c mice infected 6 weeks with *H.polygyrus* are below that of un-infected controls, suggesting that *H. polygyrus* is actively suppressing eosinophil numbers. This effect was reversed by administration of anti-TGF-β (Doligalska et al. 2006), indicating an involvement of TGF-β activity - an established property of HES (Maizels et al. 2012a; Graiger et al. 2010).

Whether a host-parasite system is a “natural” system, i.e. whether the helminth species is well adapted to the host, may be important in influencing their ability to mediate modulation of eosinophils. In many current model systems, mice are infected with helminhts that are not well adapted to survival in the mouse (e.g. *N. brasiliensis*, *S. ratti*). In these systems, helminths are often quickly killed and expelled, sometimes with the help of eosinophils (Daly et al. 1999; Watanabe et al. 2003). *H. polygyrus* infection is therefore a good model for analysing helminth mediated modulation of eosinophils in more detail.

5 CONCLUSION & PERSPECTIVES

5.1 CONCLUSION

The data presented within this thesis complement recent advances in our understanding of eosinophils as maintainers of intestinal health during homeostasis (V.T. Chu et al. 2014; Jung et al. 2015) and extend these findings by demonstrating three novel findings. Firstly, it shows for the first time that a dichotomy exists between the immune events taking place in the PP and the mLN with the former being the main site for IgA class switching, whilst the latter represents the primary site for Th2 induction and antibody production in response to an intestinal helminth infection. Secondly it demonstrates a novel role for eosinophils in maintaining such a dichotomy. Thirdly it reveals that eosinophils, contrary to what might be expected, can be beneficial for the fitness of intestinal helminths. These three points are summarized in figure 5.1.
It can be concluded from these data that, in contrast to the traditionally defined role of eosinophils as inflammatory effector cells, they have additional valuable functions in maintenance of intestinal homeostasis during enteric infections.

5.2 PERSPECTIVES

The findings within the scope of this thesis stimulate a range of further questions, most of which have been discussed in the previous section. The most pressing question that should be the focus of future investigation and which has already been thoroughly discussed, is the mechanism by which eosinophils may mediate regulation of Th2 immunity in the PP and whether this might be mediated by IgA. A second intriguing question that warrants further investigation is whether the observed decrease in parasite fecundity seen in eosinophil deficient mice is a result of parasite adaptation to an environment with an altered immunological pressure or whether eosinophils mediate a beneficial intestinal environment for optimal parasite development.
In addition to these questions, it will be of great interest to determine whether eosinophil mediated regulation of PP immune responses to enteric infection is limited to Th2 inducing pathogens or is a more general feature extending to Th1 associated responses. Furthermore, the effect of eosinophil deficiency during infection with a pathogen to which IgA is imperative in protection, may implicate eosinophils further as maintainers of intestinal health.

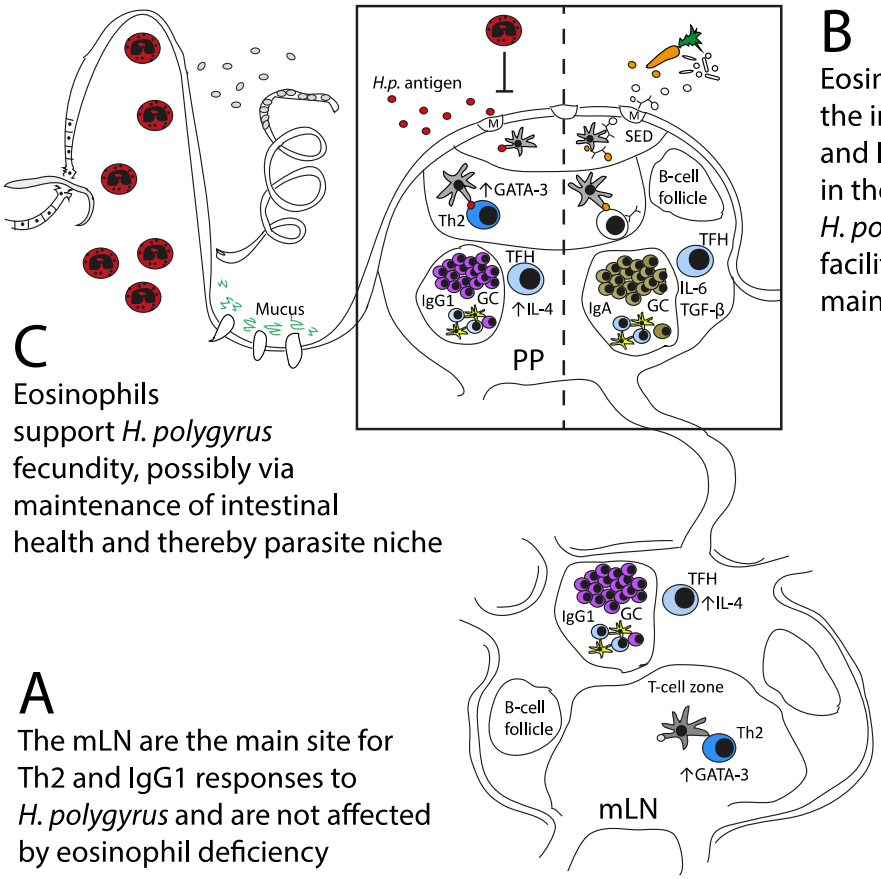

Figure 5.1 Summary of main findings
A. The mLN are the main site for *H. polygyrus* induced Th2 and IgG1 responses, with no significant contribution to these responses by the PP. Eosinophils do not influence Th2 and IgG1 responses in the mLN. **B.** The PP are the main site for IgA antibody production during homeostasis and after infection with *H. polygyrus*. Eosinophils are important in suppressing Th2 responses in the PP during *H. polygyrus* infection and ensure that IgA class switching is maintained. **C.** Eosinophils support the fecundity of *H. polygyrus*, possibly via the maintenance of a healthy mucus layer in the siLP.

5.2.1 Eosinophil regulation of PP immune responses to *Toxoplasma gondii*

Oral infection with the protozoan parasite *Toxoplasma gondii* induces a strong Th1 response in the mLN and spleen, whereas cells from the PP of infected mice proliferate only moderately in response to stimulation with *T. gondii* antigen (Chardès et al. 1993). This is despite the invasion of PP by the parasite within 1 hour of infection (Liesenfeld et al. 1997) and suggests that immune responses to *T. gondii* may be regulated in the PP. Although IL-12 and associated Th1 immune responses are essential for control of *T. gondii* (Scharton-Kersten et al. 1996), excessive IFNγ- production leads to severe immunopathology and death (Liesenfeld 2002), whereas IL-10 controls pathology (Suzuki et al. 2000). In this way BALB/c mice control *T. gondii* infection resulting in a latent infection, whereas C57BL/6 mice succumb to infection and develop severe pathology. Given the role of eosinophils in regulating immune responses to *H. polygyrus* in the PP, as demonstrated here, it would be of great interest to investigate the effect of eosinophil deficiency during *T. gondii* infection. If eosinophils regulate Th1 induction in the PP during *T. gondii* infection, their absence may have severe consequences for immunopathology and survival. Furthermore, the role of eosinophils in protection to *T. gondii* infection is contentious with conflicting results from experiments using IL-5$^{-/-}$ mice (Nickdel et al. 2001; Zhang & Denkers 1999). Thus, infection with *T. gondii* in ΔdblGATA-1 mice would further our understanding of eosinophils during protozoan parasite infection.

5.2.2 Eosinophils as maintainers of IgA during infection with *Giardia muris*

Oral administration of cysts from the protozoan parasite *Giardia muris* establish a tri-phasic infection in the mouse small intestine, with an initial latent stage taking place immediately after excystation, followed by an acute phase during which the parasite replicate and start producing cysts and a final elimination stage (Belosevic et al. 1984). A mixed Th1/Th2 response is associated with protection to *G. muris*, with comparable levels of IL-4 and IFN-γ produced in response to antigenic stimulation (Venkatesan et al. 1996; Abdul-Wahid & Faubert 2008).
As *Giardia spp*. infections are some of the few in which IgA is essential for the elimination of primary infection as well as in protection against secondary infection (Langford et al. 2002), it would be of great interest to study *G. muris* infection in the context of eosinophil deficiency. Furthermore, infection results in a significant increase in $CD4^+$ T-cells and B-cells in the PP of BALB/c mice and levels of IL-4 and IFN-γ producing cells following *G. muris* antigen stimulation are comparable between the PP and mLN of infected BALB/c mice (Abdul-Wahid & Faubert 2008). Given the role of eosinophils in maintaining a dichotomy between mLN and PP during *H. polygyrus* infection it would be of great interest to study their function in an infection where both organs participate to protective immune responses.

