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Host determinants of immunity to the gastrointestinal protozoan parasite *Giardia muris*

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LIST OF ABBREVIATIONS

ADI	Arginine deiminase enzyme
AMP	Antimicrobial peptide
APC	Antigen presenting cell
bmEos	bone marrow-derived eosinophil
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Complex of differentiation
CWP	Cyst wall protein
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EPO	Eosinophil peroxidase
ESV	Encystation-specific vesicle
F2	Second generation progeny
FLT-3L	FMS-related tyrosine 3 ligand
Foxp3	Forkhead box P3 transcription factor
GC	Germinal centre
GM-CSF	Granulocyte-macrophage stimulating factor
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IL-R	Interleukin receptor
ILC3	Innate lymphoid cell type 3
MBP	Major basic protein
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
NO	Nitric oxide
OCT	Ornithine carboxyltransferase enzyme
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PMA	Phorbol 12-myristate 13-acetate
PMD	Piecemeal degranulation
PP	Peyer’s patch
qRT-PCR	Quantitative real-time polymerase chain reaction
RORγT	RAR-related orphan receptor gamma transcription factor
SCF	Stem cell factor
sIgA	Secretory immunoglobulin A
siLP	small intestinal lamina propria
SSC	Side scatter
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cell
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell
VSP	Variant surface protein

1. INTRODUCTION

1.1 *Giardia*

1.1.1 Biology of *Giardia* parasites

As members of the order *Diplomoda*, *Giardia* parasites belong to one of the early diverging lineages of eukaryotes, often referred to as "biological fossils" (Gillin et al., 1996; Adam et al., 2001). Signature features of *Giardia* include two functional nuclei, peripheral vesicles, and a lack of a stacked Golgi apparatus or a proper aerobic mitochondrial organelle (Ankarklev et al., 2010). Importantly, *Giardia* parasite exhibit a direct life cycle and contain only two developmental stages, the dormant infective cyst stage and a motile disease-causing trophozoite stage (Fig. 1.1.1). *Giardia* cysts are oval shaped and measure on average 8-12um in length, 7-10um in width and contain a thick outer cyst wall measuring 0.3-0.5um in thickness (Ankarlev, 2010). As a result, cysts are generally resistant to a variety of environmental stress factors (DeRegnier et al., 1989) Once orally ingested by a host, *Giardia* cysts passage the esophagus and stomach and once they have reached the acidic, cholesterol-rich environment of the upper small intestinal tract (duodenum and jejunum), excystation is initiated (Gillin et al., 1996). This process involves the triggering of metabolic processes, protein expression associated with motility and the synthesis of the trophozoite adhesive disk (Ankarlev, 2010). Trophozoites are the motile disease-causing stage of the infection and typically measure 12-15um in length and 5-9um in width. Signature features of their morphology include two transcriptionally active nuclei, a median body, four pairs of flagella and the adhesive disk, which they use to attach to the intestinal epithelial layer (Gillin et al., 1996; ref). There they replicate via binary fission and colonise the host without becoming invasive. Downstream of the duodenum and jejunum, a more alkaline cholesterol-poor environment is thought to contribute to the initiation of encystation of trophozoites into cysts (Lujan et al., 1996). The flagella become internalised, the adhesive disks fragment and the trophozoite rounds up, acquires a hypometabolic state and forms encystation-specific vesicles (ESV) containing cyst wall proteins (CWP) required for the formation of the cyst wall (Gillin et al., 1996; Ankarlev, 2010). Fully-formed cysts are then excreted with the faeces of the host and are released back into the environment, thus completing the life cycle of the parasite.

A key feature of mature trophozoites is the presence of cysteine-rich proteins termed Variant Surface Proteins (VSP) covering the surface of the trophozoite. They are known to be expressed rotationally one variant at a time in a process called antigenic variation, representing an effective immune evasion strategy of the parasite. Roughly 200 variants of VSPs are thought to exist in *Giardia*'s genetic repertoire and the expression is altered every few generations (Ankarlev, 2010). Another crucial signature feature of the biology of the parasite is its arginine metabolism and hence its ability to deplete arginine in its host during infection. Specifically, trophozoites encode for the arginine-metabolising enzymes arginine deaminase (ADI) and ornithine carbamoyl transferase (OCT) involved in the breakdown of available arginine. Arginine depletion is considered as a potent immunomodulatory strategy of the parasite, as arginine is required for the synthesis of the innate immune defence molecule nitric oxide (NO) by myeloid cells. Furthermore, it has been shown to result in elevated production of the pro-inflammatory cytokine TNF-α by monocyte-derived dendritic cells in mice (Banik et al., 2013) Furthermore, both ADI and OCT enzymes are known as immunogenic factors of the parasite due to their recognition by host antibodies (Davids et al., 2006).

1.1.2 Epidemiology and clinical relevance of giardiasis

Being ubiquitous gastrointestinal pathogens, *Giardia* parasites exhibit a broad host range across all vertebrate groups. Despite continued controversies about the taxonomy of the genus *Giardia*, 6 host-adapted species are generally recognised by the scientific community, namely *G. psittaci* and *G. ardeae* in bird species, *G. microti* in voles and muscrats, *G. agilis* in amphibians, *G. muris* in murine rodents and *G. lamblia* in humans, domestic and wild

mammalian species (Adam, 2001; Ryan and Cacciò, 2013). *G. lamblia* (syn. *intestinalis, duodenalis*) exists as eight genetically distinct assemblages designated A-H, however only assemblages A and B are known to infect humans (Mayhofer et al., 1995; Horton et al., 2018). Importantly, these two assemblages are also commonly found in livestock and domestic animals, while assemblages C-G infect both domestic and wild animal species (Ankarlev et al., 2010; Ryan and Caccio, 2013; Thompson and Ash, 2016)

The route of *Giardia* transmission includes contaminated food and water, person-to-person or zoonotic transmission from livestock or companion animal species (Horton et al., 2018). Its widespread distribution in both high- and low-income countries around the world classifies giardiasis as a significant public health burden and in 2009 the World Health Organization (WHO) included giardiasis in its Neglected Disease Health initiative (Savioli et al., 2006). Prevalence rates are relatively low in industrialised countries and developing regions continue to carry a higher burden of infection with giardiasis. Recent data indicates that over 183 million cases of giardiasis occur annually across the globe and the burden of giardiasis in Disability Adjusted Life Years (DALYs) is close to 172,000 (Torgerson et al., 2015). Within high-income countries alone, *G. lamblia* accounts for about 35-37% of water-borne outbreaks, however in low-income countries lacking the necessary surveillance system the real burden of *Giardia*-associated waterborne disease outbreaks remains potentially underestimated (Baldursson and Karanis, 2011; Estratiou et al., 2017).

Giardiasis exhibits diverse, non-specific clinical symptomatology often ranging from self-resolving, asymptomatic infection to acute diarrhoea, nausea, abdominal cramps, flatulence, malabsorption and maldigestion of nutrients. Studies in recent years have associated a number of factors with the development of symptomatic giardiasis, including apoptosis of enterocytes and loss of epithelial barrier function, inhibition of brush-border enzyme activity and bile acid metabolism, and microvillus shortening among others (Andersen et al., 2006; Cotton et al., 2011; Farthing, 1997; Buret, 2007, Ankarlev, 2010).

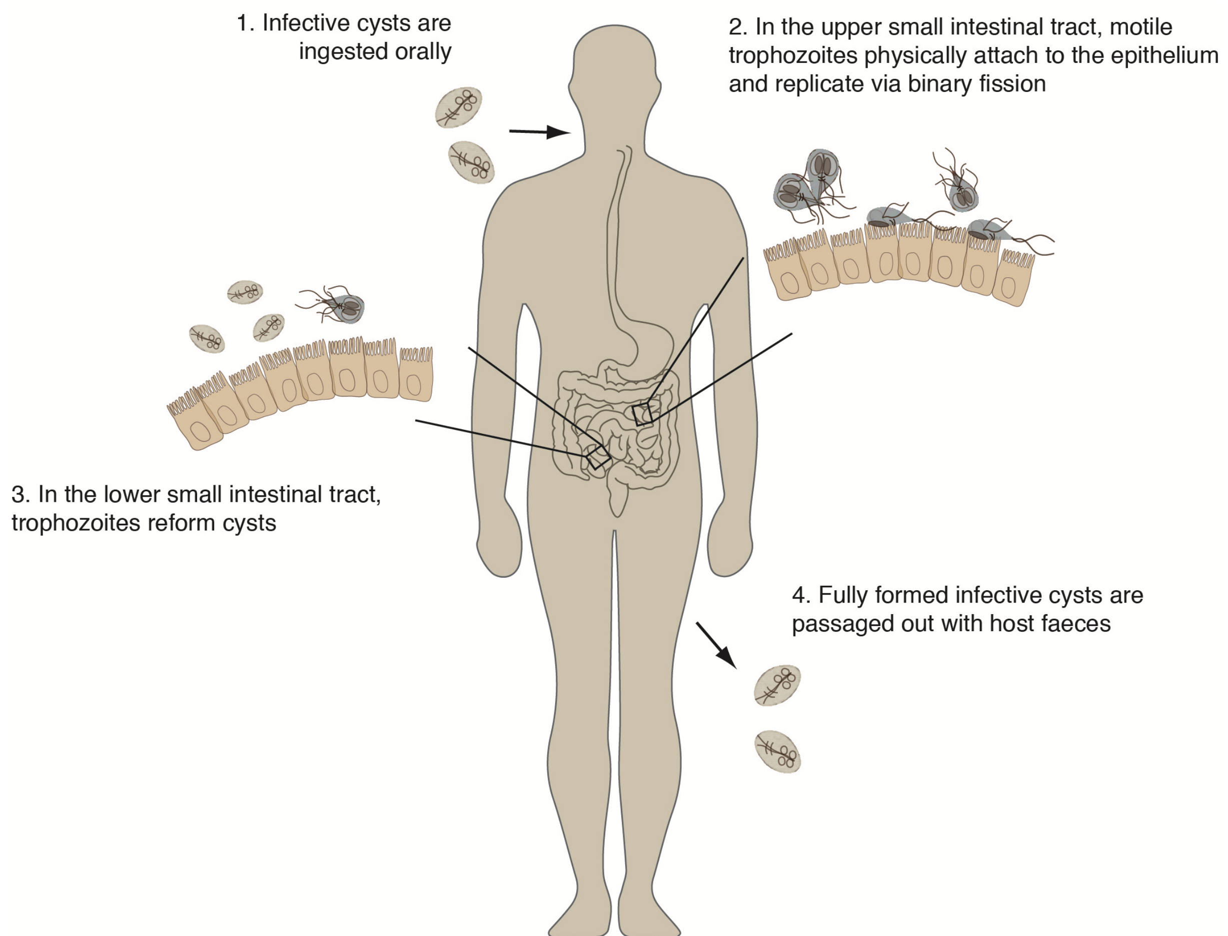

Figure 1.1 Life cycle of *Giardia* parasites. *Giardia* parasites have a direct life cycle and only two developmental stages. Infective cysts can be ingested by a host in contaminated food or water via the oral route. Once the cysts reach the upper small intestinal tract, excystation occurs and two motile trophozoites emerge from every cyst. Trophozoites physically attach to the mucus and small intestinal epithelium in the lumen, where they replicate via binary fission and form dense foci of colonised tissue. In the distal small intestine (ileum) due to unfavourable conditions such as changes in pH or availability of nutrients trophozoites lose their flagella, start depositing cyst wall proteins on the surface, alter their metabolic profile and re-form cysts. Cysts are then passaged with the faeces of the host back into the environment, where they can be picked up by another host.

1.1.3 Experimental animal models of *Giardia muris*

An experimental animal model of human giardiasis using the murine species *G. muris* was developed over 40 years ago in Swiss albino mice in attempts to better characterise disease progression and host immunity to the parasite in controlled laboratory settings (Roberts-Thomson et al., 1976). *G. muris* is a natural parasite of murine rodents and subsequently, differences in susceptibility to infection in various inbred mouse strains have been studied and reported (Adam, 2001; Belosevic and Faubert, 1983). *G. muris* is non-pathogenic to humans, has a reproducible pattern of infection in mice and a life cycle practically identical to that of the human parasite *G. lamblia* (Fig. 1.1.1). Therefore, it has been commonly used as an animal model in giardiasis research over the years to study host humoral immunity and pro-inflammatory T cell responses to infection (Finch et al., 1993; Heyworth and Vergara, 1994; Heyworth, 1986; Dreesen et al., 2014; Venkatesaan et al., 1997; Davids et al., 2006; Dann et al., 2015; Paerewijck et al., 2017). One disadvantage of this experimental model is that while *in vitro* culturing techniques have successfully been established and applied for *G. lamblia*, attempts to culture *G. muris* have been unsuccessful and maintenance of the *G. muris* life cycle requires continuous passage in mice (Adam, 2001). Nevertheless, as a natural parasite

of mice *G. muris* readily establishes infection in laboratory mice, while experimental infections of mice with *G. lamblia* generally require pre-treatment with antibiotics, potentially because *G. lamblia* is not naturally adapted to murine species (Barash et al., 2017). Therefore, *G. muris* is a suitable model for investigations of effector mechanisms of host immunity known for their cross-talk with the commensal microbiota of the host.

1.2 Host immunity to *Giardia* infection

1.2.1 Mechanical barriers to infection

1.2.1.1 Small intestinal epithelium

A characteristic feature of *Giardia* trophozoites is their localisation and attachment in high density foci in the duodenum and jejunum without becoming invasive (Barash and Maloney, 2017). The intestinal epithelial cell layer of the host therefore represents an integral physical defence mechanism against the parasite and forms a crucial part of the mucosal immune system of the host. Being only a single cell layer thick, the intestinal epithelium displays diverse anatomical structures along the length of the gastrointestinal tract and host defence mechanisms reflect the variable functionalities of the different regions of the intestinal epithelium (Ramanan and Cadwell, 2017). The small intestinal epithelial surface forms characteristic anatomical features such as finger-like villi and invaginations (crypts), serving to maximize the absorptive surface area of the gut. Intestinal epithelial cells (IEC) are known to migrate from the crypt base towards the tip of the villus in a process of continuous turn-over and thus can potentially restrain the adherence of pathogens like *Giardia* to the intestinal epithelium (Fig. 1.2A) (Marchiando, 2016). Further physical separation strategies of the intestinal epithelium include tight junction proteins like occludins, claudins and junctional adhesion molecules expressed between adjacent IECs to control epithelial permeability to small molecules and foreign antigen found in the gut lumen, to mediate paracellular communication and exchange of nutrients and more importantly to physically restrict access of lumenal pathogens to the immune cells resident in the lamina propria (Ramanan and Cadwell, 2017).

In the context of intestinal parasite infections, IL-13-mediated IEC turnover is significantly increased during infection with the gastrointestinal nematode *Trichuris* to facilitate faster expulsion of the parasite, however whether this also occurs in the context of giardiasis remains to be investigated (Cliffe et al., 2013). Importantly, IECs can also express a wide range of chemokine receptor ligands and thus serve as mediators of the recruitment of a variety of both innate and adaptive host immune cells during infection (Maaser et al., 2002). *In vitro*, attachment of *G. lamblia* trophozoites has been demonstrated to lead to the up regulation of a number of chemokine ligands on epithelial cells classically involved in the recruitment of dendritic cells, T and B lymphocytes (CCL20), neutrophils (CXCL1-3) and macrophages (CCL2) (Roxström-lindquist et al., 2005).

1.2.1.2 Mucus production

Within the intestinal epithelium resides a specific lineage of cells termed goblet cells, which specialize in the production of mucus (Fig. 1.2B). Mucus provides key protection and integrity of the gut barrier, creating a sticky environment overlaying the intestinal epithelium and serves a multitude of purposes - entrapping invading microorganisms and foreign antigen, concentrating secretory IgA antibodies and antimicrobial peptides at the lumen-epithelium interface, as well as providing an important source of nutrients for the specialized microbial communities in the gut (Pelaseyed et al., 2014; Arike and Hansson, 2017). In the context of gastrointestinal parasite infections, the mucus layer provides a crucial protective barrier by creating physical separation between lumen-dwelling pathogens and the underlying host immune system.

In order for *Giardia* trophozoites to attach and colonise the host intestinal epithelium, they must physically cross the mucus layer overlying the epithelium. The full scope of *Giardia*-mucus interactions is yet to be fully elucidated, however studies have suggested that trophozoite traversal across the mucus layer depends on a combination of flagellar motion and the secretion of proteolytic enzymes disrupting Muc2 integrity and hence the viscosity of the mucus layer (Amat et al., 2016; Cotton et al., 2015). However, whether the small intestinal mucus plays any role in the attenuation or stimulation of trophozoite growth and attachment remains an active area of investigation (Allain et al., 2017).

1.2.2 Innate immune responses

1.2.2.1 Antimicrobial peptides (AMP)

Another specialised cell type of IEC named Paneth cells are primarily located at the bottom of small intestinal crypts and specialise in the secretion of a wide range of antimicrobial peptides (AMPs) crucial for host-microbiota communication (Fig. 1.2C) (Gassler, 2017). Over 500 types of AMP have so far been identified and they broadly divide into maganins, cathelicidins and defensins (Gassler, 2017). *Giardia* infection has been demonstrated to lead to unregulated expression of a number of AMPs (Dann et al., 2015; Paerewijck et al., 2017; Tako et al., 2013). AMPs like cryptidins have been shown *in vitro* to demonstrate varying levels of killing propensities of *G. lamblia* trophozoites, as well as to partially affect cyst viability and excystation (Aley et al., 1994).

1.2.2.2 Nitric oxide synthesis

NO possesses both cytotoxic and immunomodulatory activities and is actively synthesized from L-arginine via NO synthase enzymes (NOS) by a range of epithelial and immune cells (Eckmann et al., 2000). From the three known isoforms of NOS enzymes, namely neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), the latter is the isoform most commonly expressed by myeloid and intestinal epithelial cells in response to cytokine and microbial product signaling. Elevated nitric oxide (NO) production has been observed during *G. lamblia* infection in humans (Matowicka-Karna et al., 2011), as well as elevated expression of a number of genes involved in NO synthesis in *G. muris*-infected mice (Dann et al., 2015). *In vitro* work has previously shown that NO inhibits *G. lamblia* excystation and displays cytostatic effects on trophozoites (Eckman et al., 2000). As addressed above, the parasite has evolved an efficient evasion strategy against host NO production and immune cell activation by inducing arginine depletion during infection and hence disrupting the efficient synthesis of NO from arginine (Banik et al., 2013).

1.2.2.3 Mast cells

Mast cells are a terminally differentiated granulocyte, which play key roles in inflammatory processes. Importantly, they have been previously demonstrated to play a role in protective host immunity during giardiasis. *G. lamblia*-infected gerbils and *G. muris*-infected mice display mast cell accumulation in the small intestine as a result of infection, while mast cell-deficient mice fail to control *G. muris* infection (Hardin et al., 1997; Erlich et al., 1983). Furthermore, *G. lamblia*-derived proteins such as arginine deaminase (ADI) and their potential metabolic products have been described to induce mast cell release of TNF-α and IL-6 cytokines (Fig. 1.2D) (Munoz-Cruz et al., 2018).

1.2.2.4 Macrophages

Macrophages are another type of innate immune cell previously shown to take part in host protective immunity against *Giardia*. Macrophages function via ingestion and engulfment of cellular debris, foreign antigens and microorganisms via a process termed phagocytosis. Considering the extracellular localisation of *Giardia* trophozoites in the intestinal lumen, innate immune cells such as macrophages normally have restricted access to the parasite. Nevertheless, early work has shown that macrophages derived from lymphoid organs such as

Peyer's patches and from milk can phagocytose trophozoites both *in vitro* and during infection (Radulescu et al., 1981; Kaplan et al., 1985; Owen et al., 1981; Franca-Botelho et al., 2006). Furthermore, more recent work has suggested that murine macrophages form and trigger macrophage extracellular traps (METs) in response to *G. lamblia* trophozoites in a dose-dependent manner (Li et al., 2018).

1.2.3 Adaptive immune responses

1.2.3.1 B cell and antibody responses

In the gastrointestinal tract, intestinal IgA predominates mucosal immunity and plays an essential role in shaping host-microbiota interplay under homeostasis, during infection and inflammation (Macpherson et al., 2015). In the small intestinal lamina propria (siLP), plasma B cells (PC) secrete IgA antibodies, which intestinal epithelial cells actively transport into the lumen via a transmembrane transport protein named polymeric Ig receptor (pIgR), expressed on the basolateral end of the epithelium (Fig. 1.2E) (Davids et al., 2006). Considering the extracellular lumenal localisation of *Giardia* trophozoites, host IgA responses therefore represent a potent defence mechanism against the parasite. Indeed, humoral immune responses have been demonstrated in response to *Giardia* infection in both experimental animal models and in humans. Early studies have shown that mice infected with either *G. lamblia* or *G. muris* display elevated serum and intestinal IgA and IgG antibodies capable of recognising and binding to trophozoite surface antigens (Heyworth, 1986; Heyworth and Vergara, 1994; Stager and Muller, 1997), as well as to excretory/secretory or soluble parasite extracts (Jiménez et al., 2007, 2014). Specifically, both mucosal and serum IgA antibodies appear to recognise and bind to a range of *G. muris* and *G. lamblia* antigens and the potential contributions of IgA binding to the parasite include impeding motility and attachment of trophozoites to the intestinal lumen (Heyworth et al., 1990; Heyworth and Vergara, 1994; Velazquez et al., 2005). Furthermore, studies have demonstrated that IgA-deficient (Langford et al., 2002), pIgR-deficient or B cell-deficient mice (Snider et al., 1988) fail to control infections with *G. lamblia* or *G. muris* further emphasize the importance of host humoral immunity for the efficient control of giardiasis (Langford et al., 2002; Davids et al., 2006).

1.2.3.2 T cell responses

T cell-mediated immunity is another key host protective mechanism elicited during infections with *Giardia,* as mice deficient in $CD4^+$ T cells fail to clear infection (Singer and Nash, 2000; Zhou et al., 2007). In humans, stimulation of peripheral blood mononuclear cells (PBMC), small intestinal lamina propria (siLP) lymphocytes or intestinal epithelial lymphocytes (IEL) with whole *G. lamblia* trophozoites or with antigen lysate leads to $CD4^+$ T cell activation and the production of a number of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-1β, IL-6 and IL-17 (Ebert, 1999; Saghaug et al., 2015). Furthermore, PBMC from individuals previously exposed to *Giardia* were isolated and stimulated with *Giardia* antigens 5 years after exposure to the parasite and displayed elevated activation within the $CD4^+$ T cell pool comprising IL-17 producing cells, indicating the existence of immunological memory to *Giardia* in humans (Hanevik et al., 2011). Studies of experimental infections in mice have demonstrated varying T cell responses to *Giardia* infection, with some studies reporting mixed Th1/Th2/Th17 responses. More recent work has now established that pro-inflammatory Th17 immune responses and specifically IL-17A are the crucial players in the efficient control of infection in mice with both *G. muris* and *G. lamblia* (Dann et al., 2015; Dreesen et al., 2014; Paerewijck et al., 2017; Singer, 2016).