6 MATERIALS & METHODS

6.1 MATERIALS

6.1.1 Laboratory equipment

CASY® cell counter	Innovatis, Roche, Mannheim, Germany
Cell incubator	ThermoScientific, Schwerte, Germany
Centrifuge 5810/5810R	Eppendorf, Hamburg, Germany
Centrifuge 5454R	Eppendorf, Hamburg, Germany
Centrifuge cell spin I	Tharmac, Waldsolms, Germany
FACSAria™ III cell sorter	BD Bieoscience, San Jose, CA, USA
FACSCanto™ II flow cytometer	BD Bioscience, San Jose, CA, USA
FastPrep® 24 homogenizer	MP Biomedicals, Santa Ana, CA, USA
HydroSpeed® microplate washer	Tecan, Männedorf, Switzerland
Lamina flow, Scanlaf, Mars Safety 2	LaboGene, Lynge, Denmark
Lamina flow, Heraeus LB-732-C	Thermo Fisher Scientific, Boston, MA, USA
LightCycle® 480 II	Roche, Basel, Switzerland
Mastercycler® Nexus	Eppendorf, Hamburg, Germany
Microscope, camera, Leica ICC50 HD	Leica Microsystems, Wetzlar, Germany
Microscope, compound, Leica DM750	Leica Microsystems, Wetzlar, Germany
Microscope, stereo, Leica M50	Leica Microsystems, Wetzlar, Germany
Microscope, Inverted, Primovert	Zeiss, Göttingen, Germany
NanoDrop ND 1000	PeqLab, Erlangen,Germany
Pipette, adjustable (10, 200, 1000µl)	Eppendorf, Hamburg, Germany
Pipette, multi step	Eppendorf, Hamburg, Germany
Pipette, multi channel	Eppendorf, Hamburg, Germany
Pipette controller, pipetus®	Hirschmann Laborgeräte, Eberstadt, Germany
Synergy HT plate reader	BioTek, Vermont, USA
Thermomixer 5436	Eppendorf, Hamburg, Germany
VortexGenie® 2	Scientific Industries, Bohemia, NY, USA
Waterbath	Lauda-Brinkmann, Delran, NJ, USA
Waterbath, Brunswick™ Innova® 3100	Eppendorf, Hamburg, Germany

6.1.2 Consumables

C-Chip disposable hemocytometer	Digital Bio, Seoul, Korea
Cell culture flask T75 (primary cells)	Corning, Corning, NY, USA
Cryo cooler	Kisker Biotech, Steinfurt, Germany
Cytospin, glass slide	Tharmac, Waldsolms, Germany
Cytospin, columns	Tharmac, Waldsolms, Germany
Cytospin, filter paper	Tharmac, Waldsolms, Germany
Dissection instruments	KLS Martin, Freiburg, Germany
Filter, cell strainer (70µm)	BD Bieoscience, San Jose, CA, USA
Filter, Minisart® (0.2µm)	Sartorius, Göttingen, Germany
Filter, pre-separation (70 µm)	Miltenyi Biotech, Gladbach, Germany
Filter, paper	GE Healthcare, Berlin, Germany
Flask, primary cells	Corning, Corning, NY, USA
Hypo dermic needle, (20, 23 and 27G)	Braun, Melsungen, Germany
McMaster 2 chamber counting slides	FiBL, Frick, Switzerland
Microhaematocrit capillary tubes, heparinised	A. Hartenstein Laborbedarf, Wurzburg, Germany
Petri dishes (92 and 35mm)	Sarstedt, Nümbrecht, Germany
Pipette combi tips (0,5, 2,5 and 10mL)	Eppendorf, Hamburg, Germany

Pipette tips, filtered (10, 200, 1000µl)	Biozym, Oldendorf, Germany
Pipette tips (10, 20,1000µl)	Greiner bio-one, Frickenhausen, Germany
Pipette, transfer (3.5 ml)	Sarstedt, Nümbrecht, Germany
Pipette, stripette (10, 25 and 50 ml)	Costar, Corning, Corning, NY, USA
Plate, 96 well cell culture (flat & round bottom)	Costar, Corning, Corning, NY, USA
Plate, 96 well NUNC maxisorp (flat bottom)	Fisher scientific, Schwerte, Germany
Plate, 96 well micro (flat bottom)	Greiner Bio-one, Frickenhausen, Germany
Plate, 96 well multiply PCR	Sarstedt, Nümbrecht, Germany
Plate, 96 well Lightcycler	Sarstedt, Nümbrecht, Germany
Sealing tape for 96 well PCR plate	Sarstedt, Nümbrecht, Germany
Syringe, (1, 5 and 10 ml)	Braun, Melsungen, Germany
Syringe, U40 insulin, 29G	Terumo, Eschborn, Germany
Tube, cryo	Greiner bio-one, Frickenhausen, Germany
Tube, eppendorf (1,5 & 2mL)	Eppendorf, Hamburg, Germany
Tube, flow cytometry	Sarstedt, Nümbrecht, Germany
Tube, falcon cellstar (15 & 50mL)	Greiner bio-one, Frickenhausen, Germany
Tube, FastPrep-24 lysing matrix D	MP Biomedicals, Santa Ana, CA, USA
Tube, serum, heparinised	Sarstedt, Nümbrecht, Germany
Tube, titre micro	BioRad, Hercules, CA, USA

6.1.3 Pharmaceuticals, Chemicals and Reagents

2-mercaptoethanol	Ferak Berlin, Berlin, germany
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Steinheim, Germany
Bovine serum albumin V (BSA)	AppliChem, Darmstadt, Germany
Ethanol	AppliChem, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	AppliChem, Darmstadt, Germany
Diff quick staining solution	LT-SYS, Berlin, Germany
DNA-ExitusPlus	AppliChem, Darmstadt, Germany
DNase I	Sigma-Aldrich, Steinheim, Germany
Dimethyl sufoxide (DMSO)	Applichem, Darmstadt, Germany
Dithioerythritol (DTE)	Sigma-Aldrich, Steinheim, Germany
Fixation/permeabilization concentrate	eBioscience, San Diego, CA, USA
Fixation/permeabilization diluent	eBioscience, San Diego, CA, USA
Formaldehyde 37%	Carl Roth, Karlsruhe, Germany
Foetal bovine serum (FBS)	PAN Biotech, Aidenbach, Germany
Foetal bovin serum, HyClone	GE Healthcase, South Logan, UT, USA
Gentamycin	Applichem, Darmstadt, Germany
Isoflurane	Abbott, Ludwigshafen, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
L-glutamine	PAN Biotech, Aidenbach, Germany
Liberase, TL	Roche, Basel, Switzerland
Non essential amino acids (NEAA)	PAN Biotech, Aidenbach, Germany
Penicillin/streptomycin (P/S)	PAN Biotech, Aidenbach, Germany
Percoll	GE Healthcare, Uppsala, Sweden
Permeabilization buffer	eBioscience, San Diego, CA, USA
Phosphatase tablets	Sigma-Aldrich, Steinheim, Germany
Potassium bicarbonate (KHCO3)	AppliChem, Darmstadt, Germany
Pyrantel pamoate	Sigma-Aldrich, Steinheim, Germany
Red blood cell lysis buffer, whole blood	Sigma-Aldrich, Steinheim, Germany
RNA lysis buffer	Analytic Jena, Jena, Germany