Th17 cells are an effector lineage of $CD4^+$ T cells and play prominent roles in the induction of protective immunity against extracellular bacterial, fungal and protozoan pathogen infections. Importantly, their induction and activation is at least partially dependent on commensal microbiota adhesion and signalling and they secrete a number of pro-inflammatory cytokines like IL-17 and IL-22 (Ivanov et al., 2009; Atarashi et al., 2015). IL-17 is a cytokine important for signalling epithelial cells to produce a range of chemokines, leading to neutrophil and

lymphocyte recruitment. Furthermore, IL-17 signals the expression of CCL20 on intestinal epithelial cells, which is a ligand for the chemokine receptor CCR6 commonly expressed on Th17 cells themselves (Minegishi et al., 2009). Importantly, Th17 responses have been implicated to play a role in a number of autoimmune conditions ranging from psoriasis, inflammatory bowel disease and asthma to multiple sclerosis (Yang et al., 2014).

Mice infected with *G. muris* display elevated mRNA expression of IL-17A, while IL-17 receptor knock-out mice fail to control infection and excrete significantly higher numbers of cysts than wild-type mice (Dreesen et al., 2014). IL-17A is further known to stimulate the secretion of antimicrobial peptides and stimulation of pIgR expression, and hence to influences IgA transport into the lumen (Fig. 1.2F) (Davids et al., 2006). Indeed, one study has demonstrated that IL-17A deficiency leads to elevated trophozoite loads, decreased defensin-b expression and a significant reduction in faecal IgA (Dann et al., 2015). Overall, these studies highlight pro-inflammatory Th17 responses and IL-17A secretion as a key immune effector mechanism linking humoral and cellular immune responses during *Giardia* infection.

Even though infections with *Giardia* can lead to the development of significant immunopathological sequelae, disease progression is most commonly asymptomatic and the parasite typically causes minimal mucosal intestinal damage (Oberhuber et al., 1997). While pro-inflammatory Th17 responses are key to the efficient control of infection, there is no clear correlation between exacerbated inflammation in the host and elevated Th17 activity in response to giardiasis. This therefore raises the possibility that counteracting anti-inflammatory immune responses are potentially also at play during infection and could be responsible for the commonly observed lack of immunopathology in the host. Given the importance of regulatory T cells (Treg) in controlling overt inflammatory events, their role in host protective immunity during giardiasis remains elusive and understudied to date. Therefore, the involvement of Tregs in host immunity to *Giardia* and their potential implications for efficient host control and disease progression merit further investigations.

1.2.4 Intestinal microbiota

Due to their extracellular localisation, *Giardia* trophozoites colonising the small intestinal lumen reside in close proximity to the commensal microbial communities of the host and may therefore alter host immunity-parasite-microbiota interactions. *Giardia* infection is known to increase the secretion of bile acid derivatives and other metabolites, thus altering the availability of metabolic byproducts in the gut and hence acting as a potential contributor to commensal microbiota alterations during infection (Fig. 1.2G) (Barash and Maloney, 2017).

Indeed, *Giardia* infections in mice have been demonstrated to lead to localised shifts in the host microbiota composition. During the acute stage of infection with *G. lamblia,* elevated numbers of mucus-associated bacteria and translocation of commensal bacteria from the intestine systemically to the spleen and liver have been shown, possibly resulting from epithelial cell-related damage (Chen et al., 2013; Halliez et al., 2016). Moreover, acute giardiasis leads to significant alterations in the structure of the gut microbiota (Barash et al., 2017). Importantly however, the influence of commensal microbiota on host protective immunity to *Giardia* infection remains largely elusive.

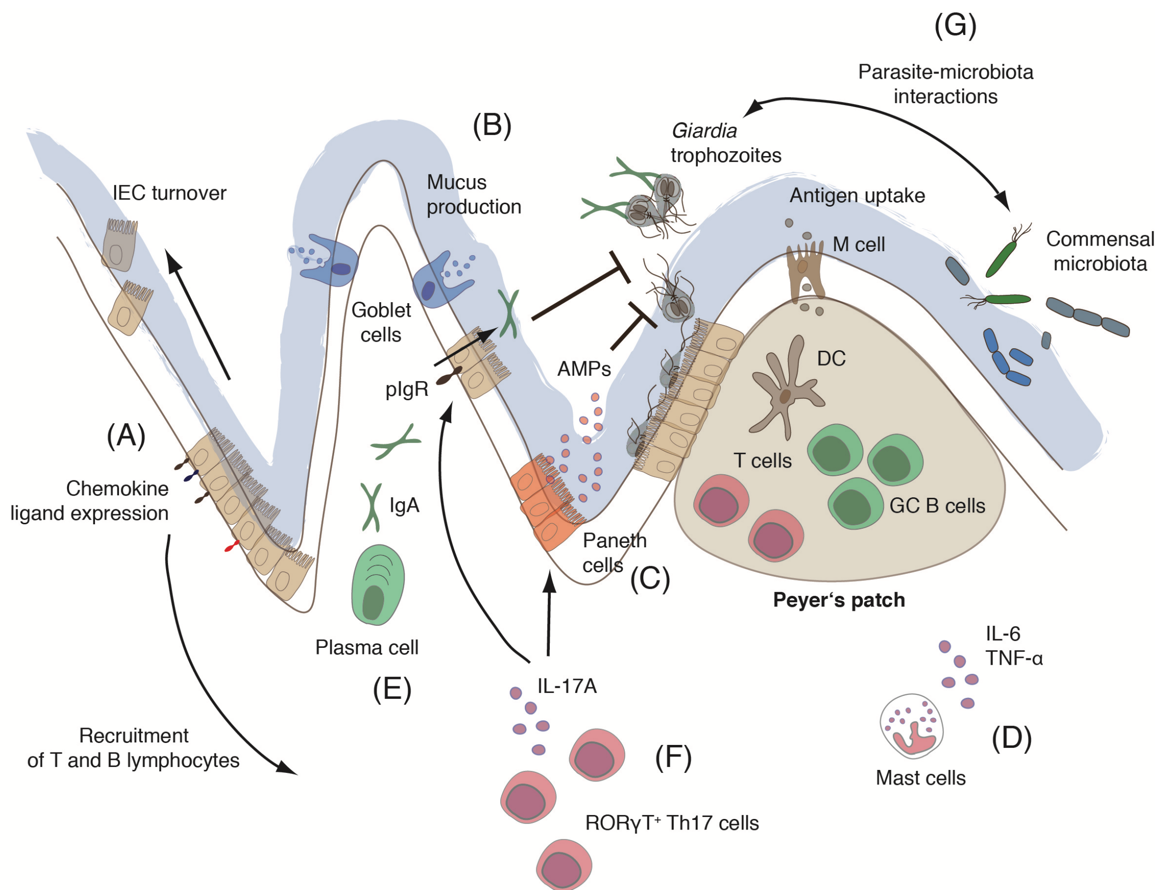

Figure 1.2. Host immunity to *Giardia* infection. (A) The small intestinal epithelium acts as a physical barrier preventing the invasion of pathogenic organisms like *Giardia* trophozoites located in the lumen. Furthermore, intestinal epithelial lymphocytes (IEC) undertake turnover and continuous renewal in an upward direction from the crypt, thus impeding the attachment of trophozoites during infection. IECs also express numerous chemokine ligands and hence participate in the recruitment of T and B lymphocytes to the site of infection. **(B)** Specialised goblet cells located in the intestinal epithelium secrete mucin proteins forming the protective mucus layer lining the small intestinal epithelium. The mucus serves as a physical barrier to gut pathogens, entraps foreign antigen and concentrates secretory IgA and AMPs at the lumen-epithelium interface. **(C)** Diverse antimicrobial peptides (AMPs) with detrimental properties to trophozoites are secreted from Paneth cells, located at the crypt base of the small intestinal epithelium. **(D)** Mast cells resident in the lamina propria release IL-6 and TNF-α during giardiasis. **(E)** IgA-secreting plasma B cells resident in siLP produce IgA antibodies, which are then transported across the intestinal epithelium via active transport of the polymeric Ig receptor (pIgR) expressed on epithelial cells. Once in the intestinal lumen, secretory IgA (sIgA) can directly target and bind to trophozoites, potentially inhibiting their motility and ability to attach to the gut epithelium. **(F)** RORγt$^+$ Th17 cells in siLP release pro-inflammatory cytokines like IL-17A, which plays a role in neutrophil recruitment, stimulation of AMP production and pIgR expression on epithelial cells and thus support IgA transport into the lumen. **(G)** Trophozoites reside in close proximity to the commensal microbiota of the host. *Giardia* infection is known to cause shifts in the composition of the microbiota, while certain commensals are known to induce and maintain innate and adaptive cellular immune responses. Parasite-microbiota interactions also potentially include competition for available nutrients in the intestinal environment and altering the availability of metabolic byproducts.

1.3 Intestinal Treg/Th17 balance

1.3.1 Regulatory T cell and Th17 cell differentiation

The current immunological dogma broadly recognises the existence of four effector T cell lineages (Th1, Th2, Th9 and Th17), T follicular helper cells and regulatory T cells (Treg). Th17 cells secreting the pro-inflammatory cytokines IL-17A and IL-22 are prominent players in

inflammatory responses in the context of autoimmunity, bacterial and certain protozoan parasite infections.

Despite that Treg and Th17 cells subserve starkly opposite anti- versus pro-inflammatory roles during infection and inflammation, respectively, research over the years has established that the two T cell subsets share a number of key signaling factors driving their differentiation (Fig. 1.3). Moreover, both Treg and Th17 cells display developmental plasticity, making Treg/Th17 balance an important factor in host immune responses during inflammation and infection. TGF-β and IL-2 are two of the critical signaling factors for the generation of Tregs, with IL-2 playing a role in stabilising TGF-β - induced Foxp3 expression (Li and Flavell, 2008; Marie et al., 2005). In contrast, in the presence of pro-inflammatory cytokines like IL-1β or IL-6, low concentrations of TGF-β and IL-2 preferentially drive Th17 cell differentiation (Chorro et al., 2018).

The regulatory T cell (Treg) pool comprises two distinct populations with defined functional differences. The majority of $Foxp3^+$ $CD4^+$ Treg originate in the thymus (tTreg) in response to TCR stimulation by self-antigens and hence play a key role in maintaining immunological tolerance to self-antigens and in preventing the development of overt autoimmune inflammatory events. Tregs utilise a number of effector mechanisms to implement immunosuppression, including the secretion of anti-inflammatory cytokines like IL-10, IL-35 and TGF-β, engagement of inhibitory receptors like CTLA-4, deprivation of IL-2 or granzyme B production (Rothstein and Camirand, 2015). Importantly, studies in recent years have identified the existence of phenotypical heterogeneity within the Treg compartment, namely $Foxp3^+$ Tregs expressing the T helper cell transcription factors Tbet (Th1), Gata3 (Th2) or RORγT (Th17) (Luu et al., 2017). RORγt-expressing Tregs are peripherally-induced and highly enriched in the small intestinal and colonic lamina propria and their induction has been shown to depend largely on microbiota signaling from mixtures of commensal bacterial groups of *Clostridia* (Solomon and Hsieh, 2016; Ohnmacht et al., 2015; Atarashi et al., 2013). Furthermore, $ROR\gamma t^+$ Treg display a TCR repertoire more similar to that of Th17 cells than of conventional Tregs, indicating a potential antigen-specific capacity of specific targeting of Th17 cells (Yang et al., 2016; Solomon and Hsieh, 2016; Kim et al., 2017; Neumann et al., 2019). Nevertheless, the heterogeneity of intestinal Treg and associations between Treg phenotypes and Th17 activity have not been addressed in the context of *Giardia* infections.

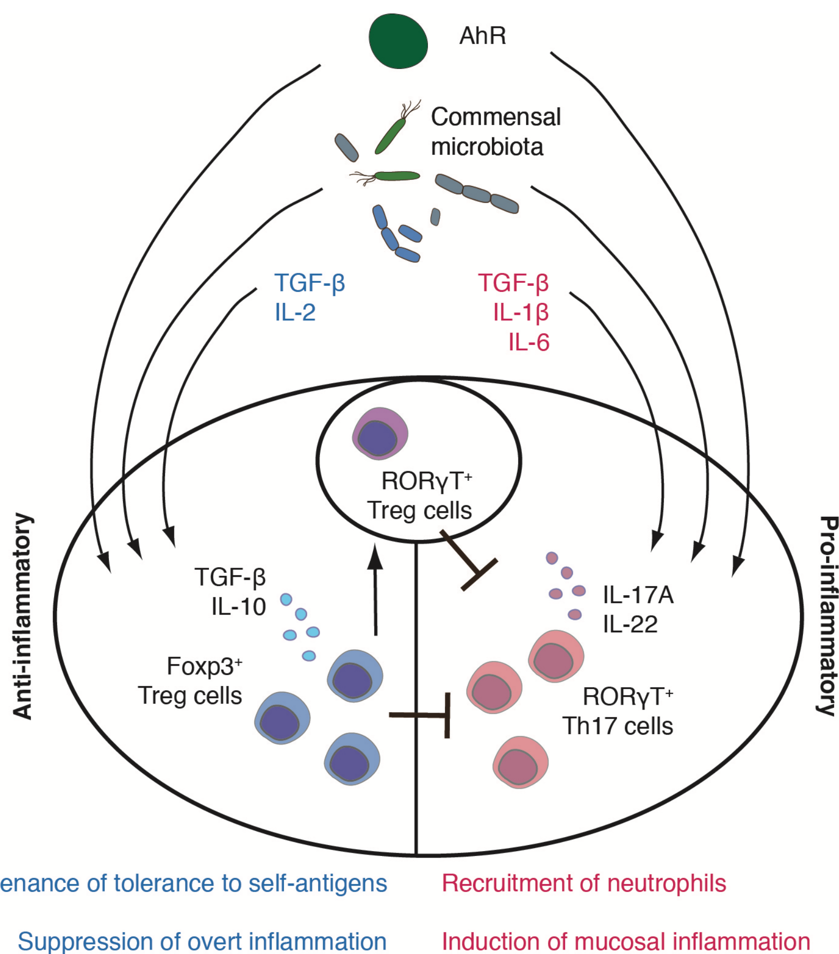

Figure 1.3 Intestinal Treg/Th17 balance. Treg and Th17 cells display certain developmental plasticity, making Treg/Th17 balance an important factor in host immune responses during inflammation and infection. TGF-β and IL-2 are critical signalling factors for the generation of Tregs, with IL-2 playing a role in stabilising TGF-β-induced Foxp3 expression. In contrast, pro-inflammatory cytokines like IL-1β or IL-6 and low concentrations of TGF-β preferentially drive Th17 cell differentiation. Further factors influencing Treg/Th17 differentiation include microbiota signaling and the aryl hydrocarbon receptor (AhR). RORγT$^+$ Tregs represent a stable Treg subset with superior suppressive capacities to RORγT$^-$ Tregs, and have been demonstrated to specifically control pro-inflammatory Th17 cells during intestinal inflammation and during infection. Microbiota signaling is known to drive the generation of RORγT$^+$ Tregs under steady state.

1.4 Effector and regulatory functions of intestinal eosinophils

Eosinophils are a type of terminally-differentiated, multifunctional granulocyte, which develop from pluripotent hematopoietic stem cells in bone marrow and their development depends on a number of transcription factors (PU.1, C/EBP, GATA-1, FOG-1) and cytokines (GM-CSF, IL-3, IL-5) (Hogan et al., 2008). Phenotypically, eosinophils possess a highly polymorphic nucleus and contain numerous primary and secondary intracellular granules, sombrero vesicles and lipid bodies. Eosinophil granules store a vast range of bioactive proteins including chemokines, enzymes and growth factors, which can be selectively released via a process known as degranulation. Signature granule contents of eosinophils, however, are cationic proteins like Major Basic Protein (MBP), eosinophilic cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (Khoury et al., 2016).

Importantly, eosinophil granule proteins like MBP display cytotoxic properties, stimulate tissue remodelling factors and alter smooth muscle function (Rothenberg and Hogan, 2006) and can

activate complement component pathways, as well as to induce mediator release from neutrophils, basophils and mast cells (Hogan et al., 2008). EPO, on the other hand, mediates the formation of Reactive Oxygen Species (ROS) and nitrogen metabolites toxic to invading pathogens (Rothenberg and Hogan, 2006; Jong et al., 1981; Borelli et al., 2003). Furthermore, eosinophils can release a range of pro- and anti-inflammatory cytokines including IL-1β, IL-4, IL-5, IL-6, IL-10 and TGF-β (Khoury et al., 2016).

The clinical relevance of eosinophils in the context of allergic inflammation and during helminth infection has long been recognised as central to their functional activities in different tissues (Strandmark et al., 2016). Importantly, the highest numbers of eosinophils reside in the gastrointestinal tract at steady state. Recent studies have demonstrated that eosinophils exhibit a range of homeostatic functions, including the maintenance of IgA class switching in PP and maintenance of IgA-producing plasma cells in the lamina propria under steady state and during infections with the small intestinal nematode *H. polygyrus* (Chu et al., 2014; Strandmark et al., 2017). Furthermore, eosinophils were reported to constrain Th17 cell differentiation and activity via the secretion of IL-1R antagonist (Chu et al., 2011; Sugawara et al., 2016; Jung et al., 2015). In the context of giardiasis, a single report so far has demonstrated evidence of eosinophilia in a patient with *G. lamblia*, but nevertheless despite recent progress in understanding the role of eosinophils, there remains a lack of studies investigating the role eosinophils in *Giardia* infections (Suzuki et al., 2010).

2. AIMS

At present, giardiasis in humans is still among the most common food- and water-borne diarrhoeal diseases worldwide. Over the years, research has attempted to attribute the high prevalence of asymptomatic cases and the wide range of immunopathology observed in symptomatic patients to a number of factors, including the genetic assemblage of *G. lamblia*, intestinal barrier function disruptions or commensal microbiota composition among others. Intestinal IgA and adaptive Th17 responses have been established as important factors driving host protective immunity against the parasite, however the role of immune regulatory mechanisms, their potential role in constraining Th17 immunity and how that correlates with efficient control of infection also remain elusive. The primary aims of this thesis have therefore been the following:

1. To ascertain whether inbred mouse strains of different genetic backgrounds known to display differences in their control of *Giardia* infection also differ in Th17 activity.
2. To establish whether *Giardia* infection leads to differences in Treg responses or an imbalance in Treg/Th17 ratios in these hosts and how that correlates with control of infection.
3. To determine whether small intestinal eosinophils play a role in the induction and maintenance of intestinal IgA and Th17 responses during *G. muris* infection.
4. To establish whether murine eosinophils can directly recognise and respond to *Giardia* trophozoite antigens.

These aims are summarised in Figure 2.

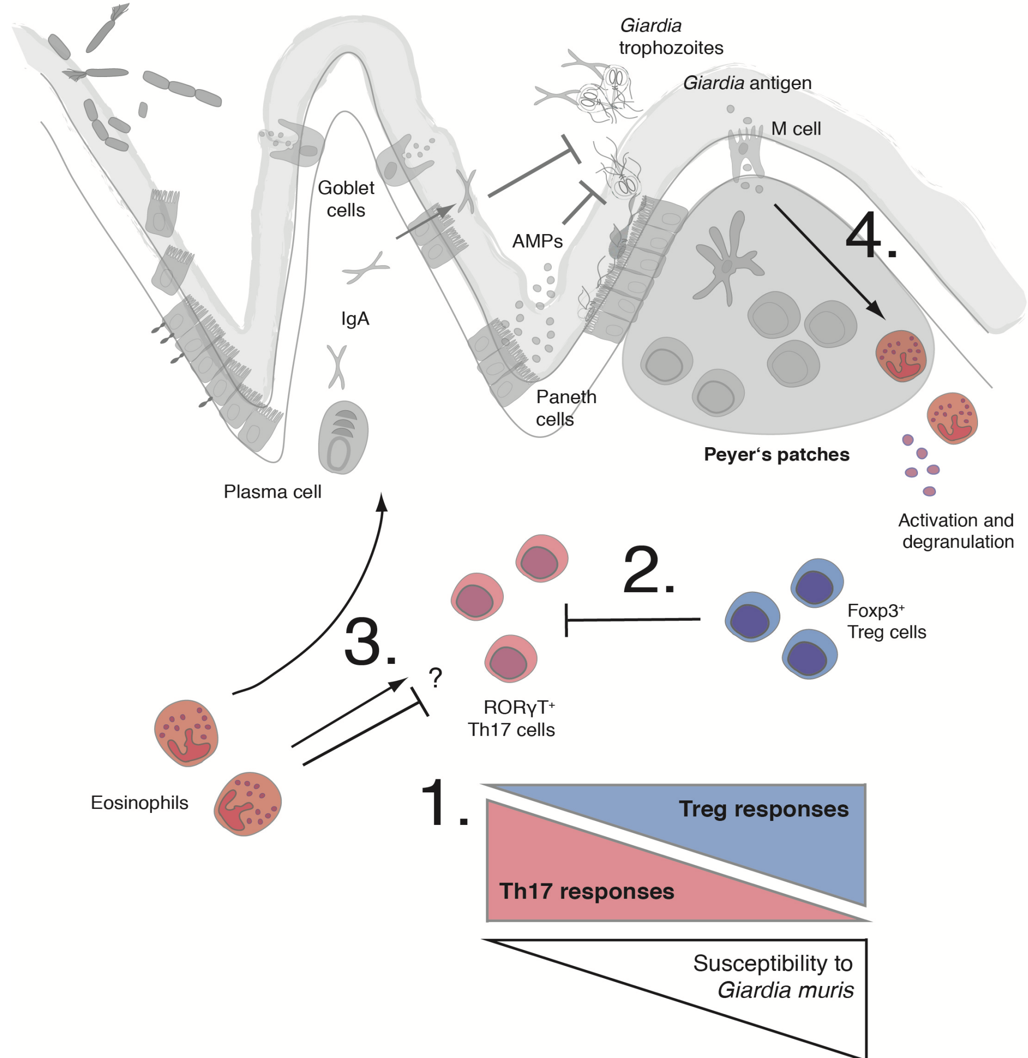

Figure 2. Project aims. Four main aims for this PhD project are defined and highlighted in colour. **1.** To ascertain whether inbred mouse strains of different genetic backgrounds, known to display differences in their control of *Giardia* infection and in their propensity for Th17 responses, also display variations in their intestinal Treg phenotype. **2.** To establish whether *Giardia* infection leads to differences in Treg responses or an imbalance in Treg/Th17 ratios in these hosts and how that correlates with efficient control of infection. **3.** To determine whether small intestinal eosinophils play a role in the induction and maintenance of intestinal IgA and Th17 responses during *G. muris* infection. **4.** To establish whether murine eosinophils can directly recognise and respond to *Giardia* trophozoite antigens.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 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 Bioscience, San Jose, CA, USA
FACS Canto II flow cytometer	BD Bioscience, San Jose, CA, USA
FastPrep 24 homogenizer	MP Biomedicals, Santa Ana, CA, USA
HydroSpeed microplate washer	Tecan, Mannedorf, Switzerland
Lamina flow, Scanlaf, Mars Safety 2	LaboGene, Lynge, Denmark
Lamina flow, Heraeus LB-732-C	Thermo Fischer 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
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

3.1.2 Pharmaceuticals, chemicals and reagents

2-mercaptoethanol	Ferak Berlin, Berlin, Germany
Bovine Serum Albumin (BSA)	AppliChem, Darmstadt, Germany
Ethanol	AppliChem, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	AppliChem, Darmstadt, Germany
DNase I	Sigma-Aldrich, Steinheim, Germany
Dithioerythritol (DTE)	Sigma-Aldrich, Steinheim, Germany
FACS lysis solution	BD Bioscience, San Jose, CA, USA
Fixation/Permeabilisation concentrate	eBioscience, San Diego, CA, USA
Fixation/Permeabilisation diluent	eBioscience, San Diego, CA, USA
Formaldehyde 3.7%	Carl Roth, Karlsruhe, Germany
Fetal Calf Serum (FCS)	PAN Biotech, Aidenbach, Germany
Fetal Calf Serum (FCS) HY Clone	GE Healthcare, South Logan, UT, USA
Gentamycin	AppliChem, Darmstadt, Germany
Isofluorane	Abbott, Ludwigshafen, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
L-glutamine	PAN Biotech, Aidenbach, Germany
TL Liberase	Roche, Basel, Switzerland
Non-essential amino acids (NEAA)	PAN Biotech, Aidenbach, Germany
Penicillin/Streptomycin (Penn/Strep)	PAN Biotech, Aidenbach, Germany
Percoll	GE Healthcare, Uppsala, Sweden
Permeabilisation buffer	eBioscience, San Diego, CA, USA
RNA lysis buffer	Analytic Jena, Jena, Germany
Sodium bicarbonate (NaHCO3)	Carl Roth, Karlsruhe, Germany
Sodium carbonate (Na2CO3)	Carl Roth, Karlsruhe, Germany

Sodium pyruvate	PAN Biotech, Aidenbach, Germany
Sulfuric acid (H2SO4)	Carl Roth, Karlsruhe, Germany
Tween-20	Carl Roth, Karlsruhe, Germany
Water, Millipore	Millipore, Darmstadt, Germany
Water, molecular biology grade	AppliChem, Darmstadt, Germany

3.1.3 Commercial kits

FastStart universal SYBR Green	Roche, Mannheim, Germany
High Capacity RNA to cDNA kit	Applied Biosystems, Darmstadt, Germany
InnuPrep RNA kit	Analytik Jena, Jena, Germany

3.1.4 Cytokines, growth factors, stimulators and inhibitors

IL-5, recombinant murine	R&D Systems, Minneapolis, MN, USA
IL-33	Peprotech, Hamburg, Germany
GM-CSF	Peprotech, Hamburg, Germany
FLT-3L	Peprotech, Hamburg, Germany
SCF	Peprotech, Hamburg, Germany
Brefeldin A	eBioscience, San Diego, CA, USA
Ionomycin	Sigma-Aldrich, Steinheim, Germany
Phorbol-12-myristate-acetate (PMA)	eBioscience, San Diego, CA, USA

3.1.5 Buffers and media

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

3.1.6 Cell isolation and culture

Bone marrow eosinophil (bmEos) culture medium	RPMI 1640 20% HY Clone FCS 25mM HEPES 100U/mL penicillin + 100U/mL streptomycin (Pen/Strep) 2mM L-glutamine 1x non-essential amino acids (NEAA) 1mM sodium pyruvate 50uM 2-mercaptoethanol
CMF (Calcium, Magnesium-free)	HBSS (w/o Ca2+ and Mg2+) 2% FCS 10mM HEPES 25mM NaHCO3
CMF/DTE	CMF 8% FCS (final 10%) 0.154mg/mL DTE
HBSS/EDTA	HBSS 10% FCS 15mM HEPES 5mM EDTA
HBSS/HEPES	HBSS 15mM HEPES
complete RPMI (cRPMI)	RPMI 1640 10% FCS 2mM L-glutamine 100U/mL penicillin + 100U/mL streptomycin (Pen/Strep)
complete RPMI siLP (gut cRPMI)	RPMI (w/o NaHCO3) 5% FCS 15mM HEPES 100ug/mL gentamycin

digest RPMI	complete RPMI siLP 0.1mg/mL TL liberase 0.1mg/mL DNase I
wash RPMI	RPMI 1640 1% FCS 100U/mL penicillin + 100U/mL streptomycin (Pen/Strep)

3.1.7 ELISA

Sodium carbonate buffer (coating buffer)	pH 9.5 distilled water 0.1M NaHCO3 0.1M Na2CO3
Assay diluent	PBS 3% BSA
Wash buffer	PBS 0.05% Tween-20

3.1.8 Flow cytometry

FACS buffer	PBS 1% BSA 2mM EDTA
Fixation/Permeabilisation concentrate	eBioscience Cat. #00-5123-43
Fixation/Permeabilisation diluent	eBioscience Cat. #00-5223-56
Permeabilisation buffer	eBioscience Cat. #00-8333-56
BD FACS lysis solution 10x	BD Biosciences Cat. #349202

3.2 METHODS

3.2.1 Animals and ethical statement

Female BALB/c and C57BL/6 wild-type mice were purchased from Janvier Labs (Saint-Berthevin, France). dblGATA-1 (Jackson Laboratory, CA) and dblGATA-1xBALB/c littermate control mice were bred in-house at the Institute of Immunology, Freie Universität Berlin under standard specific pathogen-free (SPF) conditions. To collect faeces, mice were placed in individual sterile beakers and 3-4 fresh faecal pellets per mouse were collected in eppendorf tubes or cryotubes in liquid nitrogen at designated time points during the course of infection. To collect serum, 0.2-0.5mL of blood was collected in a heparinized serum tube from the

submandibular vein using a disposable lancet and without the use of anesthesia. Mice were sacrificed at the designated time points via isofluorane inhalation, followed by cervical dislocation. All animal experiments were performed in accordance with the National Animal Protection Guidelines and approved by the German Animal Ethics Committee for the Protection of Animals (G0113/15, H0438/17, ZH171, T0407/17).

3.2.2 *G. muris* life cycle and infection experiments

G. muris cysts were originally purchased from Waterborne, Inc. (USA) and were later maintained by serial passage in BALB/c mice. For this, cysts were administered via oral gavage. Faeces of infected mice were collected starting 7 days post-infection. For cyst isolation, the faeces were homogenised in sterile PBS and were layered on a 2M sucrose gradient for density gradient separation and isolation, as previously described by Roberts-Thomson et al., 1976. Cyst suspensions were kept in PBS at 4degrees until needed. For infection experiments, 1,000 *G. muris* cysts suspended in 200uL distilled water were administered to the mice via oral gavage. Faeces were collected at designated time points as described above, cysts were counted in a Neubauer chamber and were expressed as number of cysts per gram faeces.

3.2.3 Preparation of single cell suspensions

3.2.3.1 Secondary lymphoid organs

Spleen, Peyer's patches (PP) and mesenteric lymph nodes (mLN) were isolated and kept in cold RPMI 1640 medium, containing 1% FCS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Pen/Strep). To prepare single cell suspensions, PP were pre-digested in medium with 0.1 mg ml^{-1} liberase TL (Roche, Basel, Switzerland) and 0.1 mg ml^{-1} DNase (Sigma, St LoisGerman) at 37ºC on a shaker for 30 min. Cells were washed one time and erythrocytes were lysed in spleen following a 5min incubation of the cell suspension in BD FACS lysis solution (diluted 1:10 with distilled water) on ice, followed by one more washing step. Spleen, PP and mLN tissues were then forced through 70 µm cell strainers (BD Bioscience, San Jose, CA, USA). Cell numbers were determined using an automated CASY cell counter.

3.2.3.2 Small intestinal lamina propria (siLP)

To obtain a single cell suspension of siLP, the entire small intestine was excised and placed in ice-cold CMF medium. Following removal of the mLN and PP, the intestine was opened longitudinally and the contents and excess mucus was removed by scraping off with a forceps. The intestinal tissue was then thoroughly washed in HBSS/HEPES medium, followed by cutting in 1cm pieces which were placed in 20mL HBSS/DTE. The tissue samples were incubated once on a shaker at 37 degrees, 200rpm for 15 minutes. Next, the intestinal pieces were transferred in 20mL HBSS/EDTA and were consecutively shaken three times on a high-speed shaker at room temperature for another 15 minutes. Residual EDTA was removed by rinsing the intestinal pieces in RPMI medium, followed by placing them in 10mL RPMI digest medium at 37 degrees. The samples were then incubated on a shaker at 37°C, 200rpm for 30 minutes. Following this incubation step, the tissue samples were vortexed to disrupt remaining tissue pieces and the entire suspension was forced through a 20G needle and was filtered through a 70um cell strainer. The cell suspension was then washed twice in HBSS/HEPES. Cell suspensions were layered on a Percoll gradient, lamina propria cells were carefully collected at the 40/70% interface and following one final washing step in complete RPMI medium, the samples were counted on an automated CASY counter.

3.2.4 Flow cytometry

Single cell suspensions were adjusted to $10x10^6$ cells/mL and 200uL were added to cone-bottomed 96 well plates. Cells were washed one time in RPMI medium and prior to staining with fluorescent antibodies, cells were blocked with anti-mouse FcγRII/III to prevent unspecific binding. The following leukocyte populations were defined and analysed:

Th17 cells: $CD4^+Foxp3^-ROR\gamma t^+$ and $CD4^+IL\text{-}17A^+$
Regulatory T cells (Treg): $CD4^+Foxp3^+ROR\gamma t^-$ and $CD4^+Foxp3^+ROR\gamma t^+$
Innate lymphoid cells type 3 (ILC3): $CD4^-ROR\gamma t^+IL\text{-}17A^+$ and $CD3e^-CD4^-CD90^{+/-}NKp46^{+/-}CCR6^{lo}Tbet^{lo/int}$
Intestinal B cells (PP, mLN): $B220^+PNA^{hi}$ and IgA^+, $IgG1^+$ or IgM^+; $B220^+IgA^+$
Intestinal B cells (siLP): $B220^-IgA^+$
Eosinophils (Spleen, PP, mLN, siLP): $SSC^{hi}CD45^+GR\text{-}1^{lo}CD11b^+Siglec\text{-}F^+$, $CCR3^+$ or $CD63^+$
Eosinophils (blood): $SSC^{hi}CD45^+GR\text{-}1^{lo}CD11b^+Siglec\text{-}F^+MHC\text{-}II^{lo}$
Eosinophils (bone marrow culture): $SSC^{hi}CD45^+GR\text{-}1^{lo}CD11b^+Siglec\text{-}F^+CD63^+$

To detect intracellular cytokines and intranuclear transcription factor expression, cells were stimulated for 30 minutes with PMA (500ng/mL) and Ionomycin (1ug/mL), followed by the addition of Brefeldin A (1:1000) to each well for an additional 3 hours. The cells were then washed and underwent surface and intracellular staining. To quantify the expression of cell surface markers, cells were incubated for 10 minutes at 4°C with the respective fluorescent antibody cocktail in FACS buffer, after which they were fixed in 100uL of Fixation/Permeabilisation buffer (eBioscience, 1 part concentrate to 4 parts diluent) for 20 minutes at 4 degrees. Following fixation, the cells were washed in Permeabilisation buffer (eBioscience, diluted 1:10 in distilled water) to permeabilise the cell membrane and were further stained with the appropriate fluorescent antibody cocktail for detection of intracellular cytokines and intra-nuclear transcription factors for 20 minutes at 4°C. Finally, cells were washed one time in Permeabilisation buffer and were analysed on a BD Canto II flow cytometer. Results were analysed using FlowJo software Version 10.

3.2.5 Blood typing of BALB/c x dblGATA-1 littermate control mice

To determine the phenotype of F2 generation dblGATA-1xBALB/c littermate control mice, 2-3 drops of fresh blood from the submandibular vein were collected in 1mL FACS buffer and were kept on ice. The blood samples were centrifuged at 2000rpm for 2 minutes and the supernatant was aspirated carefully, without disturbing the cell pellet. The cells were incubated in 300uL BD FACS lysis solution for 5 minutes at room temperature to remove erythrocytes, followed by one washing step in FACS buffer. The cells were then transferred to a conical 96-well plate and surface staining was performed to determine the presence of blood eosinophils and hence the phenotype of each mouse.

3.2.6 Bone marrow extraction

The tibia and femur bones from both hind legs were removed and cleaned from excess tissue using sterile cotton gauges. The bones were then opened from both ends and flushed with wash RPMI medium using a 27G needle into a clean Petri dish. The bone marrow suspension was then disrupted by passing up and down a 1mL syringe using a 20G needle and was passed through a 70um cell strainer. The bone marrow cell suspension was washed one time in RPMI medium, followed by a 10 minute incubation in BD FACS lysis solution to remove erythrocytes as described above for spleen and blood samples. Finally, the cells were washed one more time, followed by resuspension in complete RPMI medium and counting on an automated CASY cell counter.

3.2.6.1 Bone marrow derived eosinophil culture

Fresh bone marrow cell suspensions were used for the generation of eosinophils *in vitro*. Cells were placed in blue-capped T75 primary cell culture flasks at a concentration of $1x10^6$/mL in 10mL pre-warmed bmEos medium containing 100ng/mL stem cell factor (SCF) (Peprotech) and 100ng/mL FLT-3 ligand (Peprotech). The flasks were incubated for 2 days at 37degrees. On day 2 old medium was removed and pre-warmed fresh medium containing 100ng/mL SCF and 100ng/mL FLT-3 ligand, adjusting the concentration of cells to $1x10^6$/mL. Starting on day 4, every 2 days old medium was exchanged for fresh medium containing 10ng/mL recombinant mouse IL-5 (R&D), always adjusting the concentration of cells to $1x10^6$/mL. On days 8, 10 and 12 the culture flasks were exchanged due to the presence of plastic-adherent cells. On day 13-14 the purity of the bone marrow eosinophil culture was surveyed via flow cytometry and cytospin.

3.2.6.2 Generation of *G. lamblia* trophozoite antigen

G. lamblia trophozoite isolates from assemblage A (WB6) and assemblage B (GS) were cultured axenically in filter-sterilised TYI-S-33 medium at the Robert Koch Institute in Berlin, as previously described (Keister, 1983). Frozen trophozoite pellets were transported to the Institute of Immunology, Freie Universität Berlin, where they were sonicated and filter-sterilised by passing through a 0.2um filter. Protein concentrations in the trophozoite lysate were determined via a BCA assay, as per the manufacturer's instructions.

3.2.6.3 *In vitro* stimulation with *G. lamblia* trophozoite antigen

bmEos were plated out on a 96-well round-bottom cell culture plate at a concentration of 2-$3x10^5$ cells per well in bmEos medium. Cells were challenged with WB6 and GS *G. lamblia* trophozoite antigens at concentrations of 5ug/mL, 10ug/mL and 20ug/mL. Negative control wells received just medium and positive control well were stimulated with PMA/Ionomycin (1:1000), IL-5 (10ng/mL), IL-33 (10ng/mL) and GM-CSF (20ng/mL) and all stimulations were performed in duplicates or triplicates. Following a 24 hour incubation at 37°C, 5% CO_2, cell culture supernatants were removed and stored at -20°C for further analysis. Cell pellets were washed in FACS buffer, transferred to 96-well conical-bottom plates and stained using fluorescent antibodies for cell surface marker expression analysis via FACS, as described above.

3.2.7 Antibody detection by enzyme-linked immunosorbent assay (ELISA)

Total IgA and IgG2b antibody titres in blood serum were quantified via sandwich ELISA. Briefly, 96-well flat bottom Maxisorp plates (Thermo Fischer Scientific, MA, USA) were coated with 50uL goat anti-mouse IgA (Southern Biotech, AL, USA) and incubated overnight at 4C. Plates were washed using a Tecan Hydrospeed microplate washer and blocked with 200uL 3% BSA in PBS for 1h before 50uL samples and standards were added. Plates were incubated with samples and standards for 2h at room temperature, after which 50uL goat anti-mouse AP-coupled IgA detection antibody (Southern Biotech, AL, USA) was added for 1h. 50uL phosphatase substrate was then added and plates were incubated for 30 minutes at 37°C. To stop the enzymatic reaction, 25uL 100mM EDTA was added and the signal was measured at 405nm minus reference wavelength 630um on a Biotek Synergy H1 Hybrid Reader.

3.2.8 RNA extractions, reverse transcription and real-time quantitative PCR (qRT-PCR)

At necropsy, 0.5cm tissue snips were excised from duodenal, jejunal and ileal sections of the small intestinal tract. RNA was isolated using the innuPREP RNA kit according to the manufacturer's instructions (Analytic Jena, Germany). 2µg of RNA was then reverse-transcribed to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Relative gene expression was determined via quantitative real-time PCR (qRT-

PCR) using 10 ng of cDNA and FastStart Universal SYBR Green Master Mix (Roche). Primer pairs used for gene amplification are described in Supplementary Table X. Efficiencies for each primer pair were determined by generating a standard curve, mRNA expression was normalized to the housekeeping gene β-Glucuronidase (GUSB) and were calculated by the Roche Light Cycler 480 software.

3.2.9 Molecular analysis of faecal microbiota composition

Fresh fecal pellets collected from naïve controls and mice infected with *G. muris* were immediately snap-frozen in liquid nitrogen and stored at –80°C until further processing. DNA was extracted from fecal samples. Faecal microbiota analysis was performed at the Institute of Microbiology, Infectious Diseases and Immunology at the Charite Universitätsmedizin, Berlin. In brief, DNA was quantified by using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng/µl. Then, the main bacterial groups abundant in the murine intestinal microbiota including Enterobacteria, Enterococci, Lactobacilli, Bifidobacteria, *Bacteroides/Prevotella* spp., Mouse Intestinal Bacteroides, *Clostridium coccoides* group, and *Clostridium leptum* group as well as total Eubacterial loads were assessed by quantitative real-time PCR (qRT-PCR) with species-, genera-, or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as described previously (Heimesaat et al., 2017). Numbers of 16S rRNA gene copies per nanogram DNA of each sample were determined.

3.2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). Results are displayed as mean ± SD and significance is displayed as *p<0.05, **p<0.01, ***p<0.001. Results were tested for normal distribution using the Shapiro-Wilk normality tests, followed by ANOVA or Kruskal-Wallis combined with Tukey’s or Dunn’s multiple comparison testing. For the correlation analysis between $ROR\gamma t^+$ Treg:Th17 ratios, $FLC16^+$ Th17 and $Ki\text{-}67^+$ Th17 cell frequencies, Spearman’s rank correlation coefficient was calculated.

3.2.11 Funding

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4. RESULTS

4.1 Intestinal Treg/Th17 balance during *G. muris* infection

4.1.1. Small intestinal Th17 cell responses during acute *G. muris* infection

In line with earlier findings, *G. muris*-infected BALB/c and C57BL/6 mice displayed differences in cyst excretion rates and hence of efficiency in controlling the infection (Belosevic et al., 1984). Over the course of 6 weeks, BALB/c mice displayed higher fluctuations in cyst shedding with two peaks of cyst excretion at 2 and 5 weeks post-infection, while C57BL/6 mice displayed lower and more stable cyst shedding (Fig. 3.1.1A, B). Previous studies have indicated that the efficient control of *Giardia* infection depends on Th17 cells secreting IL-17A (Dreesen et al., 2014; Dann et al., 2015). To establish if the observed differences in susceptibility to *Giardia* infection depend on the host genetic background, the activity of Th17 cells in naïve and *G. muris*-infected BALB/c and C57BL/6 mice was surveyed. In siLP, C57BL/6 mice harboured significantly higher frequencies of $CD4^+$ $ROR\gamma t^+$ $Foxp3^-$ Th17 cells in steady state and during infection than BALB/c mice. However, the overall frequencies of small intestinal Th17 cells remained stable in both mouse lines upon infection (Fig. 3.1.1C). Similarly, the frequencies of $IL\text{-}17A^+$ Th17 cells were constitutively higher in both naive and infected C57BL/6 compared with BALB/c mice (Fig. X). This finding was mirrored by $CD4^+$ T cells isolated from Peyer's patches (PP, Fig. 3.1.1D). In order to assess Th17 cell activity, the intracellular expression of the proliferation marker Ki-67 in small intestinal $ROR\gamma t^+$ Th17 cells was also surveyed. Interestingly, *G. muris* infection led to significantly elevated $Ki\text{-}67^+$ expression by Th17 cells in the siLP of infected C57BL/6 mice compared with naive controls and with BALB/c mice (Fig. 3.1.1E). Furthermore, quantitative real-time PCR analysis revealed low *Il17a* mRNA expression in duodenal and jejunal tissue, irrespective of the host background and infection status of the mice (Fig. 3.1.1F). In contrast, *Il17a* expression was significantly higher in the ileum of naive C57BL/6 compared with naïve BALB/c mice. However, upon *G. muris* infection, *Il17a* expression was impaired in the distal small intestine of infected compared to naïve C57BL/6 mice (Fig. Fig. 3.1.1F)

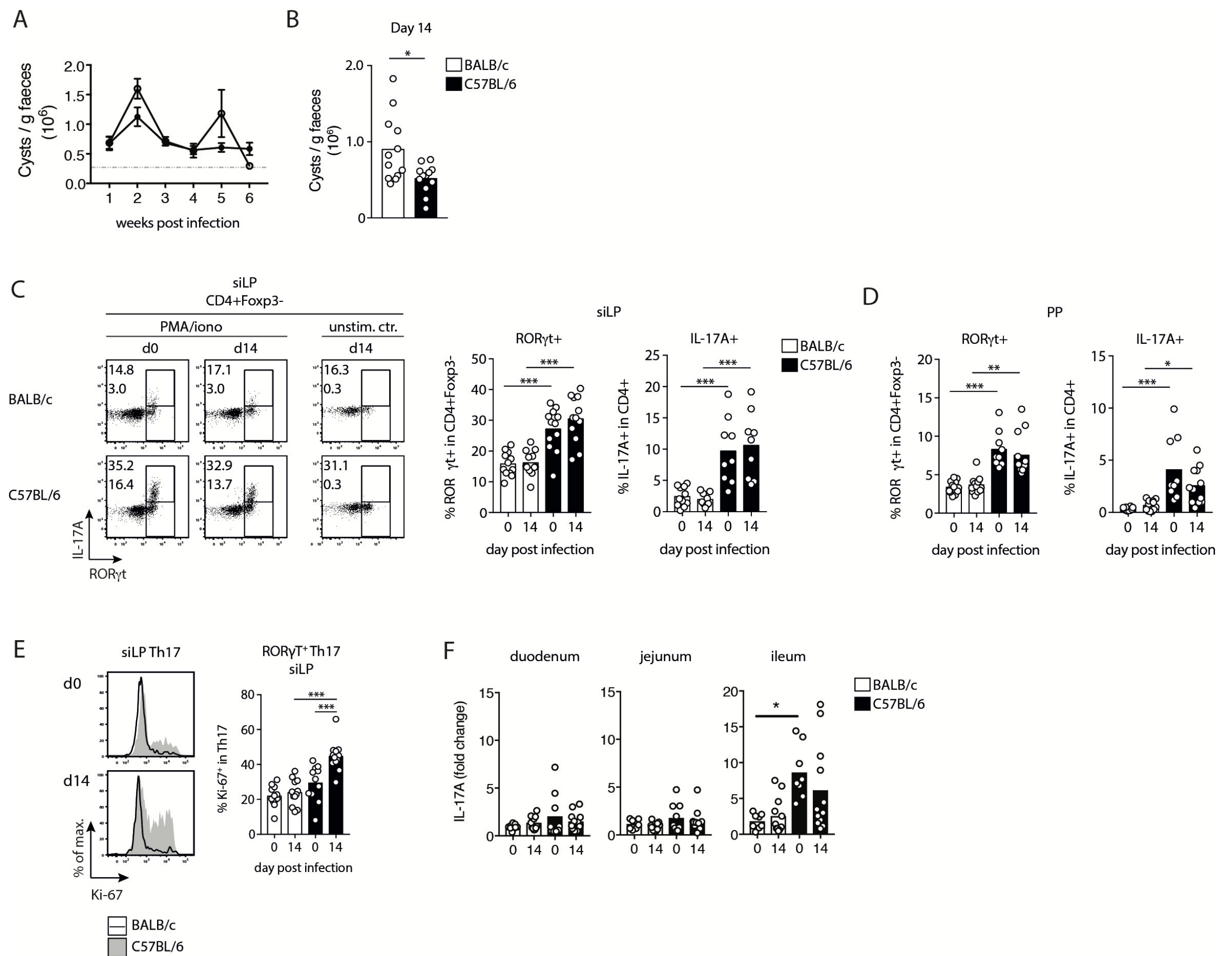

Figure 3.1.1 Differential Th17 capacity in naive and *G. muris*-infected BALB/c and C57BL/6 mice. **(A)** Cyst shedding dynamics over the course of the first 6 weeks of *G. muris* infection in wild-type BALB/c and C57BL/6 mice. **(B)** Cyst excretion 14 days post-infection. **(C)** Representative plots of RORγt and IL-17A expression by CD4⁺ cells isolated from the small intestinal lamina propria (siLP) of naïve and infected mice. Cells were stimulated for 4h with PMA/ionomycin or left untreated and Foxp3⁺ Tregs were excluded from analysis. Regular numbers report the frequencies of RORγt cells, bold numbers refer to IL-17A⁺ cells **(C, D)** Frequencies of RORγt⁺ and IL-17A⁺ Th17 cells in CD4⁺ T cells isolated from **(C)** siLP and **(D)** Peyer's patches. **(E)** Cell surface expression of Ki-67 on RORγt⁺ Th17 cells in siLP. **(F)** IL-17A expression relative to GUSB determined in small intestinal tissue samples. Data are pooled from 2-3 independent experiments, with n=3-5 mice/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's or Tukey's multiple comparison test **(B-D)** or unpaired t test **(E)**. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.2. Restricted Th17 responses correlate with elevated frequencies of intestinal RORγt⁺ Treg

We next asked if the poor Th17 cell activity in BALB/c at steady state and during *G. muris* infection was associated with differences in Tregs. A survey of the frequencies of total Foxp3⁺ Treg revealed no significant differences between the two mouse lines under homeostasis or during *G. muris* infection (Fig. 3.1.2A). Within the Treg pool however, peripherally-induced Foxp3⁺ Treg co-expressing RORγt have previously been reported to display superior suppressive activity compared to RORγt⁻ Treg and to specifically target the constriction of Th17 cell responses (Ohnmacht et al., 2015; Yang et al., 2016; Neumann et al., 2019). Assessment of RORγt expression by intestinal Foxp3⁺ Treg revealed that BALB/c mice harboured significantly more peripherally-induced RORγt⁺ Tregs compared with C57BL/6 mice,

suggesting that the more susceptible BALB/c line harbour higher frequencies of peripherally-induced Treg subsets than hosts displaying better control of infection (Fig. 3.1.2B,C).