RNase-ExitusPlus	AppliChem, Darmstadt, Germany
Sodium bicarbonate ($NaHCO_3$)	Carl Roth, Karlsruhe, Germany
Sodium carbonate (Na_2CO_3)	Carl Roth, Karlsruhe, Germany
Sodium Pyruvate	PAN Biotech, Aidenbach, Germany
Sulfuric acid (H_2SO_4)	Carl Roth, Karlsruhe, Germany
Tetramethylbenzidine (TMB) substrate solution	eBioscience, San Diego, CA, USA
Tween-20	Carl Roth, Karlsruhe, Germany
Water, Millipore	Millipore, Darmstadt, Germany
Water, molecular biology grade	AppliChem, Darmstadt, Germany

6.1.4 Commercial kits

Cytokine ELISA kits, IL-6/IL-10	BD Bieoscience, San Jose, CA, USA
Cytokine ELISA kit, IL-1β	eBioscience, San Diego, CA, USA
Diff quick staining kit	LT-SYS, Berlin, Germany
FastStart universal SYBR green	Roche, Mannheim, Germany
High Capacity RNA to cDNA kit	Applied Biosystems, Darmstadt, Germany
InnuPREP RNA kit	Analytikjena, Jena, German

6.1.5 Cytokines and growth factors

IL-5	recombinant, murine	R&D Systems, Mineaapolis, MN, USA
FLT-3L	recombinant, murine	Peprotech, Hamburg, Germany
SCF	recombinant, murine	Peprotech, Hamburg, Germany
IL-33	recombinant, murine	Peprotech, Hamburg, Germany

6.1.6 Stimulators and inhibitors

BrefeldinA	eBioscience, San Diego, CA, USA
Ionomycin	Sigma-Aldrich, Steinheim, Germany
phorbol-12-myristate-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany

6.1.7 Software

Adobe Illustrator CS6	v. 16.0.0	Adobe Systems, San Jose, USA
FASCDiva™	v. 8	BD Bioscience, San Jose, CA, USA
FlowJo	v. 8.8.7	Tree Star, Ashland, USA
Gen5™	v. 2.04	BioTek, Vermont, USA
GraphPad Prism	v. 6.0	GraphPad Software, San Diego, USA
LightCycler™ 480	v.1.5.1	Roche, Basel, Switzerland
Mendeley	v 1.15	Mendeley, London, UK

6.1.8 Buffers and media

Phosphate buffer saline (PBS)	PAN Biotech, Aidenbach, Germany
RPMI 1640	PAN Biotech, Aidenbach, Germany
HANKS Buffered Salt Solution (HBSS)	PAN Biotech, Aidenbach, Germany

6.1.8.1 *Cell isolation and culture*

BmEos (As received from Dr. K. Dyer)	RPMI 1640 20% HyClone FBS 25mM HEPES

	100U/mL Penicillin 100µg/mL Streptomycin 2mM L-glutamin 1 x NEAA 1mM Sodium pyruvate 50µM 2-mercaptoethanol
CMF (Calcium, magnesium free)	HBSS (wo Ca^{2+} and Mg^{2+}) 2% FBS 10mM HEPES 25mM $NaHCO_3$
CMF/DTE	CMF 8% FBS (= final 10%) 0,154mg/mL DTE
HBSS/EDTA	HBSS 10% FBS 15mM HEPES 5mM EDTA
HBSS/HEPES	HBSS 15mM HEPES
PEC isolation	See FACS buffer
RPMI complete	RPMI 1640 10% FBS 2mM L-glutamin 100U/mL Penicillin 100µg/mL Streptomycin
RPMI complete freezing	RPMI complete 10% DMSO
RPMI complete siLP	RPMI (w/o $NaHCo_3$) 5% FBS 15mM HEPES 100ug/mL gentamycin
RPMI digest	RPMI complete siLP 0,1mg/mL Liberase, TL 0,1mg/mL DNase I
RPMI wash	RPMI 1640 1% FCS 100U/mL Penicillin 100µg/mL Streptomycin
RPMI wash, 2x P/S (*H.p*. female culture)	RPMI 1640 1% FCS 200U/mL Penicillin 200µg/mL Streptomycin

6.1.8.2 *ELISA*

Sodium carbonate coating (pH 9,5)	Distilled water 0,1M $NaHCO_3$ 0,1M Na_2CO_3
e-bioscience x10 coating	eBioscience # 00-0000-53
Phosphatase substrate solution (IgG1 Ab)	10mL Sodium carbonate coating buffer 1 tablet phosphatase substrate 10µl 1M MgCl
TMB substrate solution	eBioscience
Assay diluent Ab ELISA	PBS 3% BSA
Assay diluent cytokine ELISA (BD)	PBS 10% FBS
Assay diluent x5 cytokine ELISA (e-bio)	eBioscience # 00-4202-55
Wash buffer	PBS 0,05% Tween-20

6.1.8.3 *Flow Cytometry*

FACS buffer	PBS 1% BSA 2mM EDTA
Fixation/permeabilization concentrate	eBioscience # 00-5123-43
Fixation/permeabilization diluent	eBioscience # 00-5223-56
Permeabilization buffer	eBioscience # 00-8333-56

6.1.8.4 *Other*

Erythrocyte lysis buffer (pH 7.5)	Distilled water 0.02 M KHCO3 0.155 M NH4Cl 0.1 mM EDTA

Table 6.1 Antibodies and dyes used in flow cytometry, antibody isotype sandwich ELISA and *in vivo* depletion of eosinophilsFLOW CYTOMETRY

Antibody	Dilution	Conjugate	Clone/species	Company
anti-mouse-B220	500	PE-Cy7	RA-B2	e-bioscience
anti-mouse-PD-1	200	eFluor780	J43	e-bioscience
anti-mouse-IL-10	150	eFluor660	JES5-16E3	e-bioscience
anti-mouse-GATA-3	50	eFluor660	TWAJ	e-bioscience
anti-mouse-CD45	500	eFluor450	30-F11	e-bioscience
anti-mouse-IFNγ	200	eFluor450	XMG1.2	e-bioscience
anti-mouse-FoxP3	300	eFluor450	FJK-16s	e-bioscience

anti-mouse-IgM	200	eFluor450	eB121-15F9	e-bioscience
anti-mouse-CXCR5	50	biotin	2G8	e-bioscience
anti-mouse-IL-4	100	PE	11B11	BD Bioscience
anti-mouse-Siglec-F	300	PE	E50-2440	BD Bioscience
anti-mouse-CCR3	200	Alexa647	83103	BD Bioscience
anti-mouse-CD4	200	PerCP	RM4-5	BioLegend
anti-mouse-IgG1	600	APC	RMG1-1	BioLegend
anti-mouse-IgA	300	FITC	Goat	Southern Biotech
Peanut agglutinin (PNA)	100	Biotin		Biozol
Streptavidin	200	PE		e-bioscience
Streptavidin	200	FITC		e-bioscience
Fixable cell viability dye	300	eFluor 506		e-bioscience
Fixable cell viability dye	1000	eFluor780		e-bioscience
Anti-mouse FcRγII/III	10µg/mL		2.4G2	DRFZ in house
isotype ratIgG2b		eFluor450	eB149/10H5	e-bioscience
isotype mouse IgG1		PE-Cy7	P3.6.2.8.1	e-bioscience
isotype ratIgG1		FITC	eBRG1	e-bioscience
isotype ratIgG1		APC	eBRG1	e-bioscience
isotype ratIgG1		PE	eBRG1	e-bioscience
Isotype ratIgG2a		APC-Cy7	RTK2758	Biolegend

ELISA

Antibody	Dilution	Conjugate	Clone/species	Company
anti-mouse-IgG1 (capture)	250	-	Goat	Southern Biotech
anti-mouse-IgG1 (detection)	5000	Alkaline phosphatase (AP)	Goat	Rockland
mouse IgG1 (Standard)	250	-	Mouse	BD Bioscience
anti-mouse IgE (capture)	250	-		BD Bioscience
anti-mouse IgE (detection)	2000	Horseradish peroxidase (HRP)	Rat	BioRad
mouse IgE (standard)	500	-	mouse	BD Bioscience

***IN VIVO* EOSINOPHIL DEPLETION**

Antibody	Concentration	Clone	Company
anti-mouse Siglec-F	20ug	238047	R&D Systems
RatIgG2a	20ug	54447	R&D Systems

6.2 Methods

6.2.1 Animals and ethics statement

ΔdblGATA-1 mice (Jackson Laboratory, CA) were bred under SPF conditions at the Institute of Immunology, Freie Universität Berlin. PHIL mice were provided by Alexander Beller, AG Dr. Claudia Berek from the German Arthritis Research Center, Berlin. Age and sex matched BALB/c and C57BL/6 wild type mice were purchased from Janvier Labs (Saint-Berthevin, France). To collect serum, mice were anesthetized with isoflurane and blood collected with

microhematocrit tubes form the peri-orbital sinus into heparinized serum tubes. Mice were sacrificed by isoflurane inhalation followed by cervical dislocation
All experiments were performed in accordance with the National Animal Protection Guidelines and approved by the German Animal Ethics Committee for the protection of animals at the Landesamt fuer Gesundheit un Soziales (LAGeSo) under the registration number GO113/15.