In order to assess if the differences in the proportions of RORγt⁺ Treg in BALB/c and C57BL/6 mice correlated with the differential activity of Th17 cells upon *G. muris* infection, as well as the effect of *G. muris* infection on RORγt⁺ Treg frequencies, we surveyed the uptake of the fluorescently-labelled palmitate fatty acid Bodipy FL16 and Ki-67 expression by effector Th17 cells as measures of cell metabolic activity and proliferation, respectively. Within the total small intestinal CD4⁺ T cell compartment, C57BL/6 but not BALB/c mice, harboured a population brightly labelled by FLC16 and marked by the expression of the activation marker CD69. These cells predominantly originated from RORγt⁺ cells, hence representing proliferating, metabolically active Th17 cells (Fig. 3.1.3A). Moreover, *G. muris* infection led to a significant elevation of FLC16⁺ Th17 cells in C57BL/6 mice, while naive and infected BALB/c mice maintained markedly lower frequencies of these cells (Fig. 3.1.3B). The quantification of RORγt⁺ Treg:Th17 ratios in the siLP revealed that BALB/c mice display significantly higher RORγt⁺ Treg:Th17 ratios under steady state compared with C57BL/6 mice. This ratio increased further in response to *G. muris* infection in BALB/c, but not C57BL/6 mice (Fig. 3.1.3C). Finally, a highly significant negative correlation between RORγt⁺ Treg:Th17 ratios, the metabolic activity and proliferation of intestinal Th17 cells was detected in siLP of *G. muris*-infected mice (Fig. 3.1.3D,E). Therefore, the above data reveal that phenotypic differences in the intestinal Treg population correlate with the differential local activity of Th17 cells of genetically distinct *Giardia* infected BALB/c and C57BL/6 mice.

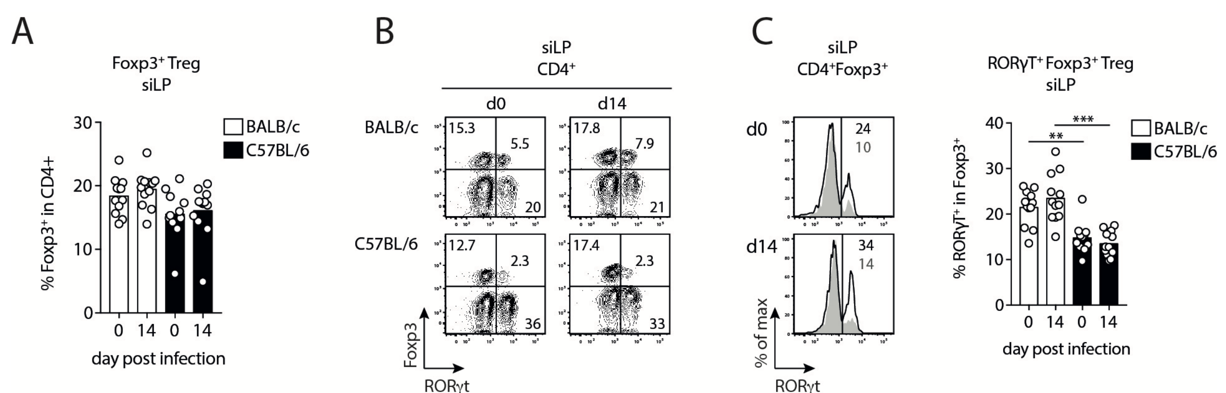

Figure 3.1.2 BALB/c mice harbour higher frequencies of RORγT⁺ Tregs under steady state and during *G. muris* infection. (A) Frequencies of Foxp3⁺ Treg in CD4⁺ cells isolated from the small intestinal lamina propria of naïve and infected BALB/c and C57BL/6 mice. **(B)** Representative plots of RORγt and Foxp3 expression by small intestinal CD4⁺ T cells. Bold numbers report frequencies of Foxp3⁺RORγt⁺ Treg. **(C)** RORγt expression by Foxp3⁺ Treg isolated from naïve and infected BALB/c and C57BL/6 mice. Data are pooled from 2-3 independent experiments, with n=3-5 mice/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's or Tukey's multiple comparison test. ** $p \leq 0.01$, *** $p \leq 0.001$.

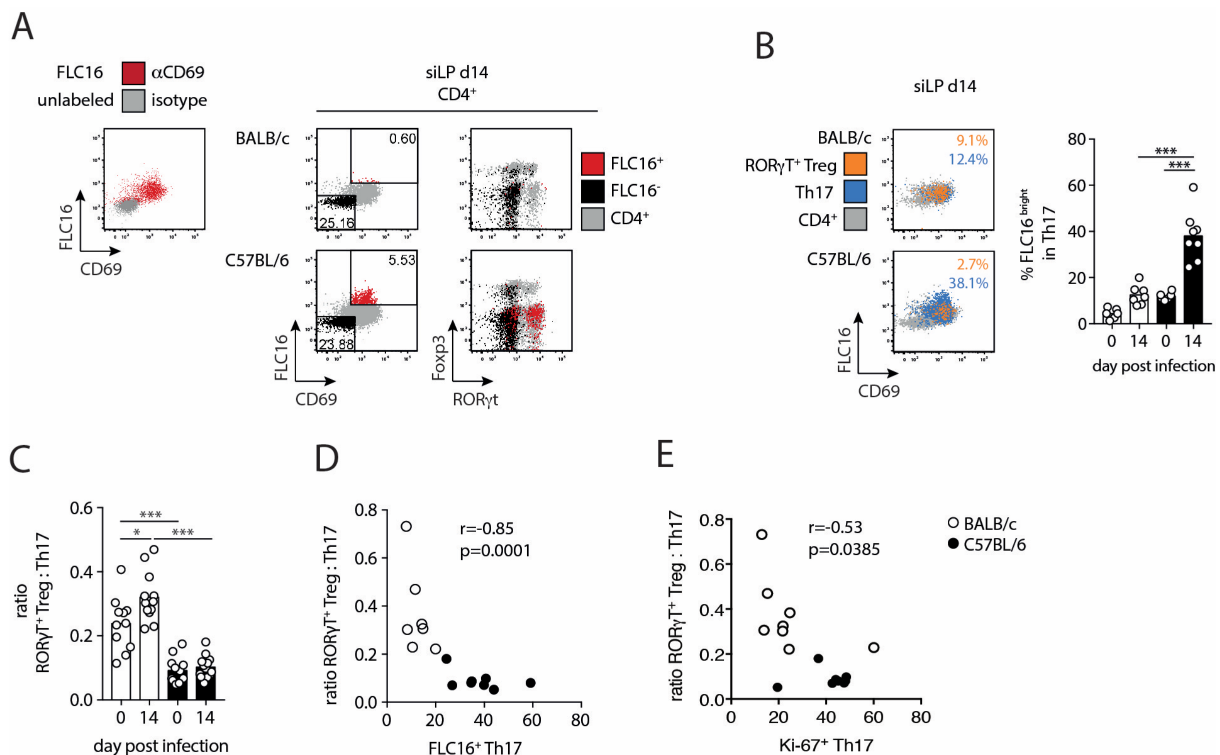

Figure 3.1.3 Higher frequencies of RORγt^{+} Tregs correlate with poor intestinal Th17 activity in *G. muris-infected* mice. (A) Uptake of fluorescently labelled palmitate (Bodipy FLC16) by CD4^{+} T cells isolated from the small intestine of mice infected with *G. muris* for two weeks. Following Bodipy FLC16 labeling *in vitro*, cells were surveyed for CD69, Foxp3 and RORγt expression. Representative overlays of CD69^{+}FL16^{bright} and CD69^{-}FLC16^{low} cells on the CD4^{+} population are shown. **(B)** Frequencies of FLC16^{bright} cells within CD4^{+}RORγt^{+} Th17 cells isolated from the small intestine after *in vitro* labelling (left). Right: representative overlays of Foxp3^{+}RORγt^{+} Treg, RORγt^{+} Th17 and total CD4^{+} cells. Numbers report frequencies of RORγt^{+} Treg and Th17 cells in CD4^{+} T cells. **(C)** RORγt^{+} Treg:Th17 ratios in small intestinal CD4+ cells. Correlation between RORγt^{+} Treg:Th17 ratios and **(D)** the frequency of FLC16^{+} Th17 cells and **(E)** Ki-67^{+} Th17 cells in intestinal cell isolates. Data are pooled from 2-3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's or Tukey's multiple comparison test **(A-G)** and Spearman correlation test **(H).** * p≤0.05, *** p≤0.001.

4.1.3 Retention marker expression by Treg and Th17 cells

CD103, the a_E subunit of the integrin a_Eb_7, is a membrane-associated receptor constitutively expressed on a range of immune cell subsets, including activated Treg and effector T helper cells. CD103 is known to play an important role in the retention of lymphocytes in epithelial tissues via its recognition and binding to E-cadherin, which itself is abundantly expressed on the basolateral side of epithelial cells (Schön et al., 1999; Cepek et al., 1994). Furthermore, upregulated CD103 expression in Tregs is a hallmark of increased Treg activity and superior suppressive capacity during nematode infections (Rausch et al., 2008; Filbey et al., 2014).

In order to establish whether the observed differences in Th17 activity and RORγt^{+} Treg frequencies in BALB/c and C57BL/6 mice during *G. muris* infection correlate with differences in Treg or Th17 retention in the intestine, the expression of CD103 on Th17 cells, total Foxp3^{+} Treg and RORγt^{+} Treg in siLP of naive and infected BALB/c and C57BL/6 mice was assessed via flow cytometry (Fig. 3.1.4)

In PP, naïve C57BL/6 mice displayed higher frequencies of CD103^{+} Th17 cells, however BALB/c mice responded to infection with a significant elevation in CD103 expression within

the Th17 pool, reaching comparable frequencies to those observed in infected C57BL/6 mice (Fig. 3.1.4B). With respect to CD103 expression on Tregs in PP, both mouse strains displayed comparable frequencies of total CD103$^+$Foxp3$^+$ Tregs (Fig. 3.1.4D) and CD103$^+$RORγt$^+$ Tregs (Fig. 3.1.4F). Furthermore, there were mild elevations in CD103-expressing total and RORγt$^+$ Tregs in response to infection in both BALB/c and C57BL/6 mice, however this difference only reached significance in total Foxp3$^+$ Tregs in C57BL/6 mice (Fig. 3.1.4D).

In siLP, BALB/c mice harboured notably lower frequencies of CD103-expressing Th17 cells, while C57BL/6 mice responded to *G. muris* infection with a significant elevation in CD103$^+$ Th17 cells, indicative of increased activation and Th17 cell retention in the lamina propria during infection in C57BL/6, but not BALB/c mice (Fig. 3.1.4A,B). Within the total pool of Foxp3$^+$ Tregs CD103 expression remained stable in BALB/c mice and did not change with infection, however interestingly C57BL/6 mice displayed elevated CD103$^+$ Tregs in response to infection (Fig. 3.1.4C,D). Furthermore, CD103$^+$RORγt$^+$ Tregs increased significantly in *G. muris*-infected mice of both strains, suggestive of elevated retention of RORγt$^+$ Tregs in the lamina propria in siLP during *G. muris* infection, possibly linked to TGF-β activity (Fig. 3.1.4E,F).

Overall, these data highlight that BALB/c and C57BL/6 mice do not display stark discrepancies in CD103 expression on Th17 or Treg subsets under steady state. However, more small intestinal Th17 cells express the retention marker CD103 in *G. muris*-infected C57BL/6 compared to BALB/c mice, providing evidence of more efficient retention of highly activated Th17 cells in the *Giardia*-infected small intestine. Furthermore, elevated CD103 expression on RORγt$^+$ Tregs indicates that there is increased retention of this Treg subset in response to infection.

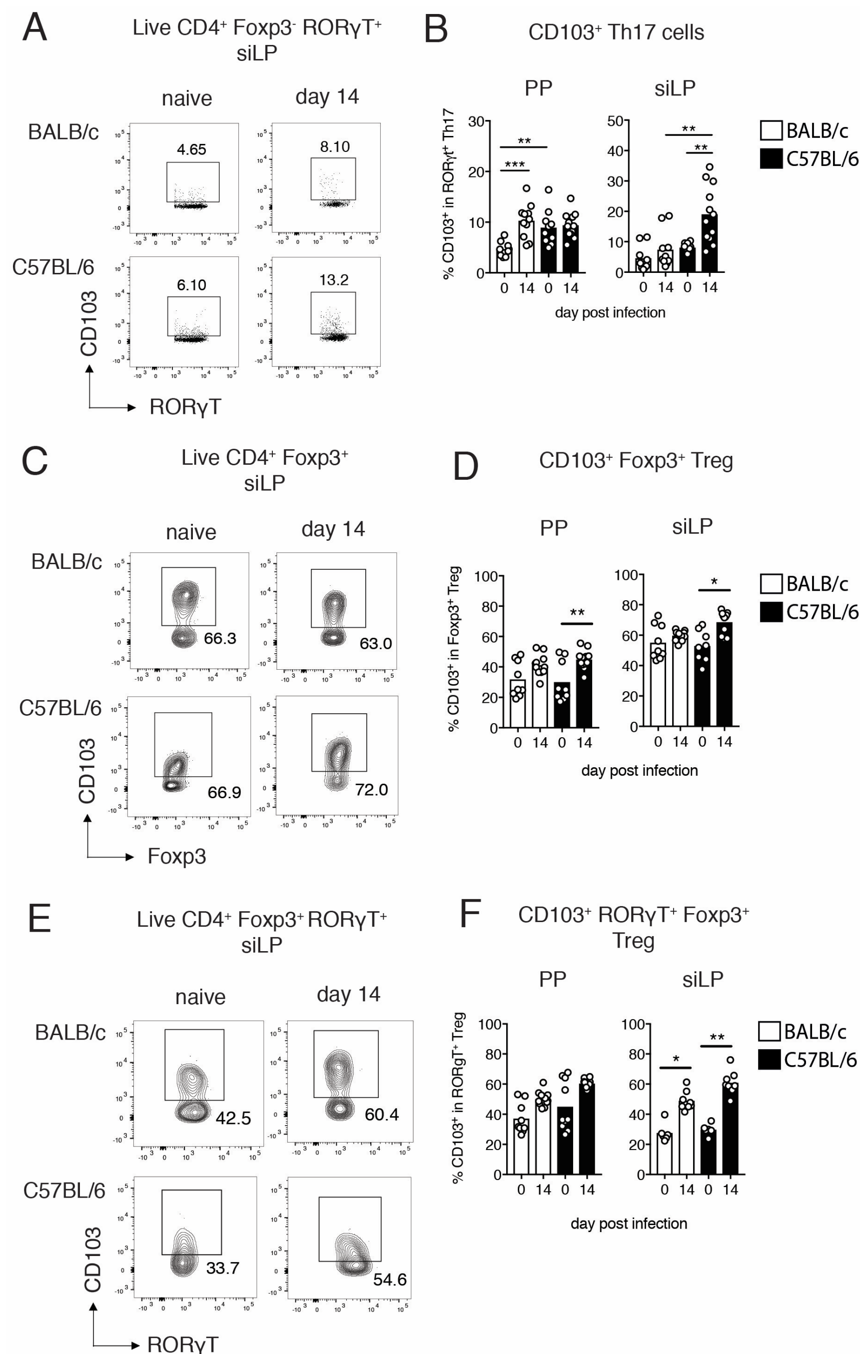

Figure 3.1.4 ***G. muris*** **infection leads to elevated CD103 expression in intestinal Th17 and Treg subsets in BALB/c and C57BL/6 mice. (A)** Exemplary FACS plots and **(B)** Frequencies of CD103 expression in CD4$^+$ Foxp3$^-$ RORγt$^+$ Th17 cells in siLP. **(C)** Exemplary FACS plots and **(D)** Frequencies of CD103 expression in total CD4$^+$Foxp3$^+$ Treg cells in PP and siLP. **(E)** Exemplary FACS plots and **(F)** Frequencies of CD103 expression in RORγt$^+$ Tregs in PP and siLP. Data are pooled from 2-3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's or Tukey's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.4 Tissue-derived cytokine expression

Since Treg and Th17 cell differentiation is regulated by the local cytokine milieu, including TGF-β, IL-1β and IL-6, it was hypothesised that the observed differences in Th17 and RORγt$^+$ Treg frequencies between BALB/c and C57BL/6 mice, as well as the shift in RORγt$^+$ Treg:Th17 ratios in response to *G. muris* infection in BALB/c mice results from the differential expression of these cytokines in the gut. To assess this, quantitative real-time PCR (qRT-PCR) was

performed to investigate the mRNA expression of TGF-β, IL-1β and IL-6 in duodenal, jejunal and ileal tissue samples of naive and infected BALB/c and C57BL/6 mice. Interestingly, gene expression levels of IL-1β and IL-6 were comparable between the two mouse strains independent of the tissue location or the mouse strain and remained stable during infection (Fig. 3.1.5B,C). However, infected BALB/c mice displayed significantly elevated TGF-β expression in the duodenum compared with C57BL/6 mice, while in jejunum and ileum TGF-β expression was comparable between the two mouse strains and remained unaffected by the infection (Fig. 3.1.5A). Importantly, the elevated TGF-β gene expression observed in the duodenum of infected BALB/c mice correlates with what is known about the preferential localisation of *Giardia* trophozoites in the upper small intestinal tract of the host and with the higher frequencies of RORγt$^+$ Tregs (Barash et al., 2017). However, whether the elevated TGF b levels in the duodenum of BALB/c mice are responsible for driving the higher frequencies of intestinal RORγt$^+$ Tregs or whether this Treg subset is the source of TGF-β itself would merit further investigations.

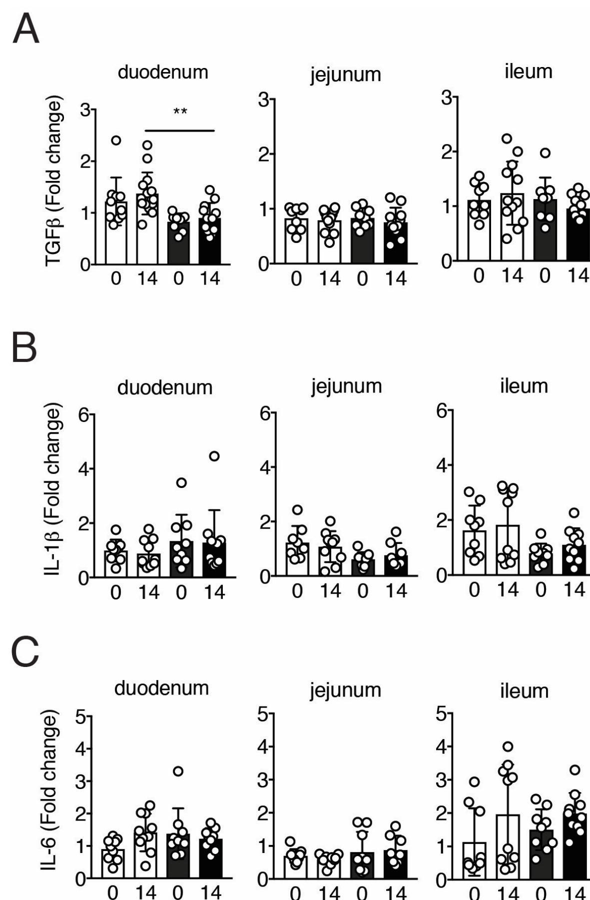

Figure 3.1.5 *G. muris* infection does not alter Treg/Th17 differentiation cytokines in intestinal tissue. Relative mRNA expression in duodenal, jejunal and ileal tissue of **(A)** TGF-β **(B)** IL-1β and **(C)** IL-6. Data are pooled from 3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's or Tukey's multiple comparison test. ** $p \leq 0.01$.

4.1.5 Poor ILC3 activity in BALB/c mice under homeostasis and during *G. muris* infection

A previous study reported on increased IL-17A expression by innate immune cell sources alongside CD4$^+$ T cells during *G. muris* infection in C57BL/6 mice (Dann et al., 2015). We hence asked if BALB/c and C57BL/6 mice differed in their IL-17A responses by non-CD4$^+$ cells. Contrasting the finding of higher Th17 cell frequencies in the small intestines of C57BL/6 mice, we detected significantly more CD4$^-$CD90$^+$RORγt$^+$ type 3 innate lymphoid cells (ILC3)

in the small intestines of naïve and infected BALB/c mice (Fig. 3.1.6A,B). Similar to Th17 cells, ILC3 in siLP of naïve and infected BALB/c mice displayed poor capacity for IL-17A production following *ex vivo* stimulation, compared with those in C57BL/6 mice (Fig. 3.1.6C). The IL-17A response pattern by small intestinal ILC3 was further mirrored by ILC3 isolated from Peyer's patches (Fig. 3.1.6D). In contrast, IL-17A responses by mLN-derived ILC3 were similar in both mouse lines (Fig. 3.1.6E). Thus, relatively high *Giardia* cyst excretion by BALB/c mice coincides with poor IL-17A production by Th17 cells and ILC3 in the local vicinity of the parasites.

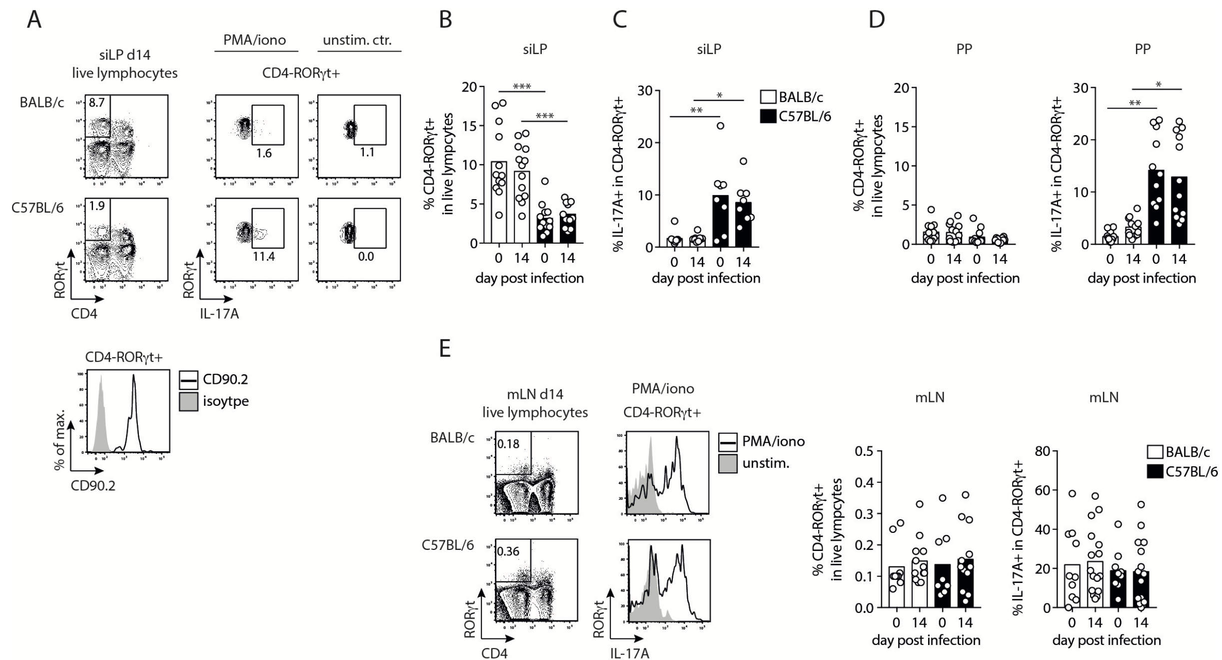

Figure 3.1.6 Naive and infected BALB/c mice display elevated ILC3 frequencies, but poor capacity for IL-17A production. (A) Representative plots of CD4 and RORγt expression by small intestinal cells. Histogram shows CD90 expression by $CD4^-ROR\gamma t^+$ cells, center and right plots report IL-17A production by PMA/ionomycin stimulated and unstimulated $CD4^-ROR\gamma t^+$ cells. **(B)** Frequencies of $CD4^-ROR\gamma t^+$ cells in siLP cells. **(C)** IL-17A expression by small intestinal $CD4^-ROR\gamma t^+$ cells after PMA/ionomycin stimulation. **(D)** $CD4^-ROR\gamma t^+$ cell frequencies and IL-17A+ responses in Peyer's patch cell isolates. **(E)** Representative plots and statistics of $CD4^-ROR\gamma t^+$ cells and IL-17A expression in cells isolated from mLN. Data are pooled from three independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.6 Commensal microbiota composition is comparable between naive and infected BALB/c and C57BL/6 mice

The intestinal induction and accumulation of Th17 cells, as well as $Foxp3^+$ regulatory T cells has previously been shown to depend on the presence and composition of the intestinal microbiota. Commensal members of the genus *Clostridium* have been shown to induce the differentiation of Tregs, including $Foxp3^+ROR\gamma t^+$cells controlling inflammatory processes in the gut at steady state and upon experimentally induced inflammation (Ohnmacht et al., 2015). On the other hand, segmented filamentous bacteria (SFB) and other microbes attaching to gut epithelial support the induction of Th17 cells and ILC3 activity (Atarashi et al., 2015; Ohnmacht et al., 2015; Sefik et al., 2015). We hence assessed if naïve and *G. muris* infected BALB/c and C57BL/6 mice - displaying differences in intestinal $ROR\gamma t^+$ Treg frequencies and Th17/ILC3 activity - differed in the composition of the commensal gut microbiota. Quantifying the total eubacterial load as well as the abundance of the main bacterial groups present in the murine intestine, we detected no significant differences in BALB/c mice compared to C57BL/6 mice

at steady state or after infection with *G. muris* (Fig.3.1.7). Of note, *G. muris* infection did not affect the abundance of any of the investigated genera and species as compared to the respective naïve control mice (Fig. 3.1.7. Hence, the differential phenotypical composition of the small intestinal Treg population and the discrete activity patterns of Th17 cells and ILC3 detected in BALB/c and C57BL/6 mice were not associated with prominent differences in the composition of the gut microbiota.

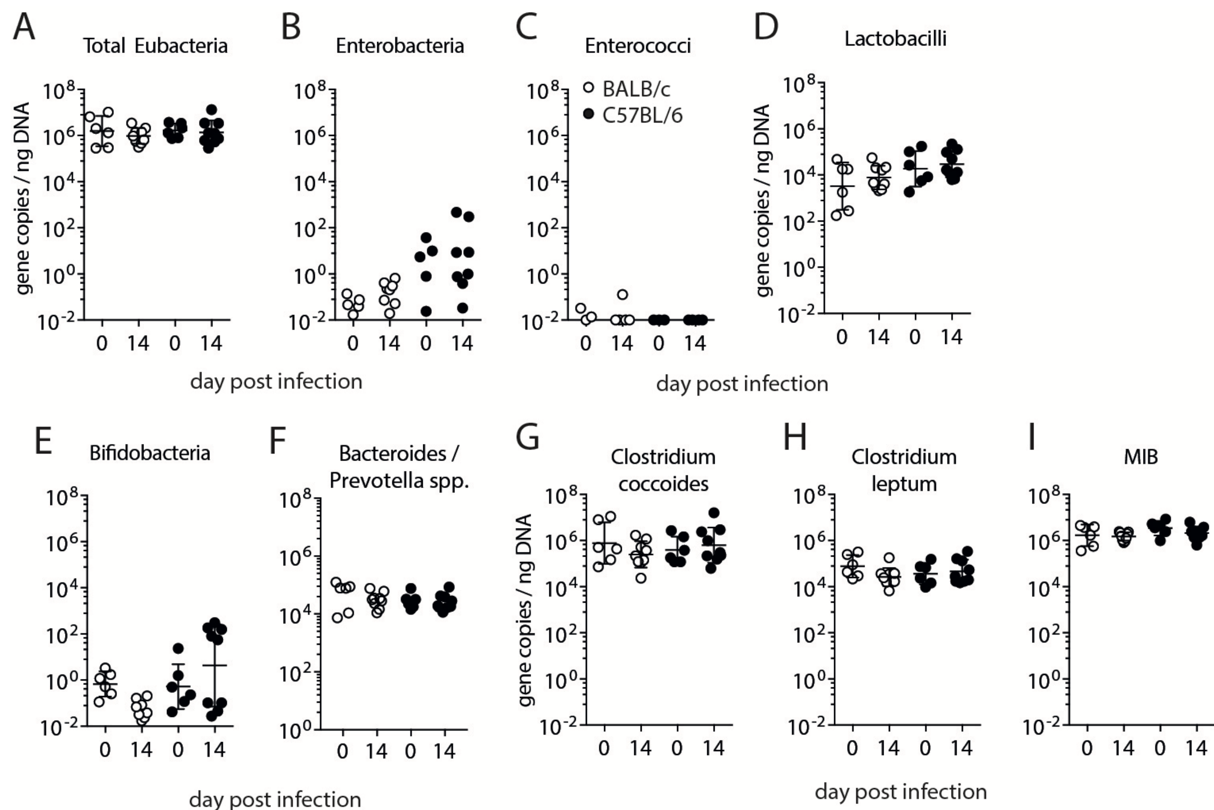

Figure 3.1.7. Commensal microbiota composition of naive and infected BALB/c and C57BL/6 mice is comparable. Fecal gene numbers of the main bacterial groups abundant in the murine intestinal microbiota detected by qPCR in naïve and *G. muris* infected BALB/c and C57BL/6 mice. Individual fecal loads of total eubacteria **(A)**, *Enterobacteria* **(B)**, *Enterococci* **(C)**, *Lactobacilli* **(D)**, *Bifidobacteria* **(E)**, *Bacterides*/*Prevotella* spp. **(F)**, *Clostridium coccoides*/*leptum* **(G, H)** and mouse intestinal bacteroides **(I)** are as expressed as 16s RNA gene copy numbers per ng DNA. Data are pooled from two independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD.

4.1.7 Local and systemic antibody levels mirror small intestinal Th17 activity at steady state and during *G. muris* infection

Previous studies have demonstrated that Th17 cells may convert to follicular T helper cells supporting IgA class switching by B cells in the Peyer's patches (Hirota et al., 2013). We hence asked if the generally higher frequencies of Th17 cells in siLP and PP of C57BL/6 compared to BALB/c mice coincided with differences in IgA^+ plasma cell (PC) numbers in the small intestine. Indeed, small intestinal cell isolates of C57BL/6 mice tended to comprise elevated frequencies of $B220^-IgA^+$ PC compared to BALB/c cell isolates, reaching significance two weeks after *G. muris* infection (Fig. 3.1.8A). Similar frequencies of IgA^+ B cells were detected in the PP of both mouse lines at steady state and following infection (Fig. 3.1.8B). Systemically, serum IgA levels did not change in infected mice compared with naive controls, however both naive and infected C57BL/6 mice displayed significantly higher serum IgA and IgG2b titres than BALB/c mice (Fig. 3.1.8C,D). Importantly, C57BL/6 mice additionally displayed a significant elevation in serum IgG2b in response to infection (Fig. 3.1.8D). Both IgA and IgG2b class switching are supported by TGF-β (Coffman et al., 1989; Deenick et al., 1999), however

the observed low TGF-β mRNA expression in duodenum of C57BL/6 mice contradicts their elevated systemic IgA and IgG2b antibody levels. Therefore, the factors behind this observation would merit further investigations. Thus, overall elevated Th17 and ILC3 numbers in the small intestine of C57BL/6 mice coincide with higher intestinal IgA^{+} PC and increased serum IgA, as well as elevated IgG2b levels during *G. muris* infection.

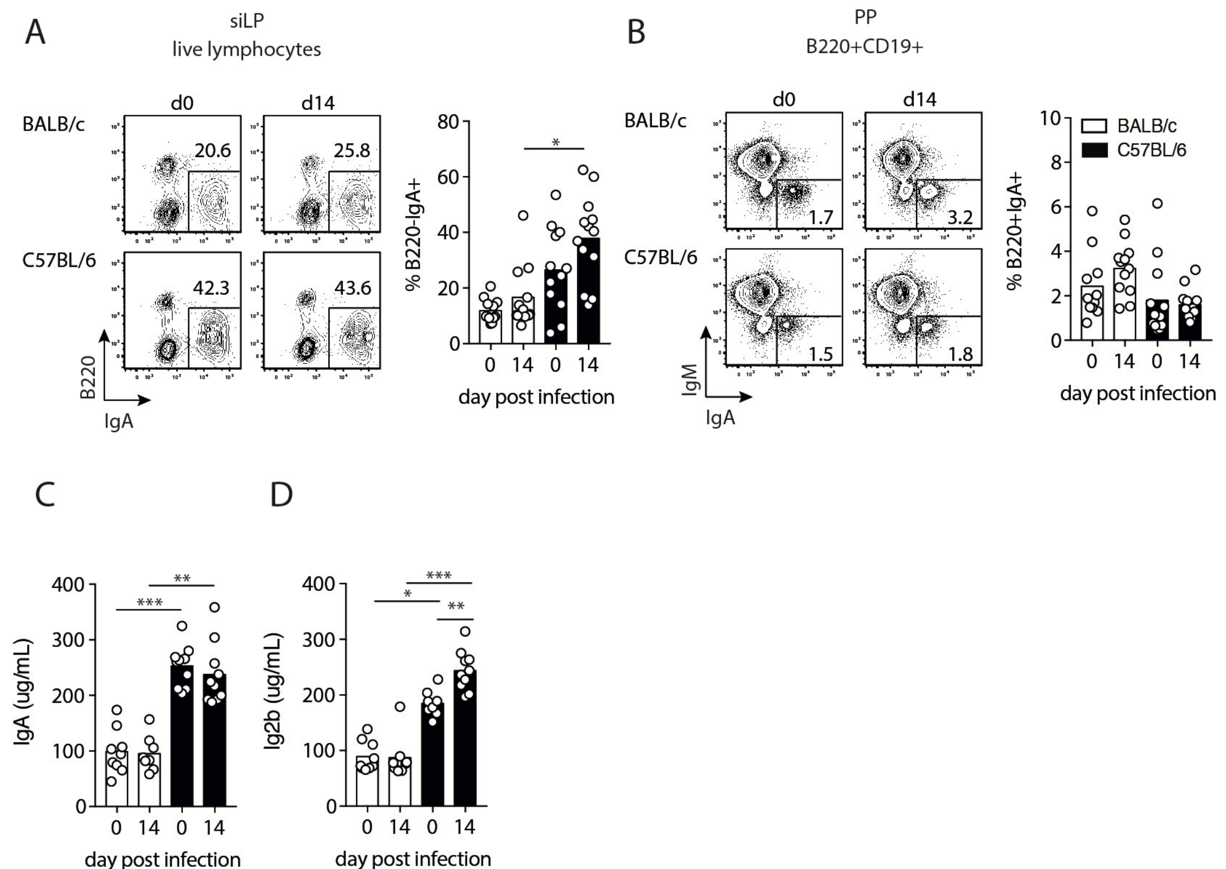

Figure 3.1.8 C57BL/6 mice display elevated intestinal IgA^{+} plasma cells and systemic IgG1 levels in response to *G. muris* infection. **(A)** Representative plots of IgA and B220 expression by small intestinal cells. Frequencies of $IgA^{+}B220^{-}$ plasma cells are reported in bar graph. **(B)** Representative plots of IgA and IgM expression by $B220^{+}CD19^{+}$ B cells and frequencies of $B220^{+}IgA^{+}$ B cells in Peyer's patches. **(C, D)** Serum levels of **(C)** IgA and **(D)** IgG2b. Data are pooled from 3 independent experiments with n=3-4/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.2 *G. muris* infection in eosinophil-deficient dblGATA-1 and wild-type BALB/c mice

4.2.1 Accumulation and activation of eosinophils during *G. muris* infection

Under homeostasis, eosinophils are known to provide support for T cell-independent IgA class switching in PP, as well as for IgA-producing plasma cells in bone marrow and siLP (Chu et al., 2011, 2014; Jung et al., 2015). Eosinophils do so in part by providing various B cell survival factors such as APRIL and BAFF, however they are capable of also producing pro-infammatory cytokines like IL-1β and IL-6 (Chu et al., 2014; Jung et al., 2015). Considering the central role of both IL-1β and IL-6 in the induction and maintenance of intestinal Th17 cells, eosinophils therefore potentially also exert an influence on Th17 responses. However, eosinophils have also been demonstrated secrete an IL-1R antagonist blocking IL-1β mediated signalling to Th17 cells and hence to rather constrict Th17 responses (Sugawara et al., 2015). Considering this contrasting evidence of the abilities of eosinophils to regulate intestinal IgA and Th17 responses and the importance of both to host protective immunity to

Giardia infection, the effect of eosinophil deficiency on *G. muris* infection and the consequent IgA and Th17 responses were therefore assessed in eosinophil-deficient dblGATA-1 mice and in wild-type BALB/c mice infected with *G. muris.*

To assess the progression of infection in the presence and absence of eosinophils, cyst shedding was monitored in BALB/c and dblGATA-1 mice over a 6-week period. Results indicate similar shedding rates during the first 2 weeks of infection, however at weeks 3, 5 and 6 dblGATA-1 mice shed significantly more cysts per gram faeces than wild-type control mice, indicating poorer control of infection (Fig. 3.2.1A).

Flow cytometric analysis of siLP cell isolates confirmed the absence of $SSC^{hi}CD45^{+}GR\text{-}1^{lo}$ $CD11b^{+}Siglec\text{-}F^{+}$ eosinophils in the gut of dblGATA-1 mice lacking these cells due to a double deletion in the high affinity GATA-1 binding site within the GATA-1 promoter confirmed that $SSC^{hi}CD45^{+}GR\text{-}1^{lo}CD11b^{+}Siglec\text{-}F^{+}$ are indeed eosinophils, due to their absence in dblGATA-1 mice (Yu et al., 2002; Fig. 3.2.1B). This gating strategy was therefore applied to quantify the frequencies of eosinophils in PP, mLN and siLP of naive and wild-type BALB/c mice 18 and 40 days post-infection with *G. muris*. Eosinophils were present at low frequencies in PP and mLN and remained unchanged during infection (Fig. 3.2.1C). In contrast, BALB/c mice displayed elevated eosinophil frequencies in siLP following *G. muris* infection, reaching significance by 40 days post-infection, the difference in eosinophil frequencies between infected mice and naive controls reached significance (Fig. 3.2.1C).

The eotaxin receptor CCR3 (CD193) is constitutively expressed on mature eosinophils and is known to bind to a variety of chemokines, including CCL7, CCL11, CCL13, CCL26 and RANTES (CCL5). Previous studies demonstrated that CCR3 gene knock-out impairs eosinophil recruitment in experimental models of asthma (Fulkerson et al., 2006). To further assess eosinophil recruitment to the small intestine of wild-type BALB/c mice during *G. muris* infection, the surface expression of CCR3 on eosinophils in PP, mLN and siLP was analysed via flow cytometry. *G. muris* infection led to a significant upregulation of CCR3 expression on eosinophils 18 days post-infection in PP, mLN and siLP (Fig. 3.2.1D). In contrast, 40 days post-infection CCR3 expression was comparable to baseline levels observed in naive control mice. Collectively, these data indicate that acute *G. muris* infection leads to significantly unregulated expression of CCR3 on eosinophils, a marker typically associated with eosinophil recruitment and chemokine-mediated degranulation (Fujisawa et al., 1999).

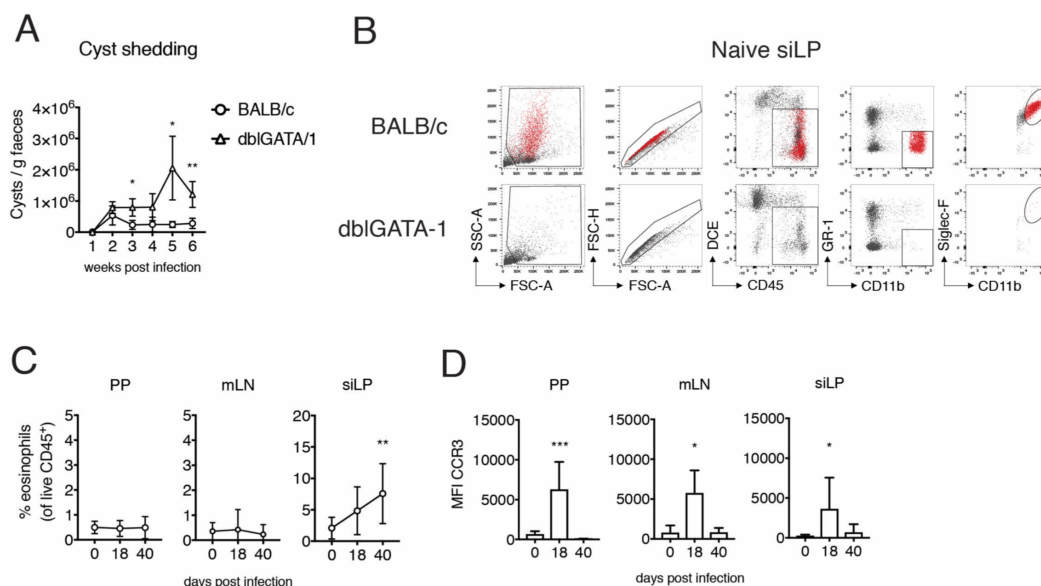

Figure 3.2.1 *G. muris* infection leads to eosinophil recruitment in siLP. (A) Cyst shedding dynamics of wild-type BALB/c and eosinophil-deficient dblGATA-1 mice over the course of 6 weeks of infection with *G. muris* **(B)** Exemplary FACS plots of $SSC^{hi}CD45^{+}GR\text{-}1^{lo}CD11b^{+}Siglec\text{-}F^{+}$ small intestinal eosinophils, present in wild-type BALB/c mice and absent in dblGATA-1 mice. **(C)** Frequencies of eosinophils in PP, mLN and siLP of naive and *G. muris*-infected BALB/c mice. **(D)** Mean fluorescence

intensity (MFI) of the eotaxin receptor CCR3 expressed on eosinophils in PP, mLN and siLP in BALB/c mice. Data are pooled from 3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4.2.2 Germinal centre antibody class switching

Peyer's patches located along the small intestine contain B cell lymphoid follicles and germinal centres, making them important sites of continuous humoral immune activation under homeostasis and during infection. PP are a key site of IgA antibody class switching and therefore are an important source of IgA-secreting plasmablasts (Mowat and Agace, 2014). Importantly, previous work has demonstrated that small intestinal eosinophils play an important role in the maintenance of IgA antibody class switching in PP under steady state and during infection with the small intestinal nematode *H. polygyrus* (Chu et al., 2014; Strandmark et al., 2017).

At steady state, dblGATA-1 mice displayed significantly lower frequencies of B220^{+}PNA^{hi} GC B cells in PP (Fig. 3.2.2A,B) and lower frequencies of IgA^{+} GC B cells, paralleled by significantly more IgG1^{+} cells in steady state (Fig 3.2.2C). Acute *G. muris* infection transiently restored the frequencies of GC B cells as well as IgA^{+} cells within the GC population in PP, reaching values similar to those in BALB/c mice at 18 and 40 days post-infection (Fig. 3.2.2C). Of note, infection did not lead to increased IgA class switching in wild type mice, confirming the stable frequencies of IgA^{+} B cells in PP of BALB/c mice upon *G. muris* infection. Previous studies indicated that increased IgG1 class switching at the expense of IgA production in eosinophil-deficient mice at steady state and upon intestinal nematode infection is restricted to PP (Chu et al., 2014; Strandmark et al., 2017). This was confirmed in the current work, showing that wild type and dblGATA-1 mice displayed similar GC B cell frequencies (Fig. X), a minor and transient increase in IgA CS and stable frequencies of IgG1^{+} as well as IgM^{+} B cells in the gut draining mLN (Fig. 3.2.2D). Specifically, in mLN, eosinophil deficiency did not appear to affect GC B cell frequencies, which remained stable and comparable between BALB/c and dblGATA-1 mice during infection (Fig. 3.2.2B). BALB/c mice did display a significant increase in IgA^{+} GC B cell frequencies 18 days post-infection, indicative of IgA class switching in response to infection and similar results were observed in dblGATA-1 mice (Fig. 3.2.2D). In contrast, IgG1^{+} and IgM^{+} GC B cell frequencies in mLN were similar between both mouse strains and remained unchanged during infection.