6.2.2 *H. polygyrus* life cycle and infection experiments

6.2.2.1 *Life cycle*

H. polygyrus was maintained by serial passage in C57BL/6 mice (LAGeSo reg. nr H0099/13). For this, L3 larvae were given by oral gavage. Faeces were collected starting at day 9 post infection, mixed with water to achieve a damp, but not wet consistency. Faeces was spread on mist filterpaper in glass petri dishes to allow eggs to hatch. L3 larvae were collected at the edge of the filter paper approximately one week later and washed with distilled water. L3 larvae were kept in distilled water at 4°C until needed.

6.2.2.2 *Infection experiments*

6-13 week old male and female mice were infected by oral gavage with 200 L3 larvae. Faeces were collected from day 8 p.i. and parasite eggs counted using McMaster chambers. Adult parasites were removed from duodenum with forceps and counted using a stereo microscope. Parasite fecundity was determined by counting eggs shed by female worms kept individually for 24 h (8 per donor mouse) on 96 well plates in RPMI wash 2x P/S (see “buffers and media”). For challenge infections experiments, mice were treated with the anti-helminthic Pyrantel Pamoate (2.5 mg/mouse), allowed to rest for 3 weeks, followed by a secondary infection at day 35. Challenged mice were killed 11 days after challenge infection.

6.2.3 Preparation of single cell suspensions

6.2.3.1 *Secondary lymphoid organs*

Spleens, mLN and PP were isolated and placed in cold RPMI Wash buffer. PP were pre-digested in 1mL RPMI digest at 37°C on a shaker for 30min. Spleens, mLN and pre-digested PP were forced through 70µm cell strainers to obtain single cell suspensions. Cells were washed once and red blood cells in spleen cell preparations were lysed in 3mL erythrocyte lysis buffer for 5 minutes followed by one more washing step. Cells were re-suspended in RPMI complete and counted using a CASY automated cell counter.

6.2.3.2 *Small intestine lamina propria*

The small intestine was excised and placed in ice-cold CMF media. Intestines were flushed with 20mL cold CMF using a 20G needle before removal of mesenteric fat and Peyer’s patches. After opening the intestines longitudinally, adult parasites were removed and excess mucus scraped off using forceps. Tissues were thoroughly washed in HBSS/HEPES followed by cutting into 1cm pieces and placed in 20mL HBSS/DTE. Tissues were incubated under shaking (200rpm at 37°C) for 15 minutes. This was repeated twice before adding intestinal pieces to 20mL HBSS/EDTA followed by vigorous shaking at room temperature for 15 minutes. This was repeated three more times. Residual EDTA was removed by rinsing intestinal pieces in RPMI before placing them in 10mL 37°C RPMI digest. They were then incubated under shaking at 37°C, 200rpm for 30 minutes. Following incubation, tubes were vortexed vigorously to disrupt remaining tissue pieces and the whole suspension was forced up and down through a 20G needle, then filtered over a 70um cell strainer and washed twice with HBSS/HEPES. Cell suspensions were layered on a percoll gradient and lamina propria

cells collected from the 40/70% interface. Cells were washed in RPMI complete SiLP and counted.

6.2.3.3 *Bone marrow*

Tibia and femur from both hind legs were isolated and cleaned with paper towels. Bones were opened at both ends and flushed with RPMI wash media using a 27G needle. Bone marrow was disrupted by passing up and down a 1mL syringe and cells were washed once followed by lysing of red blood cells, as described above for spleen. Cells were washed once more and counted on a CASY automated cell counter.

6.2.3.4 *Peritoneal exudates cells (PECs)*

To extract cells from the peritoneal cavity, 3mL cold FACS buffer was injected with a 23G needle to the cavity. Cell-containing fluid was extracted using the same needle, cells were washed once and counted on an automated CASY cell counter.

6.2.4 Flow cytometry and cell sorting

6.2.4.1 *Flow cytometry*

Single cell suspensions were adjusted to $10x10^6$/mL and 200ul was added to cone-bottomed 96 well plates. Before staining with fluorescent antibodies cells were blocked with anti-mouse-FcγRII/III to prevent unspecific binding. Antibodies and dyes used for surface and intra cellular staining can be found in table 6.1. The following leukocyte populations were defined:

Eosinophils (SiLP, PP and mLN, BmEos): $SSC^{hi}CD45^+Siglec\text{-}F^+CCR3^+$
Eosinophils (Spleen, PEC, BM): $SSC^{hi}Siglec\text{-}F^+$
Th2 cells: $CD4^+GATA\text{-}3^+$ or $CD4^+IL4^+$
T-regulatory cells: $CD4^+FoxP3^+$
TFH: $CD4^+CXCR5^+PD\text{-}1^+$, $IL\text{-}4^+$ or $IL\text{-}4^-$
GC B-cells: $PNA^{hi}B220^+$, IgA^+, IgM^+ or $IgG1^+$
DC: $CD45^+CD11c^{hi}$, $CD11b^+$ or $CD103^+$

6.2.4.2 *Surface stain*

To detect surface molecules, cells were stained for 10 minutes on ice with the appropriate antibody cocktail. The exception to this was TFH cells, as detection of CXCR5 requires 30 minutes staining at 37°C.

6.2.4.3 *Intracellular cytokines*

To detect intracellular cytokines, cells were stimulated for an initial 30 minutes with PMA (50ng/mL) and ionomycin (1µg/mL), after which brefeldinA (concentration as per manufacturers instructions) was added to the wells to prevent cytokine export. Cells were incubated for a further 2.5 hours, making the total incubation time 3 hours. Cells were washed and stained for surface molecules, after which they were fixed in 3,7% formalin for 10 minutes then washed twice in perm wash to permeabilize the plasma membrane. Cells were then stained intra-cellularly for 30 minutes on ice in perm wash buffer. Finally, cells were washed twice in perm wash buffer.

6.2.4.4 *Intranuclear transcription factors*

The e-bioscience T-reg kit (see buffers and media) was used to detect intra-nuclear transcription factors. After staining of surface molecules, cells were fixed with Fix Perm for 30 minutes followed by 2 x wash in Perm buffer. Cells were stained for 30 minutes in Perm wash buffer followed by two washes in perm wash buffer.
Cell were aquired using a Canto II flow cytometer and results analysed with FloJo software.

6.2.4.5 *Eosinophil cell sorting*

Small intestine lamina propria single cell suspensions were prepared as described above and pooled from 3-4 mice. Surface staining was performed as described above and eosinophils ($CD45^+SSC^{hi}Siglec\text{-}F^+CCR3^+$) were sorted with a FACSAria cell sorter. Purity of

sort was checked on a FACSCanto II flow cytometer and by cytospin.

6.2.5 Cytospins

$5x10^4$ sorted SiLP cells or BmEos were added to cytospin columns and centrifuged for 3 minutes at 1000rpi. Slides were allowed to air-dry before staining with Diff quick solution according to manufacturer instructions. Photographs taken with a compound microscope with built-in camera (see laboratory equipment).