Overall, *G. muris* infection appears to lead to localised differences in IgA class switching, with BALB/c mice displaying elevated IgA class switching in mLN, but not in PP, while dblGATA-1 mice displayed significant IgA class switching in both PP and mLN in response to infection in the absence of eosinophils. Furthermore, both mouse strains displayed comparable elevated IgM class switching in PP in response to infection.

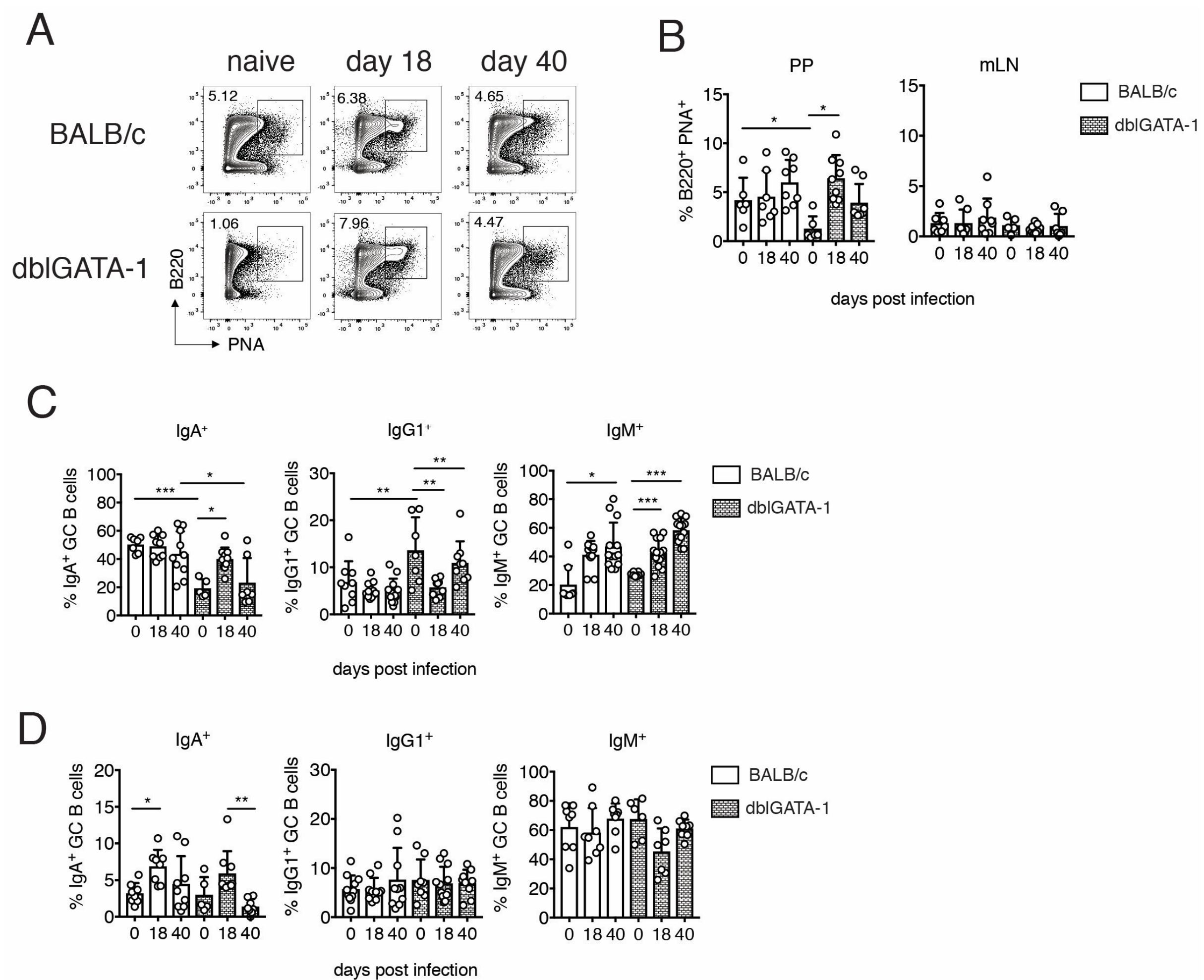

Figure 3.2.2 Eosinophil deficiency does not affect antibody class switching in PP and mLN during *G. muris* infection. (A) Exemplary FACS plots of live lymphocytes in PP. **(B)** Frequencies of $B220^+PNA^+$ germinal centre (GC) B cells in PP and mLN. Frequencies of IgA^+, $IgG1^+$ and IgM^+ GC B cells in **(C)** PP and **(D)** mLN of naive and infected BALB/c and dblGATA-1 mice. Data are pooled from 2-3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05, **p≤0.01, ***p≤0.001.

4.2.3 Antibody production in siLP

Under homeostasis, the siLP harbours high numbers of plasma cells predominantly secreting IgA antibodies, while fewer plasma cells dedicate to IgG and IgM secretion (Mowat and Agace, 2014). Previous studies reported that impaired IgA CS in PP of dblGATA mice as well as several other eosinophil-deficient mouse lines results in the reduced accumulation of small intestinal IgA^+ plasma cells (Chu et al., 2011, Jung et al., 2015). We hence assessed if eosinophil deficiency affects IgA-secreting plasma cells during *G. muris* infection. In contrast to previous findings however, under steady state both BALB/c and dblGATA-1 mice displayed similar frequencies of IgA^+B220^- cells in live lymphocytes isolated from siLP (Fig. 3.2.3A). In line with the transiently increased IgA class switching in PP and mLN of infected dblGATA-1 mice (Fig. 3.2.2), *G. muris* infection led to elevated frequencies of IgA^+ cells in siLP, which was also observed in infected wild type mice (Fig. 3.2.3A). Fewer $IgG1^+$ and IgM^+ cells were detected in the siLP , with no significant changes depending on background and infection status (Fig.3.2.3B,C). The data presented here therefore confirm the previously reported defect in steady state GC B cell production and IgA class switching in PP of eosinophil deficient mice. *G. muris* infection transiently increases GC B cell differentiation as well as IgA CS in PP in absence of eosinophils and supports the accumulation of IgA^+ B cells in the small intestine.

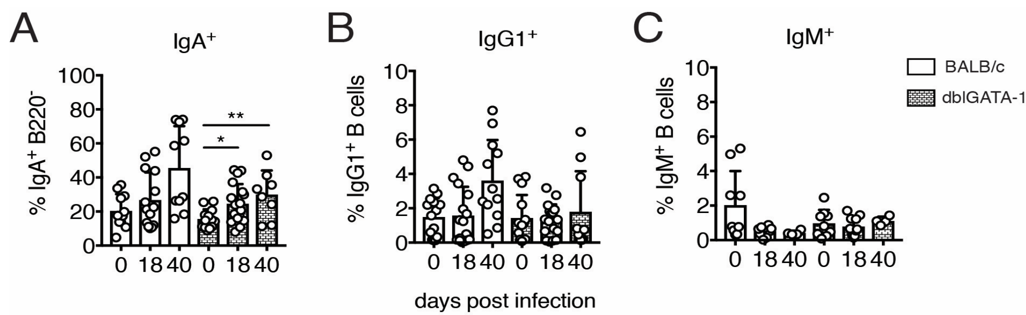

Figure 3.2.3 *G. muris* infection induces elevated frequencies of IgA-secreting B cells in siLP of BALB/c and dblGATA-1 mice. Frequencies of $B220^{-}$ cells in siLP of naive and infected BALB/c and dblGATA-1 mice secreting **(A)** IgA, **(B)** IgG1 and **(C)** IgM. Data are pooled from 2-3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05, **p≤0.01.

4.2.4 Intestinal Th17 responses

Next to the regulation of isotype class switching in PP, small intestinal eosinophils were shown to display contextual functions with relation to intestinal Th17 responses. Namely, they appear able to either support (Esnault et al., 2012) or restrict (Sugawara et al., 2016) Th17 cell responses via secreting IL-1β or an IL-1 receptor antagonist (IL-1Ra) under steady state, respectively.

To establish whether eosinophil deficiency affects the induction and maintenance of intestinal Th17 responses in the context of *G. muris* infection, $ROR\gamma t^{+}$ Th17 cells and IL-17A production in PP and siLP were quantified in both naive and infected BALB/c and eosinophil-deficient dblGATA-1 mice. In PP, the frequencies of $ROR\gamma t^{+}$ Th17 cells were comparable between the two mouse strains under homeostasis and remained stable during the acute and chronic stage of *G. muris* infection (Fig. 3.2.4A,B). At day 40 post infection, dblGATA-1 mice displayed impaired IL-17A production comparable to that observed in wild-type mice (Fig. 3.2.4A,B).

In siLP, *G. muris* infection led to a mild increase in Th17 cells in BALB/c mice, while dblGATA-1 mice displayed a transient elevation in Th17 cells during infection (Fig. 3.2.4C,D) and significantly lower Th17 cell frequencies at the chronic stage of infection. This was, in trend, reflected by small intestinal IL-17A responses in both strains with poor IL-17A production in chronically infected dblGATA-1 mice (Fig. 3.2.4C,D). Hence, Th17 activity seemed to be impaired in eosinophil-deficient mice chronically infected with *G. muris.* Overall, analysis of the intestinal Th17 responses indicated that even though eosinophil deficiency does not appear to significantly affect the frequencies of intestinal Th17 cells, dblGATA-1 mice are unable to mount an appropriate IL-17A response to *G. muris* infection.

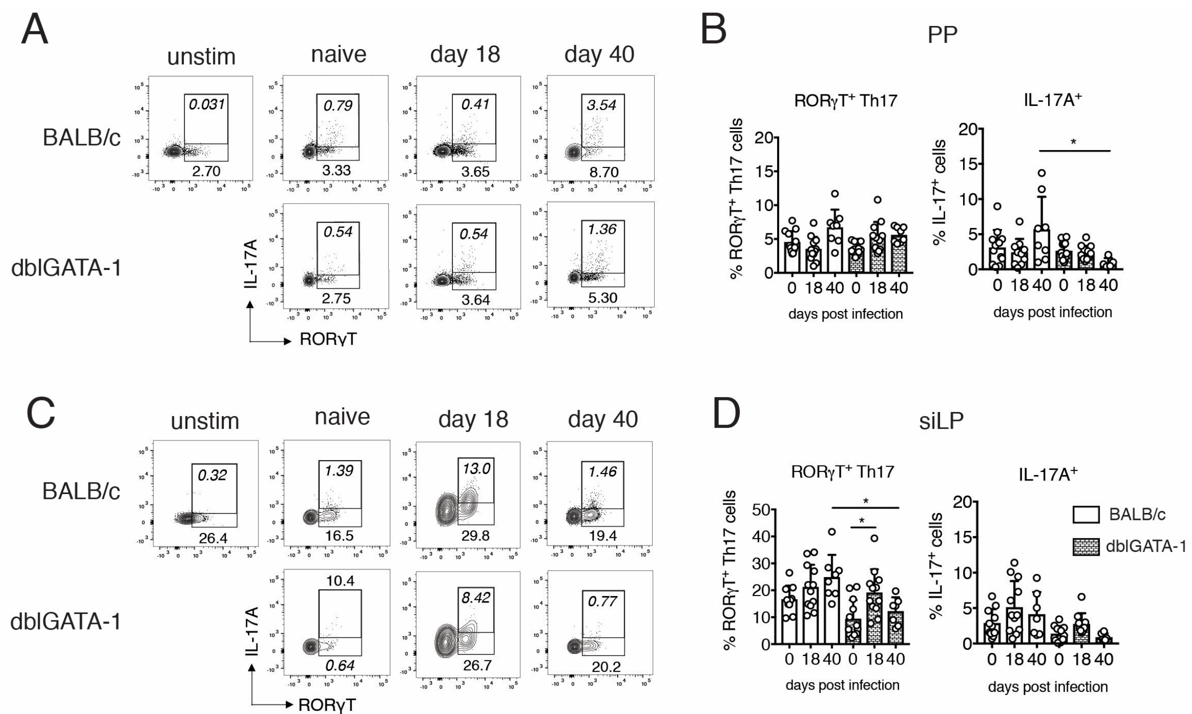

Figure 3.2.4. Infected dblGATA-1 mice display transient increase in intestinal Th17 cells, but poor IL-17A production in PP and siLP. (A) Exemplary FACS plots and **(B)** Frequencies of RORγt$^+$ Th17 cells and IL-17A$^+$ T cells in PP. **(C)** Exemplary FACS plots and **(D)** Frequencies of RORγt$^+$ Th17 cells and IL-17A$^+$ T cells in siLP. Regular numbers report the frequencies of RORγt$^+$ Th17 cells, italicized numbers refer to IL-17A$^+$ cells. Data are pooled from 2-3 independent experiments with n=3-5/group. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05.

4.3 *G. muris* infection in littermate control mice

To verify that the observed immunophenotypical differences between wild-type and dblGATA-1 mice resulted from eosinophil-deficiency and not from other confounding factors such as possible differences in the intestinal microbiota composition, the control of *G. muris* infections and associated immune responses were surveyed in wild-type and eosinophil-deficient littermate control mice. As IgA CS in PP as well as Th17 activity in PP and siLP appeared impaired in dblGATA-1 mice at the chronic stage of infection, this late time point of infection was chosen for further investigations.

4.3.1 Breeding of BALB/c x dblGATA-1 littermate control mice

The expression of the transcriptional factor GATA-1 is important for the development of eosinophils. A double palindromic deletion within the GATA-1 site leads to the generation of a viable, fertile mouse strain (dblGATA-1) displaying otherwise normal frequencies and functionalities of other immune cell types, but lacking eosinophils (Yu et al., 2002). Being an X-linked gene, eosinophil deficiency in this transgenic mouse line occurs in homozygous females and hemizygous males, with heterozygous females displaying a wild-type phenotype and hence the presence of eosinophils (Yu et al., 2002). To establish the breeding of littermate control mice, female dblGATA-1 and male wild-type BALB/c mice were bred together. The F1 progeny of these breeding pairs yielded expected ratios of the different genotypes, where all females displayed a wild-type phenotype due to being heterozygous, while all males were hemizygous for the deletion and hence lacked eosinophils (Fig. 3.3.1A). To obtain an even sex ratio of the two phenotypes, F1 females and males were further bred to yield an F2 generation, where 50% of females and 50% of males presented with an eosinophil-deficient phenotype. Both the F1 and F2 generation appeared healthy and demonstrated no

abnormalities. Their phenotype was confirmed via analysis of the presence of eosinophils in peripheral blood (Fig. 3.3.1B).

4.3.2 Cyst shedding dynamics in *G. muris*-infected littermates

Evaluation of cyst shedding in faeces of littermate control mice infected with *G. muris* over a 6-week infection period revealed that in contrast to BALB/c and dblGATA-1 mice, the wild-type and eosinophil-deficient littermate mice were shedding very similar numbers of cysts, which remained stable and low from week 1 until week 6 (Fig. 3.3.1C). The cyst shedding rates in the littermate control mice were similar to those observed for wild-type BALB/c mice after week 3 post-infection (Fig. 3.1.1A).

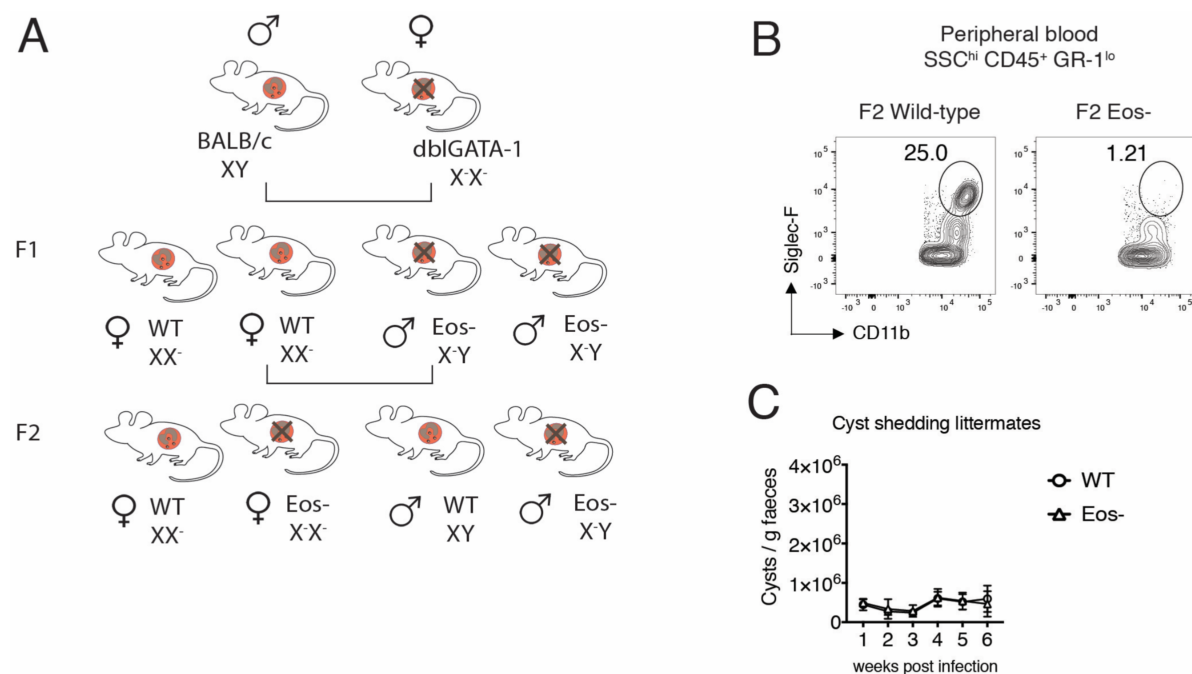

Figure 3.3.1 BALB/c x dblGATA-1 littermate control breeding. (A) Genetic breeding scheme for the generation of wild-type and eosinophil-deficient littermate control mice. **(B)** Exemplary FACS plots of an F2 generation wild-type and an eosinophil-deficient mouse showing the presence and absence, respectively, of $SSC^{hi}CD45^{+}GR\text{-}1^{lo}CD11b^{+}Siglec\text{-}F^{+}$ peripheral blood eosinophils. **(C)** Cyst shedding dynamics of wild-type and eosinophil-deficient littermate control mice over the course of a 6 week infection with *G. muris*.

4.3.3 Antibody class switching in PP, mLN and antibody production in siLP

Assessment of $B220^{+}PNA^{+}$ GC B cells in littermate control mice revealed mild elevations of GC B cell frequencies in response to infection in PP in both host phenotypes, while GC B cell frequencies in mLN remained low and unchanged in infected mice compared with naive controls (Fig. 3.3.2A,B). In PP, infected wild-type and eosinophil-deficient littermates displayed elevated frequencies of IgA^{+} GC B cells, however this response to infection was more pronounced in eosinophil-deficient mice (Fig. 3.3.2C). Frequencies of IgG1-switched GC B cells remained low and unaltered in infected mice, however *G. muris* infection led to elevated IgM class switching in PP similarly to that observed in BALB/c and dblGATA-1 mice (Fig. 3.3.2C). In contrast, analysis of antibody class switching in mLN did not reveal clear differences in IgA, IgG1 or IgM class switching in response to infection in neither wild-type, nor in eosinophil-deficient mice (Fig. 3.3.2D). Analysis of the frequencies of antibody-secreting plasma cells in siLP of wild-type and eosinophil-deficient littermate mice revealed no evidence

of significant elevation in IgA, IgG1 or IgM-secreting cells in response to infection (Fig. 3.3.2E). Overall, these preliminary data suggest that eosinophil deficiency does not affect the host's capacity for antibody class switching in secondary lymphoid organs in response to *G. muris* infection as *G. muris*-infected mice display similar levels of IgA and IgM class switching in PP. Moreover, eosinophil deficiency does not affect the frequencies of IgA^+ plasma cells in siLP under steady state and during *G. muris* infection, even though littermate mice displayed overall lower frequencies of IgA^+ PC in siLP compared with BALB/c or dblGATA-1 mice. This effect was independent of eosinophil deficiency, but nevertheless would merit further investigations. Furthermore, eosinophil-deficient littermates did not display signs of elevated IgG1 class switching in PP, another phenomenon for future investigations to focus on.

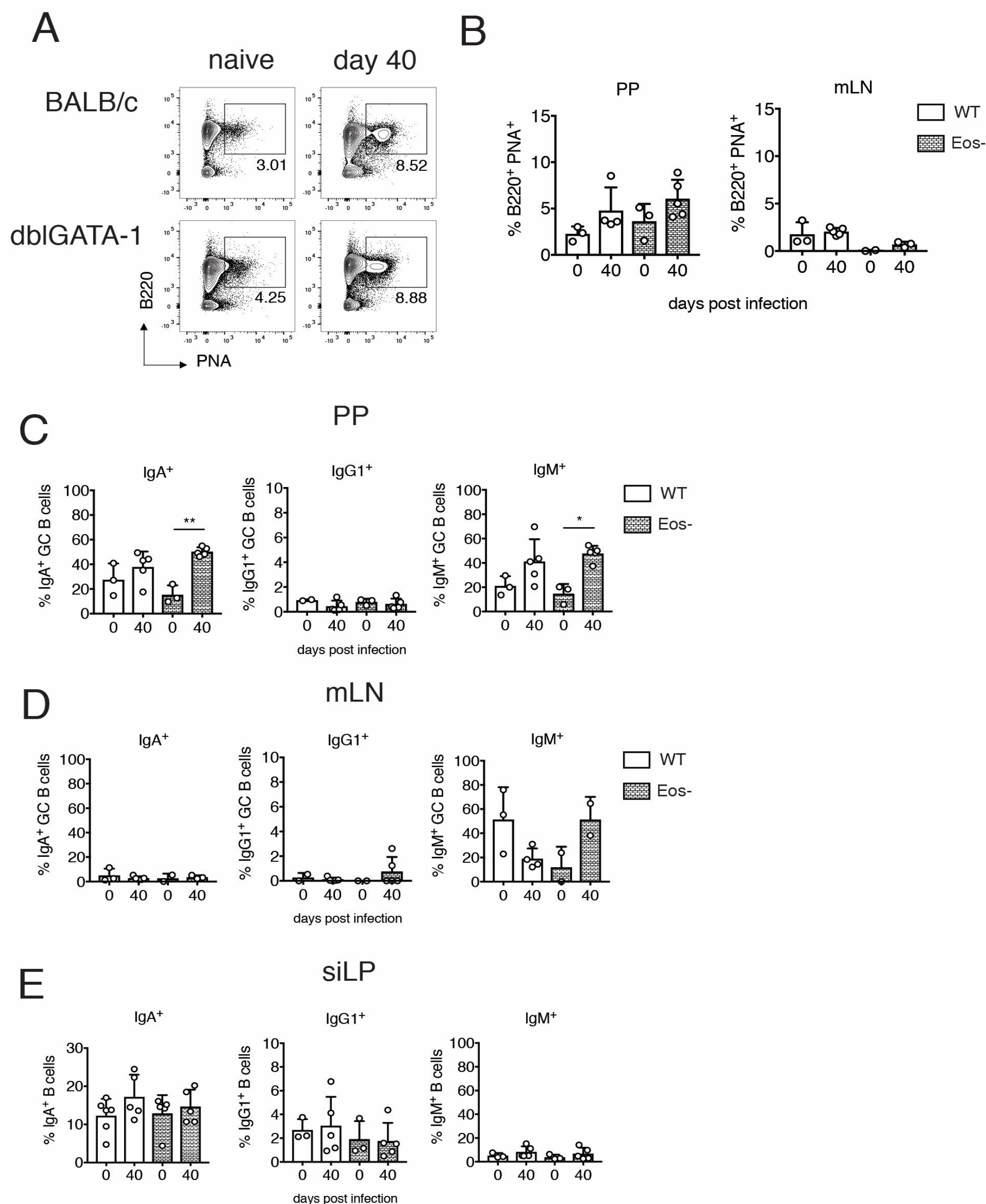

Figure 3.3.2 Antibody responses in littermate control mice infected with *G. muris.* (A) Exemplary FACS plots of live lymphocytes in PP. **(B)** Frequencies of $B220^+PNA^+$ GC B cells in PP and mLN of naïve and infected littermate mice. Frequencies of IgA^+, $IgG1^+$ and IgM^+ GC B cells in **(C)** PP and **(D)** mLN. **(E)** Frequencies of $B220^-IgA^+$, $IgG1^+$ and IgM^+ cells in siLP. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05, **p≤0.01.

4.3.4 Intestinal Th17 responses

In PP, RORγt^{+} Th17 frequencies were comparable between wild-type and eosinophil-deficient littermate mice, and remained unchanged during *G. muris* infection. This was further reflected in the stable and comparable levels of IL-17A production in PP of wild-type and eosinophil-deficient mice (Fig. 3.3.3A). In contrast, in siLP *G. muris* infection induced a significant increase in RORγt^{+} Th17 cell frequencies independent of eosinophil deficiency in the littermate control mice. Eosinophil-deficient littermates displayed notably lower frequencies of IL-17A^{+} T cells under steady state when compared with wild-type mice, however doing infection these frequencies increased to levels similar to those observed for wild-type littermates (Fig. 3.3.3B). This therefore indicated that eosinophil deficiency potentially affects intestinal IL-17A production under homeostasis, but does not impede IL-17A production during murine giardiasis. The differences in Th17 activity seen between chronically-infected BALB/c and dblGATA-1 mice and the littermate mice potentially relates to differences in their microbiota composition and would need further detailed assessment.

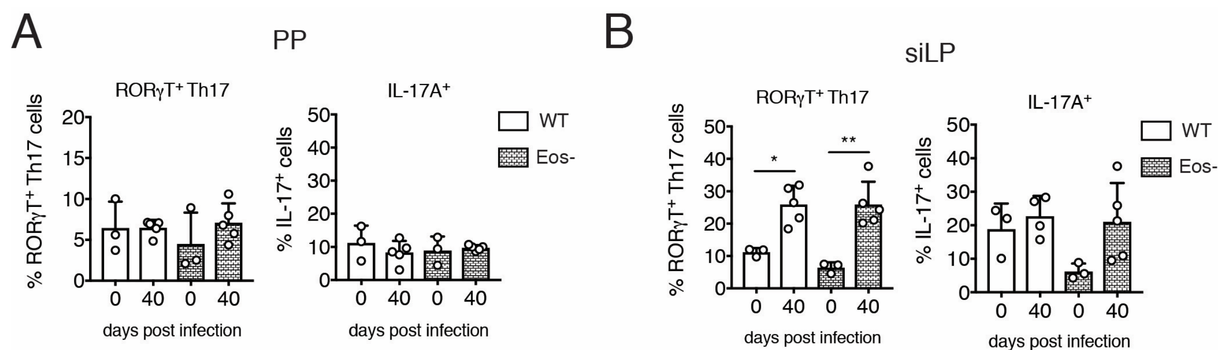

Figure 3.3.3. Intestinal Th17 responses in littermate control mice during *G. muris* infection. Frequencies of RORγt^{+}Foxp3^{-}CD4^{+} Th17 cells and IL-17A-producing cells in **(A)** PP and **(B)** siLP of naïve and infected wild-type and eosinophil-deficient littermate mice. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05, **p≤0.01.

4.4 Eosinophil activation in response to *G. lamblia*

4.4.1 Bone marrow-derived eosinophil stimulation with *G. lamblia* trophozoite antigens

Eosinophil responses during inflammation or infection classically involve the release of their granule contents in their immediate environment, a process termed degranulation. To date, three main mechanisms of degranulation have been recognised, namely cytolysis, compound exocytosis and piecemeal degranulation (PMD) (Mahmudi-Azer et al., 2002). CD63, a tetraspanin protein also known as lysosome-associated membrane protein 3, is constitutively expressed on the membranes of eosinophil secretory granules and hence normally localises in the cytoplasm of the cell. However, membrane fusion events of granules with the outer cell membrane during eosinophil degranulation lead to the translocation of CD63 to the cell surface membrane, making CD63 a commonly used marker of eosinophil degranulation (Arnold et al., 2018; Mahmudi-Azer et al., 2002; Verjan Garcia et al., 2011).

To assess whether murine eosinophils degranulate in response to the human parasite species *G. lamblia*, bone marrow-derived eosinophils (bmEos) were stimulated with varying concentrations of *G. lamblia* WB6 (assemblage A) and GS (assemblage B) trophozoite antigens (Fig. 3.4.1). Phorbol-12-myristate-13-acetate (PMA) and ionomycin stimulation was used as a positive control. GM-CSF, IL-5 and IL-33 were chosen as additional stimuli reported to induce eosinophil activation and degranulation. The cell surface expression of CD63 was quantified via flow cytometry. Preliminary results indicate that PMA/Ionomycin and IL-33

induced the highest expression levels of CD63 on bmEos, while IL-5 and GM-CSF did not induce significant levels of CD63 surface expression (Fig. 3.4.1C). Stimulation with both *G. lamblia* WB6 and GS antigens led to unregulated CD63 expression on bmEos, however only reaching statistical significance for the lowest antigen concentrations (Fig. 3.4.1C).

Together, this data indicates that murine eosinophils display partial levels of degranulation in response to *G. lamblia* antigens *in vitro*. Considering their observed accumulation in the siLP of *G. muris* infected mice shown in this study and their competence in storing and releasing a range of cytokines, chemokines and cytotoxic proteins, it would be therefore important to further elucidate the downstream immunostimulatory or regulatory effects of eosinophils and potential cytotoxicity effects on trophozoites as a result of eosinophil degranulation in the context of giardiasis.

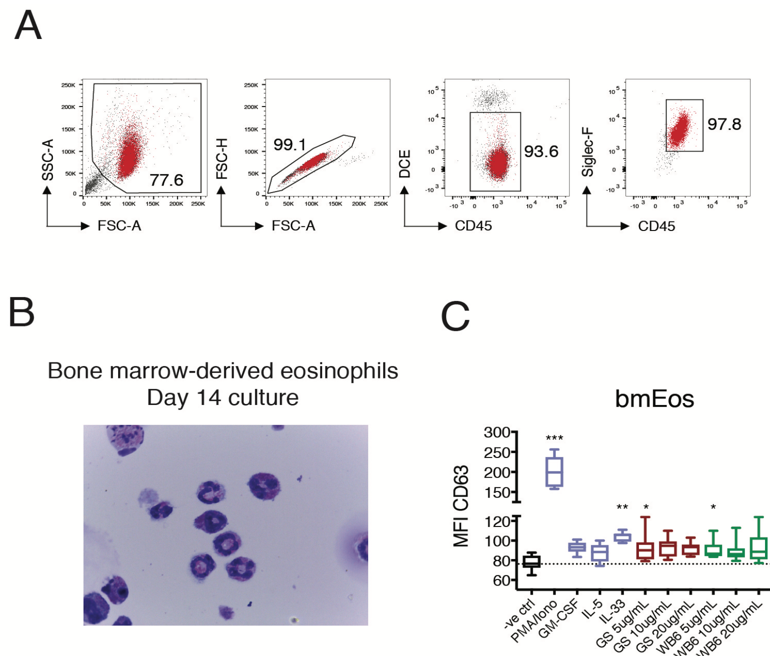

Figure 3.4.1 Bone marrow-derived eosinophil cell culture. (A) FACS analysis of a bone marrow-derived eosinophils (bmEos) on day 14 of *in vitro* culture growth with a purity of 97.8% $SSC^{hi}CD45^{+}Siglec\text{-}F^{+}$ cells. **(B)** Cytospin of bone marrow-derived eosinophils on day 14 of *in vitro* cell culture growth. **(C)** Mean fluorescence intensity (MFI) of CD63 on bmEos following in vitro stimulation with cytokines and *G. lamblia* trophozoite antigen. Data are pooled from 2 independent experiments. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05, **p≤0.01, ***p≤0.001.

4.4.2 Eosinophil activation in *G. lamblia*-infected mice

Under homeostasis, eosinophils represent between 10 and 20% of all leukocytes in the lamina propria of the gastrointestinal tract of mice and are also found at low numbers in PP and mLN (Strandmark et al., 2016). In order to further validate whether murine eosinophils indeed display signs of degranulation in response to *G. lamblia*, the surface expression of CD63 was quantified *ex vivo* in eosinophils from small intestinal PP, mLN and spleen of *G. lamblia* GS-infected mice 9 days post-infection. Preliminary data in this study shows that compared with naive controls, *G. lamblia*-infected mice display significantly elevated CD63 expression on eosinophils locally in PP and mLN, but not systemically in the spleen, indicating that *G. lamblia* infection leads to degranulation events in eosinophils in gut-associated secondary lymphoid organs (Fig. 3.4.2A). Considering the signature granular morphology of eosinophils, degranulation events typically lead to a drop in their side scatter values (SSC) as measured via FACS analysis. Therefore, we further analysed the SSC of eosinophils in PP, mLN and spleen of naive and *G. lamblia*-infected mice. In PP, side scatter values of eosinophils were

significantly lower in infected mice compared with naive controls, paralleling the elevation in CD63 expression observed in these mice (Fig. 3.4.2B). Eosinophils in mLN did not display a similar change in side scatter, whereas eosinophil side scatter values in the spleen were also significantly lower in infected mice compared with naive controls. Therefore, these data complement the preliminary observations of signs of eosinophil degranulation *in vitro* in response to G. lamblia antigens by demonstrating significantly unregulated CD63 expression and lower eosinophil granularity as measured by SSC values in *G. lamblia* infected mice. Together, the *in vitro* and *in vivo* data therefore suggest that murine eosinophils potentially degranulate in response to *Giardia* parasites and opens exciting new avenues for research into the biological significance of eosinophil degranulation during giardiasis.

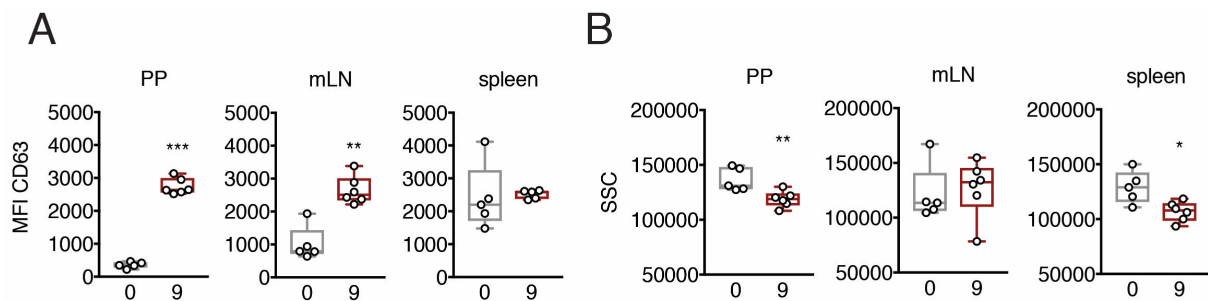

Figure 3.4.2 Murine eosinophils degranulate in response to *G. lamblia* infection. (A) Mean fluorescence intensity (MFI) of CD63 on eosinophils in PP, mLN and spleen of *G. lamblia* GS-infected mice 9 days post-infection. **(B)** Side scatter values of eosinophils in PP, mLN and spleen. Statistical analysis was done using Kruskal-Wallis test combined with Dunn's multiple comparison testing. Results are displayed as mean ± SD and significance is displayed as *p≤0.05, **p≤0.01, ***p≤0.001.

5. CONCLUSIONS AND PERSPECTIVES

5.1 Conclusions

The data presented as part of this thesis complements the current understanding of host protective immune mechanisms during infection with the gastrointestinal protozoan parasite *Giardia muris* in inbred mice in two ways. Firstly, work reported here demonstrates that hosts shedding higher numbers of cysts and hence displaying poorer control of infection harbour constitutive higher frequencies of RORγt$^+$ Tregs and importantly, these correlated negatively with Th17 cell proliferation and activity, suggesting that the presence of high numbers of RORγt$^+$ Tregs potentially influences Th17 responses to the parasite. Importantly however, the data presented here are restricted to showing correlation analysis of RORγt$^+$ Tregs, efficiency of Th17 responses and host control of infection and further in-depth functional studies would be necessary to establish with certainty the role played by RORγt$^+$ Tregs in modulating host protection against *Giardia*.

Furthermore, it demonstrates, perhaps in contradiction to the current understanding of eosinophil immunomodulation, that eosinophil deficiency does not constrain dblGATA-1 or littermate control mice lacking eosinophils from mounting adequate IgA and Th17 responses during *G. muris* infection, indicating that eosinophils are potentially redundant with respect to host protective immunity during murine giardiasis. Nevertheless, novel findings in this work indicate that eosinophils experience increased recruitment, activation and indications of degranulation in response to both *G. muris* and the human species *G. lamblia* and hence highlight exciting new insights into the versatility of small intestinal eosinophils.

5.2 Perspectives

The findings presented here stimulate a number of further open research questions to address with future work on host determinants of immunity to *Giardia*. Importantly, among the most pressing questions to address is establishing what effector mechanisms of RORγt$^+$ Tregs potentially interfere with Th17 cell activity during *G. muris* infection and whether RORγt$^+$ Tregs exhibit Th17-specific suppressive capacities as previously reported by others. Secondly, further work confirming the redundancy of eosinophils using littermate control mice observed here would be required to show in greater details whether eosinophils indeed provide little support for IgA class switching and IgA production in the gut, as well as for Th17 responses during giardiasis.

5.2.1 Treg/Th17 balance during giardiasis

Considering the high prevalence of asymptomatic infections with giardiasis in humans, it would be imperative to perform both further experimental analysis of the role of Tregs in constraining pro-inflammatory Th17 responses to infection in animal models with *G. lamblia* as well as *G. muris*. Considering their crucial role in the maintenance of tolerance and suppression of overt inflammation, Tregs display a range of suppressive mechanisms at the molecular and cellular level. These include the generation of immunosuppressive adenosine, secretion of anti-inflammatory cytokines like IL-10 and TGF-β, induction of cell death via secretion of granzyme B and direct cell-to-cell contact inhibiting TCR-induced proliferation of effector T cells or TCR signaling by antigen-presenting cells (Schmidt et al., 2012). It would therefore be important to assess cytokine production *in vivo* and *in vitro*, cell-to-cell contact via intravital microscopy, as well as similarities and differences in the TCR repertoires of RORγt$^+$ Tregs and Th17 cells from Giardia-infected hosts. Furthermore, the relevance of these findings should be oriented to its potential clinical relevance and to establish whether higher Treg subset frequencies in humans infected with G. lamblia correlate with their capacity for mounting adequate Th17 responses, with their symptomatology and cyst shedding.

5.2.2 Eosinophil recruitment, activation and degranulation in response to *Giardia*

Eosinophils are increasingly being recognised as immune mediators in host protective immunity in the context of parasite infections. Eosinophils have been demonstrated to lead to killing of *Nippostrongylus brasiliensis* infection larvae *in vitro,* while *in vivo* they associate with reduced parasite burdens in the long and impaired fitness of parasites in the intestine (Knott et al., 2007; Dent et al., 1999; Knott et al., 2009). Similar results displaying limited roles of eosinophils have also been demonstrated for infections with *Strongyloides* species (O'Connell et al., 2011; Ovington et al., 1998; Watanabe et al., 2003). In contrast, eosinophils have also been shown to promote muscle-stage larval survival of *Trichinella spiralis* (Huang et al., 2014). Furthermore, several lines of evidence over the years have associated eosinophils with protozoan parasite infections. Infections with the intracellular parasite *Leishmania amazonensis* leads to eosinophil infiltration in primary lesions and draining lymph nodes (de Oliveira et al, 2010) Some of the data presented here indicates that small intestinal eosinophils are recruited and acquire higher activation, potentially degranulating in response to both *G. muris* and *G. lamblia*. Considering the lack of understanding of the potential cytotoxic effects of eosinophils on *Giardia* trophozoites or cysts, further work focusing on eosinophil degranulation and direct effects during giardiasis merits further investigations and would potentially reveal novel functions of this versatile granulocyte.

6. DISCUSSION

The findings presented in this work shed new light on previously unexplored immunological pathways during infections with the gastrointestinal protozoan parasite Giardia muris. Specifically, this study demonstrates that higher cyst shedding rates in BALB/c mice correlate with constitutively higher frequencies of RORγt-expressing Foxp3$^+$ Tregs, low Th17 cell proliferation and metabolic activity and insufficient IL-17A production in response to G. muris infection. In contrast, C57BL/6 mice shedding fewer cysts display high Th17 cell activity, higher constitutive production of IL-17A and stable but low frequencies of RORγt$^+$ Tregs. RORγt$^+$ Treg cell have previously been associated with potent suppressive capacities in the context of intestinal inflammation and infections with the enteric bacterial pathogen *Helicobacter hepaticus* (Yang et al., 2016; Xu et al., 2018). However, despite their known ability to specifically suppress intestinal Th17 cells, the potential effect on Th17 immunity during murine giardiasis has remained unexplored to date. This study therefore contributes to the field by demonstrating that hosts on different genetic backgrounds, which display differences in control of infection with *G. muris* harbour differing frequencies of RORγt$^+$ Tregs and furthermore, that acute *G. muris* infection is associated with shifts in RORγt$^+$Treg:Th17 ratios in more susceptible hosts (BALB/c) but not in hosts displaying better control of infection (C57BL/6).

Small intestinal eosinophil responses and their potential involvement in host immunity in the context of experimental giardiasis have remained unexplored. Therefore, this study has explored for the first time the role of eosinophil-mediated support for intestinal IgA and Th17 responses to *G. muris* infection. The results indicate that eosinophil-deficient mice display unaltered IgA class switching in PP and IgA production in siLP, as well as elevated Th17 cell frequencies and IL-17A production in response to *G. muris* infection, suggestive of a redundancy of eosinophils during *G. muris* infection. Neverthelss, the findings presented in this work on eosinophil recruitment to the small intestinal lamina propria, as well as the evidence of eosinophil activation in response to *G. muris* and *G. lamblia in vivo* and to *G. lamblia* trophozoite antigens *in vitro* demonstrate an exciting and novel insight into eosinophils “behaviour” during murine giardiasis.

6.1 Treg/Th17 balance during *G. muris* infection

The first aim of this study has been to establish whether inbred mouse strains of different genetic backgrounds known to display differences in their control of *Giardia* infection also differ in their Th17 activity. In consequence, the second aim of the study was to establish whether *G. muris* infection leads to differences in Treg responses and hence to an imbalance in Treg:Th17 ratios and how that correlates with the host’s ability to control infection.

The regulatory T cell (Treg) pool comprises two distinct populations with defined functional differences. The majority of Foxp3$^+$ CD4$^+$ Treg originate in the thymus (tTreg) in response to TCR stimulation by self-antigens and hence play a key role in maintaining immunological tolerance to self-antigens and in preventing the development of overt autoimmune inflammatory events. In contrast, in mucosal tissues highly diverse in microbial, dietary and pathogenic antigens naive CD4$^+$ T cell precursors can convert to peripherally-induced Treg responsive to foreign antigen and hence take part in the control of host immunity in the context of infection and inflammation (Luu et al., 2017). Furthermore, pTreg cell display a certain degree of functional heterogeneity especially in the gastrointestinal environment, where various Treg subsets have been found to reside. In the context of Th1-driven inflammation, Treg can upregulate T-bet and CXCR3 expression, promoting Treg migration to sites of Th1-driven inflammation (Koch et al., 2009). Intestinal Tregs expressing the IL-33 receptor ST2 and the Th2 transcription factor GATA3 have also been demonstrated during intestinal inflammation in response to IL-33 signaling, leading to stabilization and increased recruitment of Tregs during intestinal inflammation (Schiering et al., 2014). Furthermore, Foxp3$^+$ Treg expressing the signature Th17 transcription factor RORgT have also been demonstrated to reside in the gastrointestinal environment and they represent a stable Treg lineage with superior suppressive capacities compared to conventional Treg (Yang et al., 2016). RORγt$^+$

Treg display a TCR repertoire indicative of peripherally-induced Treg and moreover, recent evidence suggests that they share some of their most common TCR variants with Th17 cells, indicating that their capacity to specifically target and restrict Th17 cells potentially relates to shared antigen specificity between RORγt^{+} Tregs and Th17 cells (Yang et al., 2016; Solomon and Hsieh, 2016; Kim et al., 2017; Neumann et al., 2019).

In the context of *Giardia* infections, conflicting evidence from cattle has demonstrated elevated expression of the Treg signature transcription factor Foxp3 in CD4^{+} T cells stimulated with *G. lamblia* trophozoite antigens, while co-culture of trophozoite antigen-loaded DC with CD4^{+} T cells rather leads to a down regulation of Foxp3 expression (Grit et al., 2014a, b). Nevertheless, these data must be interpreted with caution, as unregulated Foxp3 expression alone is not evidence of elevated Treg responses during giardiasis. Elevated secretion of the typical Treg-associated anti-inflammatory cytokine IL-10 has been demonstrated in spleen cells from *G. lamblia* infected mice challenged with E/S or soluble parasite extract (Jimenez et al., 2014). Nevertheless, more recent work has demonstrated that IL- 10 is not a critical cytokine in limiting intestinal T cell responses in *G. muris*-infected mice, but rather plays a role in the control of overt immune responses to the commensal microbiota (Dann et al., 2018).

6.1.1 Th17 cell responses during *G. muris* infection in BALB/c and C57BL/6 mice

Observations in this study confirm previous findings of differences in susceptibility to *G. muris* between BALB/c and C57BL/6 mice as evidenced by their cyst shedding patterns over the course of a 6-week infection period. Elevated cyst shedding in faeces has previously been correlated with higher trophozoite loads in the small intestinal lumen and hence cyst shedding represents an indirect, but reliable measure of intestinal parasite burden and hence host susceptibility to infection (Dreesen et al., 2014; Barash et al., 2017).

Under steady state, more robust Th17 differentiation and Th17-mediated IL-17A production have previously been reported for C57BL/6 compared to BALB/c mice, highlighting differences in Th17 propensity between the two mouse strains (Atarashi et al., 2015). The relatively poor generation of Th17 responses in response to members of the commensal gut microbiota was shown to result from minor IL-1β production by small intestinal dendritic cells, resulting in insufficient serum amyloid A (SAA) production by epithelial cells in BALB/c mice (Atarashi et al., 2015). Of note, SAA1/2 expression has been shown to be heavily upregulated in *G. muris* infected C57BL/6 mice (Dann et al., 2015) and microbiota-driven SAA1/2 responses by EC directly support IL-17A production by intestinal Th17 cells (Sano et al., 2015). Hence, insufficient IL-1β/SAA responses resulting in low numbers of intestinal Th17 cells and poor IL-17A production potentially influence the differences in control of *G. muris* and *G. lamblia* infections between BALB/c and C57BL/6 mice observed in this study, as well as by others (Belosevic et al., 1984; Li et al., 2004).