6.2.6 Sandwich ELISA

6.2.6.1 *Total IgG1 and IgE in sera*

96 well flat bottom Maxisorp plates were coated with 50µl goat anti-mouse IgG1 or monoclonal rat anti-mouse IgE and incubated at 4°C overnight. Plates were washed using a microplate washer and then blocked with 200µ 3% BSA in PBS for one hour before 50µl samples and standards were added. For dilutions of samples and generation of standard curves see table 6.2. Plates were incubated for 2 hours at room temperature, after which 50µl detection antibodies conjugated to alkaline phosphatase (IgG1) or horseradish peroxidase (IgE) was added for 1 hour. 50µl phosphatase (IgG1) or TMB (IgE) substrate solutions was added and plates incubated for 30 minutes at 37°C (IgG1) or 15 minutes at room temperature (IgE). To stop the enzymatic reaction 25µl 0,1M EDTA (IgG1) or 1M sulphuric acid was was added. IgG1-AP was read at 405nm minus reference wavelength 630nm. IgE-POX was read at 450-570nm.

6.2.6.2 HES specific IgG1 and IgE in sera

To determine parasite-specific antibody titers, plates were coated with *H. polygyrus* excretory/secretory products (HES) at a concentration of 10µg/mL and incubated at 4°C overnight. Diluted serum (see table 6.2) was added to plates and incubated for 2 hours at room temperature. Subsequent steps followed as described for total antibody detection.

6.2.6.3 *Cytokine ELISA*

Mouse IL-10, IL-6 and IL-1β were detected in the supernatants of BmEos cultured for 5 days with the use of commercial ELISA kits, following the manufacturers instructions. Briefly, plates were coated with 50µl capture antibody and incubated overnight at 4°C. Plates were washed using a microplate washer, then blocked for one hour with 10% FBS in PBS (IL-6 and IL-10) or e-bioscience assay diluent (IL-1β). 50µl undiluted samples or serially diluted standards were added and plates were incubated for 2 hours at room temperature. For IL-6 and IL-10, detection antibody and HRP were mixed as instructed and 50µl added to plates followed by 1 hour incubation at room temperature. For IL-1β detection antibody was added separately, plates incubated for one hour, washed and then incubated for an additional 30 minutes with 50µl HRP. 50µl TMB substrate was added to all plates and incubated for 30 minutes (IL-6/IL-10) or 15 minutes (IL-1β). Plates were at 450-570nm.

6.2.7 RNA extraction, reverse transcription and real time quantitative PCR

Frozen (-80C) duodenal sections, PP or mLN were placed in RNA lysis buffer and homogenized using in a Fast Prep tissue homogenizer. RNA was extracted from the supernatants with InnuPREP RNA kit, following manufacturers instructions. Extracted RNA was measured using a Nanodrop 1000 and 2µg of RNA was reverse transcribed to cDNA using a High Capacity RNA to cDNA kit. Real time PCR was performed with LightCycler480, using 10 ng of cDNA and FastStart Universal SYBR Green Master Mix. Prior to gene expression analysis, the efficiency of each primer pair was tested by generating standard curves of pooled cDNA (0,001-100ng). The amplification of unspecific product was controlled by the generation of melting curves (appendix). mRNA expression was normalized to the housekeeping gene GUSB (Wang et al. 2010) and calculated by Roche Light Cycler 480 software, using naïve BALB/c cDNA as a calibrator to which all other samples were compared.

Primer pair sequences and their calculated efficiencies are found in table 6.3. Primers were synthesized by MOLBIOL (Berlin, Germany).

Table 6.2 Dilutions of sera and generation of standard curves for detection of total and HES specific IgG1 and IgE by sandwich ELISA

SERUM DILUTIONS				STANDARD CURVES	
IgG1 total	**IgE total**	**IgG1 HES**	**IgE HES**	**Purified mouse IgG1**	**Purified mouse IgE**
1:5000 (Naive)	1:100 (Naive)	1:25	1:5	2000	1000
1:200000 (d14)	1:5000 (d14)	1:50	1:10	1000	200
		1:100	1:20	500	40
		1:200	1:40	250	8
		1:400	1:80	125	1,6
		1:800	1:160	62,5	0,32
		1:1600	1:320	31,25	0,064
		1:3200	1:640	-	-

Table 6.3 Primer sequences for RT-qPCR, their accession number and calculated efficiencies Gene	**Accession #**	**Sequence (5' – 3')**	**Efficier**
Glucuronidase-β GUSB	NM_010368	fwr: GCTCGGGGCAAATTCCTTTC rev: CTGAGGTAGCACAATGCCCA	1,88
AID (Aicda)	NM_009645	fwr: CCAGGAACCGCTACTCGTTT rev: GGTCCGTCTCAGGCACTATG	1,73
Arginase-1	NM_007482	fwr: CAGAAGAATGGAAGAGTCAG rev: CAGATATGCAGGGAGTCACC	1,84
Relm-α (Retnla)	NM_020509	fwr: TCCCAGTGAATACTGATGAGA rev: CCACTCTGGATCTCCCAAGA	1,81
Relm-β (Retnlb)	NM_023881	fwr: GGCTGTGGATCGTGGGATAT rev: GAGGCCCAGTCCATGACTGA	1,83
IL-25	NM_080729	fwr: ACAGGGACTTGAATCGGGTC rev: GGTAAAGTGGGACGGAGTTG	1,85
IL-33	NM_001164724	fwr: AGGAAGAGATCCTTGCTTGGCAGT rev: CCATCAGCTTCTTCCCATCCACA	1,76
TSLP	NM_021367	fwr: AGCAAGCCAGCTTGTCTCCTG rev: TGTGCCATTTCCTGAGTACCGTCA	2,1

6.2.8 Histology

6.2.8.1 *Staining and quantification of goblet cells and eosinophils*

All histological analyses were performed by Dr. Anja Kühl (Research Center ImmunoSciences, Charité University Medicine, Berlin). 0,5-1cm pieces of proximal duodenum were snap frozen in liquid nitrogen and stored at -80C until ready for processing. The following methods are as described by Dr. Kühl: Formalin fixed, paraffin embedded sections (1-2 µm) of duodenum were de-waxed and stained with hematoxylin and eosin (H&E) for overview, with periodic acid Schiff

(PAS) for goblet cell quantification and by Direct red 80 (Sigma) for the detection of eosinophils. PAS^+ goblet cells were counted along 5 villi per section and eosinophils were quantified in 5 high power fields (hpf, 0.237 mm^2). Images were acquired using the AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). All evaluations were performed in a blinded manner

6.2.8.2 *Histological scoring*

Enteritis was scored by Dr. Anja Kühl according to a two-parameter scale (i/ii, each from 0 to 4): (i) appearance of intestinal architecture (0, normal; 1, slight blunting of villi; 2, moderate blunting of villi, slight crypt hyperplasia; 3, strong blunting of villi and crypt hyperplasia; 4, strong villus atrophy and crypt hyperplasia); (ii) signs of inflammation (0, no signs of inflammation; 1, mild leukocyte infiltration; 2, moderate leukocyte infiltration; 3, marked leukocyte infiltration, thickening of bowel wall; 4, transmural infiltration, mucin depletion, strong bowel wall thickening, ulcerations.

6.2.9 Eosinophil depletion experiments

Lyophilized anti-Siglec-F or RatIgG12a were re-suspended in sterile PBS. BALB/c mice were injected with 20ug anti-Siglec-F or RatIgG2a intraperitonealy, starting 2 days prior to *H. polygyrus* infection and every second day thereafter up until day 12 p.i. Blood was taken before commencement of antibody treatment and every fourth day thereafter. Eosinophils were stained in whole blood by placing 50µl blood in FACS tubes containing 0,5mL FACS buffer. Cells were centrifuged and FcgγRII/III was blocked for 5 minutes followed by addition of antibody cocktail. Red blood cells were lysed with whole blood lysis buffer for 5 minutes on ice. Cells were washed twice in FACS buffer. On day 14 post *H. polygyrus* infection mice were killed and siLP, PP and mLN single cell suspensions prepared. Eosinophils were detected in the siLP and T and B-cells in the siLP, PP and mLN.

6.2.10 Generation and *in vitro* stimulation of bone marrow eosinophils

6.2.10.1 In vitro *differentiation of eosinohils from mouse bone marrow*

Bone marrow single cell suspensions were prepared aseptically as described above. Cells were counted and re-suspended in RPMI complete freezing medium at a concentration of 20 x 10^6 cells/mL. Aliquots of 1mL were placed in a freezing container and left at -80°C for up to three days at which point aliquots were moved for long term storage at -196°C and defrosted for use according to demand.
Aliquots of frozen cells were briefly placed in 37°C degrees water bath for defrosting. Immediately upon complete thawing, cells were placed in T75 cell culture flasks specifically for primary cells, containing 20mL, pre-warmed BmEos media to which 100ng/mL stem cell factor (SCF) and 100ng/mL FLT-3L were added. Flask were placed in 37°C and left for two days. On day 2 medium was changed and fresh SCF and FLT-3L given. On day 4 cells were transferred to new flasks and placed in fresh media containing 10ng/ml rIL-5. Every second day thereafter medium was changed, density adjusted to 10^6/mL and fresh rIL-5 provided. Cultures were regularly checked for contaminants using a phase contrast microscope and new flasks were provided upon appearance of adherent cells. On day 12-14, purity of BmEos was surveyed by flow cytometry and cytospin.
As the establishment of BmEos differentiation cultures was initially not straight forward, important points to consider are noted here in order to facilitate future success.
1) Cell culture flasks for primary cells, not cell lines, should be used and no smaller than T75.
2) Cells should not be kept in a volume smaller than 10ml or a density lower than 10^6/mL.
3) IL-5 should be from R&D systems, as otherwise sourced IL-5 yields sub-optimal eosinophil differentiation.
4) HyClone FBS should be used, as cells grown in standard FBS grow sub-optimally (Fig 6.1)

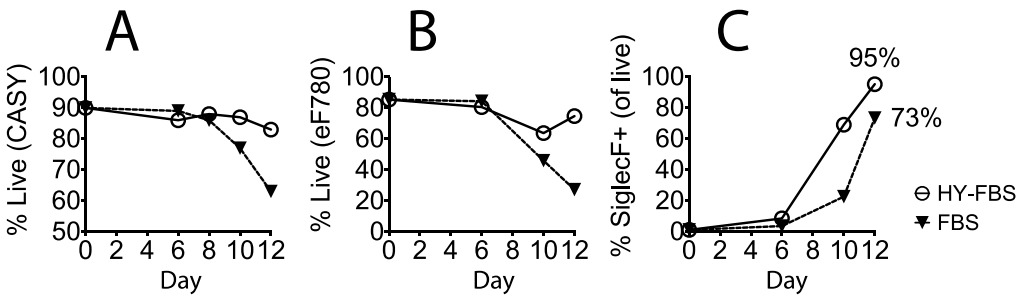

Figure 6.1 Hy-clone FBS support cell survival and eosinophil differentiation
Bone marrow was isolated from one BALB/c mouse and cells were cultured as described. Media contained either Hy-clone (open circles) or standard FBS (black triangles). **A.** Percent living cells as estimated by CASY automated cell counter. **B.** Percent living cells as estimated by viability dye eF780. **C.** Percent Siglec-F^+ cells of all live cells.

6.2.10.2 *In vitro stimulation of BmEos*

$50x10^4$ BmEos were stimulated with 2ng IL-33 2ng IL-25 or PMA/Ionomycin alone or in combination. Cells were cultured for 5 days, after which supernatants were removed and stored at -20°C until assayed for the pressence of cytokines by ELISA

6.2.11 Statistics

Experiments were performed as indicated and expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism software. Unless otherwise indicated, level of significance was tested by one-way ANOVA followed by Holm-Sidak post hoc test for multiple comparison.

6.2.12 Funding

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7 SUMMARY

Title: the role of eosinophils in regulation of T- and B-cell responses during enteric nematode infection

Background: eosinophils are implicated in B-cell maintenance and Th2 induction and are increasingly recognized for their influence on the development of Peyer's patches (PP). Moreover, it was recently discovered that eosinophils support IgA class switching in the PP, further implicating eosinophils in intestinal homeostasis. However, whether the PP participate in immune responses against intestinal helminths and whether eosinophils influences such responses has not been studied. Here eosinophil deficient mice were used to investigate the role of eosinophils in intestinal Th2 responses, using the small intestinal nematode *Heligmosomoides polygyrus*- a natural pathogen of mice and a useful model for human gastrointestinal helminth infection, but to which the role of eosinophils in host protection is understudied.

Aims:

1. Determine whether the PP participate in *H. polygyrus* induced Th2 and antibody responses.

2. Determine whether eosinophils affect Th2 and antibody responses to *H. polygyrus*

3. Assess whether eosinophil deficiency affects the survival and fecundity of *H. polygyrus*.

Results: the main immune response to *H. polygyrus* in wild type (WT) mice was found to take place in the gut-draining mesenteric lymph nodes (mLN), not the PP and eosinophils were found to maintain a divison of labor between the PP and mLN. Specifically, whereas *H. polygyrus* infection did not result in significant increases of IL-4^+ T-cells or $IgG1^+$ B-cells in the PP of WT mice, significant increases in IL-4^+ and $IgG1^+$ cells were detected in the PP of eosinophil deficient mice. This was despite Th2 induction and IgG1 class switching being unaffected by eosinophil deficiency in the mLN. The increase of IL-4 producing cells in the PP of eosinophil deficient mice was specifically evident in T-follicular helper cells (TFH), whereas TFH in PP of WT mice did not increase IL-4 production in response to *H. polygyrus*. The PP of WTmice maintained high levels of IgA production following *H. polygyrus* infection, Whereas the frequencies of IgA^+ B-cells dropped considerably in the PP of infected eosinophil-deficient mice. Finally, although *H. polygyrus* survival was not affected by the absence of eosinophils, eosinophil deficiency significantly impaired parasite fecundity. this was despite expression of molecules with known anti-helminth functions being comparable between eosinophil deficient and wt mice.

conclusion: this work demonstrates that eosinohils maintain a regulated state in the pp during infection with *h. polygyrus*. this places the eosinophil in a new, evolutionary sensible position in which it ensures that iga production is maintained despite infection with an enteric pathogen.

8 ZUSAMMENFASSUNG

Title: Die Rolle von Eosinophilen in der Regulation von T- und B- Zellantworten in einer Intestinalen Nematodeninfektion

Hintergrund: Eosinophile unterstützen die Aufrechterhaltung langlebiger B-Zell-Populationen in Knochenmark und Darm und sind, unter bestimmten Bedingungen, an der Ausbildung von T-Zellantworten des Typs 2 (Th2) beteiligt. Zusätzlich spielen sie eine Rolle für die normale Entwicklung der Peyerschen Plaques (PP) des Dünndarms. Dass Eosinophile wichtig für die Aufrechterhaltung der Homöostase intestinaler Immunreaktionen sind konnte kürzlich durch den Nachweis ihrer unterstützenden Rolle für den IgA-Isotyp-Klassenwechsel von B-Zellen in den PP nachgewiesen werden. Ob Eosinophile allerdings auch die intestinale Immunantwort gegen parasitische Darmwürmer beeinflussen, und was während solcher Infektionen in den PP geschieht, ist bisher nicht untersucht worden. In dieser Arbeit wurden Eosinophilen-defiziente Mausstämme genutzt, um eine mögliche regulative Rolle dieser Zellen in intestinalen Th2-Infektionen zu analysieren. Als Infektionsmodell diente der Dünndarmnematode *Heligmosmoides polygyrus*, ein natürlicherweise in Mäusen vorkommender Parasit, der als Modell für chronische Wurminfektionen des Menschen Verwendung findet. Die Rolle von Esoinophilen in der Immunantwort gegen diesen Parasiten ist bisher ungenügend untersucht.

Ziele:

1. Beurteilung der Beteiligung der PP an der durch *H. polygyrus* induzierten Th2- und Antikörperantwort.
2. Überprüfung, ob Eosinophile die Th2- sowie Antikörperantwort in der Infektion beeinflussen.
3. Überprüfung, ob das Fehlen von Eosinophilen das Überleben und die Fekundität von *H. polygyrus* beeinflusst.

Ergebnisse: Die Immunantwort gegen *H. polygyrus* findet in Wildtyp- (WT-) Mäusen vornehmlich in den Darm-drainierenden, mesenterialen Lymphknoten (mLK) statt, wobei Eosinophile für die Aufgabenteilung zwischen mLK und PP wichtig sind. Während die Infektion durch *H. polygyrus* in den PP von WT-Tieren nur zu einer geringfügigen Zunahme von IL-4 produzierenden Th2 Zellen und IgG1+ B-Zellen führte, nahmen beide Zelltypen in PP von Eosinophilen-defizienten Tieren nach Infektion signifikant zu. Die Zunahme von Th2-Zellen und IgG1-produzierenden B-Zellen in den mLK war interessanterweise in Eosinophilen-defizienten Tieren nicht verändert. Die Zunahme von IL-4-produzierenden Zellen war in PP besonders deutlich in der subpopulation von follikulären T-Helferzellen (TFH) nachweisbar. Im Gegensatz zur übermäßigen Zunahme von IgG1-produzierenden B-Zellen bei gleichzeitigem Verschwinden von IgA-produzierenden B-Zellen in PP von infizierten Eosinophilen-defizienten Tieren wurde in PP von infizierten WT-Tieren die Produktion von IgA beibehalten. Das Überleben von *H. polygyrus* schließlich war durch diese Veränderungen nicht beeinträchtigt, die Eosinophilendefizienz beeinträchtigte jedoch die Fekundität der adulten Würmer, messbar durch die verminderte Eiproduktion weiblicher Würmer. Die Frage nach dem für die geringere Eiproduktion verantwortlichen Faktor bleibt vorläufig offen, da die Produktion von Effektormolekülen, welche die Fruchtbarkeit der Würmer beeinträchtigen können, von der Eosinophilendefizienz nicht beeinflusst war.

Fazit: Diese Arbeit zeigt, dass Eosinophile nicht nur, wie bisher gezeigt, homöostatische B-Zell-Antworten in den PP regulieren, sondern auch wichtig für die balancierte Antikörperproduktion während einer intestinalen Nematodeninfektion sind. Eosinophile spielen für die Ausprägung von Th2- sowie assoziierten Antikörperantworten in den Darm-drainierenden mLK keine Rolle, sind jedoch wichtig, um einen Zusammenbruch der kontinuierlichen IgA-Antikörperproduktion in den PP des Dünndarmes während einer intestinalen Parasiteninfektion zu unterbinden. Diese Arbeit zeigt also, dass Eosinophile

vermutlich im Lauf der Evolution neben ihrer Effektorfunktion gegen Pathogene eine weitere, auf die Immunregulation in den PP des Darmes spezialisierte Rolle übernommen haben.

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10 APPENDIX

Table 10.1 Overview of the role of eosinophils in protection against helminths
Findings are divided into *in vitro* findings and *in vivo* responses during primary and challenge infections. + denotes a finding where a role for eosinophils in host defence could be shown in at least one scenario, ~ denotes no role of eosinophils and – denotes a finding where eosinophils appear to assist parasite survival or development

Prasite species	Model	*In vitro*	*In vivo* primary response	*In vivo* challenge response	Reference
H. polygyrus	Treatment with αIL-5 mAb (BALB/c)	~	No effect of αIL-5 treatment on adult worm recovery	No effect of αIL-5 treatment on adult worm recovery or faecal egg shedding	Urban et al. 1991, PNAS
	IL-5Tg, eotaxin$^{-/-}$ (BALB/c)	~	No difference in faecal egg shedding or adult worm recovery between IL-5Tg, eotaxin$^{-/-}$ and WT mice		Knott et al. 2009, Mol Immunol
	ΔdblGATA-1 (BALB/c)	+	GATA-1 mice higher worm numbers in primary infection d21.	HES vaccinated GATA-1 mice similarly protected as wild type.	Hewitson et al. 2015, PLoS Path
S. mansoni	Human blood eosinophils	+ Blood eosinophils cultured with HI sera damage schistosomula. Eosinophils from helminth infected patients relatively inactive.			Butterworth et al., 1975, Nature
	Rat eosinophils	+ Complement dependent killing of			Ramalho-Pinto et al. 1978, JEM

		schistosomula by eosinophils.			
	Treatment with αIL-5 mAb (C3H/HeN, BL/6)	~		Protection to challenge infection not affected by αIL-5 mAb treatment	Sher et al. 1990, JI
	IL-5 Tg (-)	–	Comparable levels of liver stage parasites	Higher levels of liver stage parasites in Tg animals	Dent et al. 1997, Parasiite Immunol
	PHIL (BL/6) and ΔdblGATA-1 (BALB/c)	~	No effect on liver pathology, egg or larval levels.		Swartz et al. 2006, Blood
T. muris	αIL-5 mAb (BALB/K)	~	No effect of αIL-5 treatment on worm expulsion		Betts & Else 1999, Parasite Immunol
T. spiralis	Mouse eosinophils	+	Eosinophils kill newborn larvae. Dependent on specific antibody.		Kazura and Grove, 1978, Nature
	Treatment with αIL-5 mAb (CF1, CBA/J)	~	No effect on muscle stage larval recovery	No effect on muscle stage larval recovery	Herndon and Kayes, 1992, JI
	IL-5 Tg (C3H/HeN)	~	No effect on adult or larval recovery.	No effect on adult or larval recovery	10.1.1.1.1.1.1 Hokibara et al. 1997, J Parasitol
	IL-5$^{-/-}$ (BL/6)	+	No effect of IL-5 deficiency on worm recovery	Higher intestinal worm burdens and slower expulsion in IL-5$^{-/-}$ mice	Vallance et al., 2000, Parasite Immunol
	PHIL (BL/6) and ΔdblAGTA-1 (BALB/c)	–	Increased killing of muscle stage larvae in eosinophil deficient mice		Fabre et al. 2009, JI

	PHIL (BL/6) and ΔdblAGTA-1 (BL/6)	–		Eosinophils produce IL-10, inhibits NO production and protects muscle stage larvae	Huang et al. 2014, JI
	ΔdblGATA-1 , (BL/6)	+		No effect of eosinophil deficiency on adult worm recovery or fecundity, but decreased migration of newborn larvae to muscle. Dependent on immune sera.	Huang et al., 2015, JI
N. brasiliensis	IL-5 Tg (-)	+		IL-5Tg mice harbour fewer, smaller and less fecund worms in the intestine. Time needed for complete worm expulsion unaffected by IL-5Tg.	Dent et al., 1999, Infect Immun Shin et al. 1997, Int Arch Allergy Immunol
	IL-5 Tg (CBA)	+		Eosinophils trap *Nb* larvae in the skin leading to decreased larval recovery from the lung	Daly et al. 1999, Infect Immun
	Peritoneal eosinophils from IL-5Tg (C3H/HeN)mice	+	Eosinophils adher to and kill *Nb* L3 via complement or zymosan on parasite surface		Shin et al., 2001, Parasite Immunol
	IL-5$^{-/-}$ and ΔdblGATA-1 (BALB/c)	+		Intestinal worm burden and parasite egg production greater in ΔdblGATA-1 and IL-5$^{-/-}$ mice	More larvae reach the lungs and intestine early following challenge, but compensatory mechanisms result in	Knott *et al*. 2007, Int J Parasitol

				comparable expulsion from intesinte	
	IL-5Tg, eotaxin$^{-/-}$ (BALB/c)	+ ~		IL-5Tg mice significantly fewer larvae in lung compared to WT or eotaxin$^{-/-}$ IL-5Tg mice. Higher number of larvae reach the intestine in eotaxin deficient mice.	Eotaxin$^{-/-}$ comparably protected against challenge infection	Knott *et al*. 2009, Mol Immunol
S. ratti	IL-5$^{-/-}$ mice (BL/6)	+		Higher worms count and higher faecal egg counts in IL-5$^{-/-}$ mice. Expulsion comparable.		Ovington et al., 1998, Immunology
	αIL-5 mAb treatment (BL/6)	+		Higher levels of migrating and intestinal worms and higher faecal egg output in αIL-5 mAb treated mice	αIL-5 mAb mice show higher levels of migrating parasites. No intestinal worms in either treated or untreated mice.	Watanabe et al., 2003, J Helminthology
S. venezuelensis	αIL-5 or αIL-5R mAb treatment (BL/6)	+ ~		No effect of IL-5 mAb treatment on faecal egg counts or intestinal worm recovery	Higher lung worm recovery in anti IL-5, but not IL-5R mAb treated mice. Intestinal expulsion not affected by mAb treatment.	Korenaga et al., 1991, Immunology
S. stercoralis	PHIL, MBP$^{-/-}$, EPO$^{-/-}$ and αCCR3 mAb treatment (BL(6)	+ ~	Eosinophil mediated killing of larvae is dependent MBP and requires complement component C3	Comparable killing of larvae in WT, EPO$^{-/-}$, MBP$^{-/-}$ and PHIL mice, but impair killing in all mouse strains treated with αCCR3 mAb.	No effect on eosinophil or eosinophil derived protein deficiencies	O'Connell et al., 2011, Infect Immun

B. phangi	IL-5$^{-/-}$ (BL/6)	+ ~	Higher recovery of worms in IL-5$^{-/-}$ mice	Comparable worm recovery in IL-5$^{-/-}$ and WT mice	Ramalingam et al., 2003, Exp Parasitol
B. Malayi	PHIL, MBP$^{-/-}$, EPO$^{-/-}$ (BL/6)	+ ~	More circulating microfilariae (Mf) in PHIL mice compared to WT, but no effect of MBP or EPO deficiencies	PHIL, MBP$^{-/-}$ and EPO$^{-/-}$ mice comparable levels of circulating Mf	Cadman *et al*. 2014, PLoS Path
L. sigmodontis	IL-5Tg (CBC/Ca)	+ –	Faster parasite development in IL-5Tg mice, but reduced recovery of adults in late infection		Martin et al., 2000, Infect Immun
	EPO$^{-/-}$ MBP$^{-/-}$ (129/SvJ)	+	MBP$^{-/-}$ EPO$^{-/-}$ mice higher worm burdens in thoracic cavity d28 p.i.		Specht et al., 2006, Infect Immun
	IL-5$^{-/-}$ and PHIL (BL/6)	–	Slower parasite development in PHIL and IL-5$^{-/-}$ mice, but comparable expulsion.		Babayan et al., 2010, PLoS Biology
A. costaricensis	IL-5 Tg (C3H/Hen)	+	Lower number and smaller sized adult worms, fewer eggs in intestinal wall and fewer larvae in faeces of IL-5Tg mice		Sugaya et al., 2002, Parasitol Res
A. cantonensis	αIL-5 mAb treatment -	+	Higher intra cranial worm survival in αIL-5 mAb treated mice		Sasaki et al., 1993, Parasite Immunol
	IL-5Rα$^{-/-}$ (128/Sv)	+	IL-5Rα deficiency results in higher worm burden and larger parasites compared to WT		Yoshida et al., 1996, Immunity

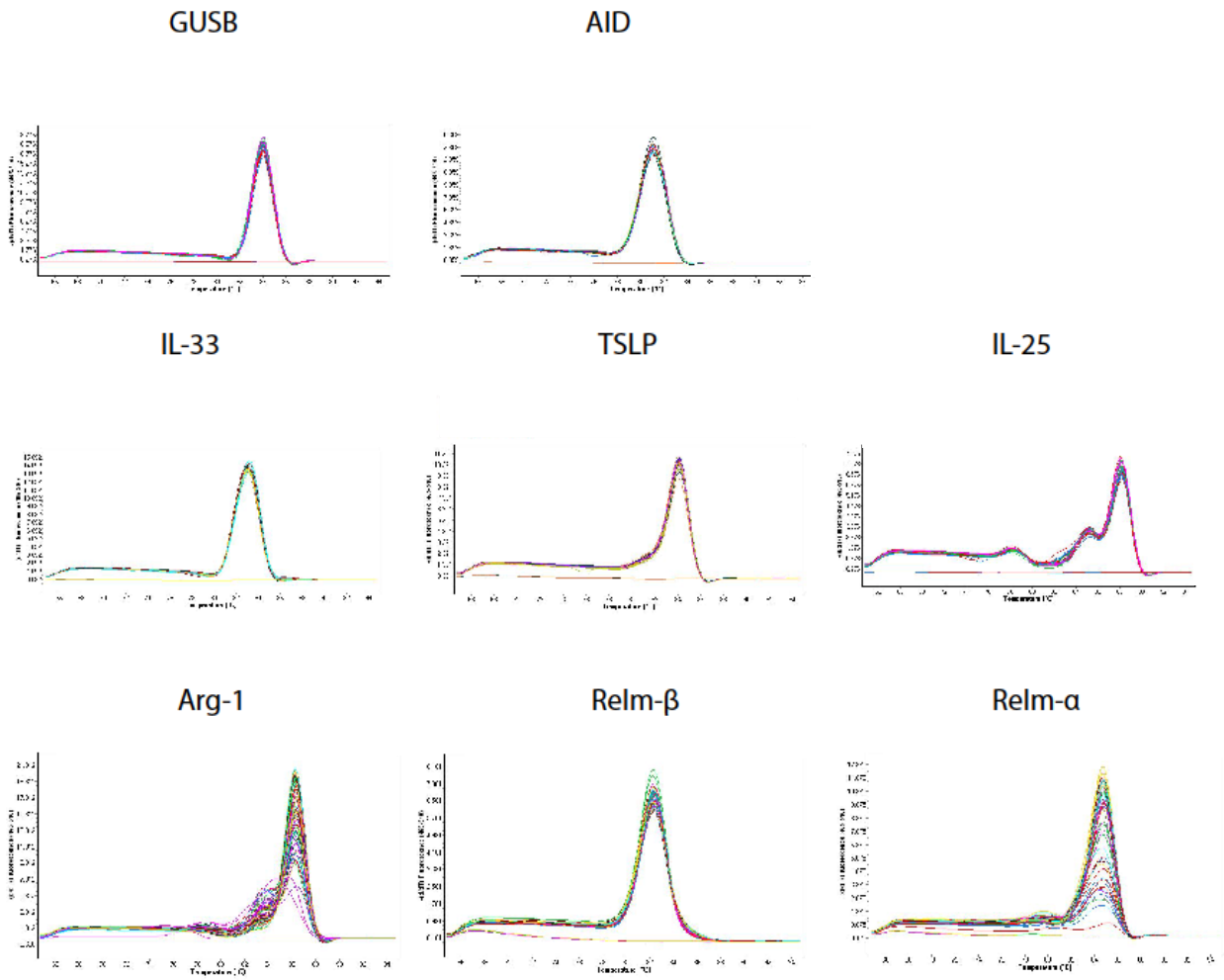

Figure 10.1 Exemplary melting curves
Melting curves were generated by Light cycler 480, for indicated genes

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12 PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

12.1 PUBLICATIONS

Strandmark, J., Steinfelder, S., Berek, B., Kühl, AA., Rausch, S., Hartmann, S., 2017. Eosinophils are required to suppressTh2 responses in Peyer's patches during intestinal infection by nematodes. *Mucosal Immunology*, 10(3), pp.661-72.

Strandmark, J., Rausch, S., Hartmann, S., 2016. Eosinophils in homeostasis and their contrasting roles during inflammation and helminth infection. *Critical Reviews in Immunology*, 36(3), pp. 193-238.

Chu, V.T., Beller, A., Rausch, S., **Strandmark, J**., Zänker M., Arbach, O., Krugolov, A., Berek, C., 2014. Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis. *Immunity*, 40(4), pp.582-93

12.2 CONTRIBUTIONS AT SCIENTIFIC MEETINGS

Strandmark, J., Rausch, S., Hartmann, S. Eosinophils guard compartmentalization of immune responses during intestinal infection. *47th Annual Meeting of the German Society for Immunology*, 27th – 30th September 2016. Hamburg, Germany. Poster presentation.

Strandmark, J., Rausch, S., Hartmann, S. Eosinophils regulate intestinal B- and T-cell responses following infection with the nematode *H. polygyrus*. *27th Annual Meeting of the German Society for Parasitology*, 9th – 12th March 2016. Göttingen, Germany. Poster presentation.

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13 DECLARATION

I hereby declare that this thesis has been written by me alone using no other sources than those listed and that the work described has been performed by me in co-operation with those mentioned in the text or acknowledgements

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Julia Strandmark, Berlin 10/07/2017