Earlier studies have reported the generation of a mixed Th1/2 response to *Giardia* infection (Djamiatun and Faubert, 1998; Jimenez et al., 2014). Nevertheless, assessment of Tbet^{+} Th1 and GATA3^{+} Th2 cell frequencies, as well as their signature cytokines revealed no elevated response of either Th1 or Th2 cells during *G. muris* infection. In contrast, more recent work has demonstrated that Th17 responses are pivotal for the control of murine *Giardia i*nfections (Dreesen et al., 2014; Dann et al., 2015). In line with these reports, in this work comparative analysis of the propensity of BALB/c and C57BL/6 mice infected with *G. muris* revealed that C57BL/6 mice constitutively harbour higher frequencies of small intestinal Th17 cells and produce more IL-17A, coinciding with their lower cyst shedding. BALB/c mice on the other hand display low RORγt^{+} Th17 cell frequencies and poor IL-17A production under steady state or during infection. A novel finding in this study has been that in C57BL/6 mice, but not BALB/c mice, *G. muris* infection leads to higher proliferation and metabolic activity of Th17 cells, evident in their elevated Ki-67 expression and uptake of Bodipy FLC16 fluorescent palmitate fatty acids in infected mice compared with naive controls. Typically, upon TCR engagement and signaling, T cell stimulation leads to elevated metabolic activity and proliferation of activated T cells (Sun et al., 2017). Considering the elevated proliferation and metabolic activity of Th17 cells observed in C57BL/6 mice it is therefore tempting to speculate that this

occurs in response to *G. muris* antigen presented to intestinal Th17 cells. However, attempts at detecting CD40L expression and hence antigen specificity of Th17 cells in response to *Giardia* trophozoite-loaded dendritc cells in vitro have so far been unsuccessful. Nevertheless, the presence of the parasite in the lumen could potentially trigger elevated activity of microbiota-specific Th17 cells, rather than parasite-specific ones. This is a possibility, as innate functions of Th17 cells rather depend on IL-1β and IL-23 signalling, rather than on pathogen-driven TCR activation (Guo et al., 2009).

A further novel finding of this study has been the upregulated expression of CD103 and hence intestinal retention of Th17 ells in response to infection with *G. muris* and the much more efficient Th17 cell retention in more resistant C57BL/6 mice than in BALB/c. Furthermore, another interesting finding has been that RORγt$^+$ Tregs expressing relatively low levels of CD103 under homeostasis strongly upregulate CD103 expression in response to infection in both mouse strains. It is therefore tempting to speculate on the migratory behavior of Th17 and Treg cells and whether RORγt$^+$ Tregs constitutively expressing lower levels of CD103 may be more motile in order to constrain the potential induction of overt microbiota-specific immune responses in gut-associated lymphoid tissues. This offers interesting directions for potential exciting future studies using intravital microscopy.

Overall however, these results indicate that BALB/c mice display lower frequencies and activity of intestinal Th17 cells, which coincide with their higher cyst shedding in faeces, while C57BL/6 mice which display lower cyst shedding and hence better control of *G. muris* infection harbour higher frequencies of Th17 cells, which respond to infection with higher proliferation and metabolic activity.

6.1.2 RORγt$^+$ Tregs in the context of murine giardiasis

In the current study, BALB/c mice infected with *G. muris* displayed constitutively higher frequencies of RORγt$^+$ Tregs in siLP, which coincides with their higher cyst shedding rates and their significantly lower frequencies of intestinal Th17 cells and IL-17A production compared with C57BL/6 mice. Furthermore, infected BALB/c mice displayed no elevated Th17 cell proliferation or metabolic activity in response to infection and their elevated RORγt$^+$ Treg:Th17 ratios negatively correlated with Th17 cell proliferation. Cell-to-cell contact inhibiting TCR-induced proliferation is one of the major mechanisms of Treg-driven immunosuppression. Considering the partially overlapping TCR repertoire of RORγt$^+$ Tregs and Th17 cells reported by Solom and Hsieh (2016), as well as our findings in *G. muris*-infected BALB/c and C57BL/6 mice, it is therefore possible that BALB/c mice harbouring constitutively higher frequencies of this Treg subset experience RORγt$^+$ Treg-driven suppression of Th17 cell proliferation. This would lead to inefficient production of intestinal IL-17A, and hence to poor control of infections.

The shared differentiation pathways of Treg and Th17 cells play a role in the tendency of these two T cell subsets to display contextual interconversion. Furthermore, intestinal Treg display phenotypic and functional heterogeneity. In recent years, studies have demonstrated that Tregs can adopt specialised phenotypes by expressing typical T helper cell transcription factors such as T-bet$^+$, Gata3$^+$ and RORγt$^+$ Tregs, paralleling Th1, Th2 and Th17 effector T cell subset respectively, hence adding a potential context-dependent regulatory functionality to Tregs (Campbell and Koch, 2011).

Their differentiation is primarily dependent on commensal microbiota signalling, aryl hydrocarbon receptor (AhR), the transcriptional factor c-MAF and the local cytokine milieu (Quintana et al., 2008; Omenetti & Pizaroo, 2015; Sefik et al., 2015; Ohnmacht et al., 2015; Wheaton et al., 2017; Neumann et al., 2019). Importantly, RORγt$^+$ Tregs represent a stable Treg subset with superior suppressive capacities to RORγt$^-$ Tregs, and have been demonstrated to specifically control pro-inflammatory Th17 cells during intestinal inflammation and during infection with the intestinal bacterial pathobiont *Helicobacter hepaticus* (Yang et al, 2016; Xu et al., 2018; Neumann et al., 2019). Furthermore, TCR sequencing has revealed that RORγt$^+$ Tregs possess a TCR repertoire significantly distinct from that of conventional RORgT-

Tregs or Th17 cells. However, a subset of RORγt$^+$ Tregs shares some of their most common TCR variants with Th17 cells, thus potentially contributing to antigen-specific Th17 cell suppression during infection or inflammation (Solomon and Hsieh, 2016).

In this study, *G. muris*-infected BALB/c mice display higher TGF-β mRNA expression in the duodenum, as well as elevated frequencies of CD103$^+$ RORγt$^+$ Tregs in siLP. Importantly, C57BL/6 mice display a very similar increase in CD103$^+$ RORγt$^+$ Tregs during infection indicating comparable levels of RORγt$^+$ Treg retention in siLP, however they also display significant expression of CD103$^+$ by Th17 cells in response to infection - an effect not seen in BALB/c mice. This indicates that even though *G. muris* infection appears to lead to comparable retention of RORγt$^+$ Tregs in siLP of BALB/c and C57Bl/6 mice, the latter additionally display elevated retention of Th17 cells. Lower TGF-β in C57BL/6 mice, as seen by their low expression of TGF-β mRNA would therefore translate in poor signalling of the resident RORγt$^+$ Treg cells and hence would allow for more efficient Th17 responses to infection than in BALB/c mice.

6.1.3 Commensal microbiota composition

Considering the evidence of microbiota-dependent Treg and Th17 induction under homeostasis and the effects of *Giardia* itself on the host commensal microbiota, this study therefore focused on analysing the commensal microbiota composition of naive and infected BALB/c and C57BL/6 mice in an attempt to establish whether any compositional microbiota differences between the two mouse strains potentially contribute to the observed differences in their immunological phenotype during *G. muris* infection. Interestingly, initial results have revealed that among the 9 bacterial groups surveyed via 16sRNA-based qPCR, BALB/c and C57BL/6 mice did not display significant differences under homeostasis. Furthermore, infection with *G. muris* did not lead to significant alterations in the microbiota composition of either mouse strain. Nevertheless, high-throughput analysis and expansion of the bacterial groups included in future microbiota analysis of this infection model in relation to Treg/Th17 balance during murine giardiasis should be performed to establish with greater certainty whether the differences in Treg and Th17 propensity between BALB/c and C57Bl/6 is microbiota-independent in the context of a *Giardia* infection.

In the context of maintenance of immunity under homeostasis or during gastrointestinal infections, the resident microbiota of the host is an essential functional player in the induction and maintenance of a range of immune responses (Yordanova et al., 2018). This holds true especially in the context of intestinal Treg and Th17 cells under homeostasis, during infection or inflammation. Species-specific adhesion of commensal microbial groups like Segmented Filamentous Bacteria (SFB), as well as enteric bacterial pathogens like *Citrobacter rodentium* and enterohemorrhagic *Escherischia coli* (EHEC) are known to induce intestinal Th17 cells in mice under homeostasis and during infection, respectively (Ivanov et al., 2009; Atarashi et al., 2015). Furthermore, bacterial groups such as *Clostridium, Bifidobacterium, Ruminococcus* and *Bacteroides* isolated from humans have been shown to have Th17-inducing properties (Atarashi et al., 2015). Bacterial members belonging to *Clostridia* cluster XIVa and IV isolated from human faeces have also been shown to induce Foxp3+ Treg and furthermore, RORγt-expressing Treg induction in mice has also been shown to depend on antigen signalling and short-chain fatty acid (SCFA) production by commensal *Clostridium* species (Atarashi et al., 2013; Ohnmacht et al., 2015).

Infection with *Giardia* itself can lead to notable alterations in the composition of the intestinal microbiota and reports have shown significant expansion of b- and g-*Proteobacteria* in the small intestine, cecum and colon and a concomitant drop in the abundance of *Clostridia* and *Melainebacteria* (Barash et al., 2017). Furthermore, *Giardia* infection elevates the excretion of bile acid derivates and shifts the availability of byproducts of glucose metabolism, this potentially contributing to commensal microbiota alterations in a metabolism-related manner.

6.1.4 Antibody responses in *G. muris*-infected BALB/c and C57BL/6 mice

Studies demonstrating that IgA-deficient (Langford et al., 2002), pIgR-deficient or B cell-deficient mice (Snider et al., 1988) fail to control infections with *G. lamblia* or *G. muris* further emphasise the importance of host humoral immunity for the efficient control of giardiasis (Langford et al., 2002; Davids et al., 2006). This work shows that under steady state and during infection BALB/c and C57BL/6 mice do not display differences in IgA class switching as assessed by the frequencies of IgA^{+} $B220^{+}$ PNA^{+} cells in PP. Nevertheless, naive and infected C57BL/6 mice harbour significantly more B220- IgA+ cells in siLP and display constitutively higher IgA levels in serum. This finding contradicts previous work showing that BALB/c mice have constitutively higher serum IgA than C57Bl/6 mice (Fransen et al., 2015), however considering the high dependency of host IgA responses on microbiota composition between different experimental animal facilities, such discrepancies are not unusual.

Between intestinal Th17 cells and IgA production there is considerable interplay under homeostasis. IL-17A supports pIgR expression on epithelial cells and hence the transepithelial transport of IgA into the intestinal lumen. Furthermore, Th17 cells have been demonstrated *in vivo* to convert to follicular T helper cells supporting IgA class switching in Peyer's patches, a main site of T cell-dependent class switching (Cao et al., 2012; Hirota et al., 2013). Furthermore, the intestinal microbiota and host IgA responses exist in a bidirectional state of tight control, however germ-free naive BALB/c mice do display conspicuous amounts of IgA independent of their lack of microbiota, indicating that host genetic drivers exist which regulate IgA production independently of microbiota signalling (Fransen et al., 2015).

Giardia trophozoites are abundantly covered by surface antigens, so-called Variant Surface Proteins (VSPs). *Giardia* possesses a repertoire of more than 200 variants of VSPs and display continuous shedding and cycling of its variants as an immune evasion strategy known as antigenic variation (Stager et al., 1997; Ankarlev, 2010). Due to insufficient *G. muris* trophozoite material and previous reports showing cross-reactivity between *G. muris* and *G. lamblia*, surveys of parasite-specific IgA antibodies in *G. muris*-infected BALB/c and C57BL/6 mice using *G. lamblia* antigen-coated ELISA plates in this study have not yielded results, indicating that either polyreactive IgA is a sufficient response to infection or that there is no *G. muris-G.lamblia* cross specificity. Unexpectedly, naive C57BL/6 mice also displayed higher levels of IgG2b in serum and showed further elevation in serum IgG2b in response to infection. Early studies have previously demonstrated the presence of parasite-specific IgG2b antibodies following both primary and challenge infection with *G. muris* (Daniels & Belosevic, 1994) and following challenge with an experimental recombinant vaccine targeting variant surface proteins (VSP) of *G. lamblia* in mice (Stäger et al., 1997). TGFb is known to play an important role in supporting both IgA and IgGb2 class switching (McIntyre et al., 1993; Jang et al., 2015), however the serum antibody levels do not correspond to the observed mRNA expression in duodenum, which was higher in BALB/c than C57BL/6 mice. Therefore, further work would have to elucidate the effector mechanisms triggering this difference in serum IgA and IgG2b in C57BL/6 mice and to establish whether IgG2b exhibits any parasite specificity, perhaps instead or in parallel to IgA.

6.2 The role of eosinophils in host immunity to *G. muris*

In general, IgA class switching is T cell dependent and requires signaling via cytokines like TGF-β, IL-6 or IL-10 and ligation of CD40 on B cells with CD40L on T cells. Nevertheless, T cell-independent class switching can also occur and it primarily relies on B cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL) (Macpherson et al., 2008; Litinskiy et al., 2002). Importantly, eosinophils have been shown to provide support for IgA class switching *in vitro* and furthermore, to support IgA-producing plasma cells in bone marrow via provision of plasma cell survival factors like APRIL and IL-6 (Chu et al., 2011). Eosinophils also support IgA-producing plasma cells in siLP under homeostasis and in the context of infection with the small intestinal nematode *H. polygyrus* (Chu et al., 2014; Jung et al., 2015; Strandmark et al., 2017). Furthermore, one study has previously demonstrated that small intestinal eosinophils can restrict Th17 cell differentiation via secretion of high levels of IL-1 receptor antagonist (IL-

1Ra), an inhibitor of IL-1β (Sugawara et al., 2016). Nevertheless, the role of eosinophils in supporting intestinal IgA responses and in controlling Th17 cells during giardiasis have not been studied to date. The third main aim of this study was therefore to establish whether small intestinal eosinophils play a role in the induction and maintenance of intestinal IgA and Th17 responses during *G. muris* infection, considering the importance for both IgA and Th17 responses in host protective immunity against the parasite.

6.2.1 Control of *Giardia* infection in the absence of eosinophils

In the context of a *G. muris* infection, this study demonstrates that dblGATA-1 mice display significantly higher numbers of cysts shed in faeces at weeks 3, 5 and 6 post-infection compared with wild-type BALB/c mice, which is suggestive of higher trophozoite loads in the small intestine and hence of poor control of infection. One novel finding of this study has been that *G. muris*-infected BALB/c mice displayed a significant elevation in eosinophil frequencies in siLP, but not in PP and mLN, in the course of a 40-day infection period. Accompanying the finding of elevated frequencies of eosinophils in siLP of *G. muris*-infected mice was the observed transient increase in CCR3 expression on eosinophils found in both PP and mLN, as well as in siLP, at day 18 post-infection. CCR3 is an eosinophil surface chemokine receptor mediating their recruitment to tissues, as it recognizes and binds to a range of chemokines such as CCL11 (eotaxin 1) and CCL24 (eotaxin 2) (Rosenberg et al., 2013). Furthermore, chemotactic responses to eosinophils in the presence of sevaral eotaxins, ligands to CCR3, has been shown to lead to eosinophil activation and degranulation responses (Alam et al., 1993; El-Shazly et al., 1998; Fujisawa et al., 2000). However, to better characterize this eosinophil recruitment to siLP in response to *G. muris* infection, it would be imperative for future studies to perform histological analysis of all three intestinal tissue examined here and to confirm the presence of higher numbers of eosinophils in siLP in response to infection, as opposed to PP and mLN. Furthermore, cell sorting of eosinophils found in siLP of infected versus naïve animals would allow for a detailed exploration of the precise effector functions carried out by eosinophils following recruitment to the lamina propria.

High eosinophilia is a common observation during helminth and some protozoan parasite infections both in the circulation and localized in different tissues, as they partake in processes such as direct parasite killing, immunomodulation and potentially also wound healing (Strandmark et al., 2016, 2017; Svensson et al., 2011; Forman et al., 2016; de Oliveira et al., 2010). Furthermore, during *H. polygyrus* infection, eosinophil deficiency does not appear to affect adult worm burden in the small intestine. In contrast however, eosinophils appear to exert a positive effect on adult worm fecundity in dblGATA-1 mice compared with wild-type controls, as *H. polygyrus* females excrete significantly higher numbers of eggs in the absence of eosinophils (Strandmark et al., 2017). Furthermore, eosinophils appear to promote muscle-stage larval survival of *Trichinella spiralis* via inhibition of NO production and hence promote progression of trichinelosis (Huang et al., 2014). In yet other infection models, eosinophil deficiency has been shown to have no significant effect on parasitological parameters, as dblGATA-1 mice display similar *T. gondii* brain cyst formation and worm burdens of *T. muris* to wild-type mice (Forman et al., 2016). The findings in this study are therefore in line with previous observations of eosinophilia in the context of gastrointestinal parasite infections and demonstrate signs of eosinophil recruitment and activation based on CCR3 expression. Nevertheless, the potential for direct parasite killing of trophozoites mediated by small intestinal eosinophils would merit further investigations in the future.

6.2.2 Antibody responses during *G. muris* infection in BALB/c and dblGATA-1 mice

As small intestinal eosinophils have previously been shown to provide support for IgA class switching in PP under homeostasis and during infections with *H. polygyrus* (Chu et al., 2014; Strandmark et al., 2017), it was important to assess IgA class switching in the context of eosinophil deficiency in *G. muris*-infected mice in order to establish the versatility of eosinophils in supporting IgA class switching. Moreover, studies demonstrating that IgA-deficient (Langford et al., 2002), pIgR-deficient or B cell-deficient mice (Snider et al., 1988) fail to control infections with *G. lamblia* or *G. muris* further emphasise the importance of host

humoral immunity for the efficient control of giardiasis (Langford et al., 2002; Davids et al., 2006).

Results in this study demonstrate that under homeostasis dblGATA-1 mice display significantly lower frequencies of IgA^{+} GC B cells in PP, which complements previous findings of insufficient IgA class switching in PP as opposed to higher IgG1 class switching in the absence of eosinophils under steady state (Chu et al., 2014; Strandmark et al., 2017). Furthermore, another novel finding in this study has been the significant IgM antibody class switching observed in PP in *G. muris*-infected mice independent of eosinophils, which serves as evidence of immune activation in response to infection. In mLN, BALB/c and dblGATA-1 mice displayed comparable elevated IgA class switching during the acute stage of infection (18dpi.), but not significant alterations in IgG1 or IgM class switching, indicating that antibody class switching events differ in PP and mLN during *G. muris* infection, with IgA class switching occurring in both PP and mLN independent of eosinophil deficiency, while wild-type mice only displayed elevated IgA class switching in mLN during infection. This study therefore supports previous findings of the essential role of eosinophils in providing support for the maintenance of adequate IgA class switching in PP during infection with *H. polygyrus*, as G. muris-infected dblGATA-1 mcie were unable to maintain IgA^{+} GC B cell frequencies comparable with wild-type mice 40 days post-infection.

Results in this study further highlight that in siLP, eosinophil deficiency does not affect IgA-producing plasma cells, as the frequencies of IgA^{+} cells in siLP increased comparably between BALB/c and dblGATA-1 mice during infection. Therefore, these findings contrast previous studies, which have reported impaired IgA CS in PP of dblGATA mice results in the reduced accumulation of small intestinal IgA^{+} plasma cells by showing that in the context of a *G. muris* infection, eosinophil deficiency does not alter the frequencies of small intestinal IgA* plasma cells (Chu et al., 2011, Jung et al., 2015).

6.2.3 Th17 responses in *G. muris*-infected wild-type BALB/c and eosinophil-deficient dblGATA-1 mice

Small intestinal eosinophils are versatile multipoint effector cells in respect to their ability to produce and store a variety of cytokines, chemokines and other bioactive molecules with immune activating and modulatory properties. Moreover, they have been demonstrated to secrete large amounts of a soluble IL-1Ra interfering with IL-1β-mediated support for Th17 cells and hence restrict intestinal Th17 responses (Sugawara et al., 2016). Importantly however, eosinophils are known to be able to store and secrete IL-1β and IL-6 cytokines, both essential for the induction and maintenance of Th17 cells. A potential dichotomy exists therefore with respect to the role of eosinophils in intestinal Th17 responses and hence this study assessed Th17 cell frequencies and IL-17A production in *G. muris*-infected mice in the presence and absence of eosinophils. Therefore, this study further focused on assessing Th17 cell responses in *G. muris* infected wild-type BALB/c and eosinophil-deficient dblGATA-1 mice.

This study has demonstrated that even though eosinophil deficiency does not affect the frequencies of $ROR\gamma t^{+}$ Th17 cells in PP and siLP under homeostasis and during *G. muris* infection, BALB/c mice display significantly higher $IL\text{-}17A^{+}$ T cells 40 days post-infection in PP and display mildly elevated $IL\text{-}17A^{+}$ in siLP compared compared with dblGATA-1 mice. This inefficient intestinal IL-17A response coincides with the higher cyst shedding observed in dblGATA-1 mice and further fits with the previous findings in this work, where C57BL/6 mice displaying more potent IL-17A production even than BALB/c mice also display much lower cyst excretion rates. Therefore, these findings are in line with previous studies demonstrating the essential role of IL-17A in controlling murine giardiasis (Dann et al., 2015; Dreesen et al., 2014; Paerewijck et al., 2017).

6.2.4 Immune responses to *G. muris* infection in littermate control mice

Even though eosinophil-deficient dblGATA-1 mice are bred on a BALB/c background, they have previously been demonstrated to possess intestinal microbiota with a different

composition from that of BALB/c mice (Jung et al., 2015; Alexander Beller, pers. commun.). Considering the now widely-recognised roles of the commensal microbiota in cross-talk with immune cells under homeostasis and during infection, this study therefore generated BALB/c x dblGATA-1 littermate control mice to exclude the potential influence of the microbiota or the housing conditions on the immunological phenotype observed in *G. muris*-infected BALB/c and dblGATA-1 mice. Furthermore, as IgA CS in PP as well as Th17 activity in PP and siLP appeared impaired in dblGATA-1 mice at the chronic stage of infection (40 dpi.), this late time point of infection was chosen for further investigations in littermate control mice and the preliminary results of these studies are discussed below.

Assessment of cyst shedding in the littermate mice revealed cyst shedding rates of eosinophil-deficient mice were comparable from those observed in WT littermates and to those observed for wild-type BALB/c mice. In other infection models, eosinophil deficiency has been shown not to affect adult worm burden during *H. polygyrus* infection and to even support adult worm fecundity (Strandmark et al., 2017). Furthermore, eosinophils appear to promote muscle-stage larval survival of *Trichinella spiralis* (Huang et al., 2014). Eosinophil deficiency has also been shown to have no significant effect on parasitological parameters during *T. gondii* or *T. muris* infections (Forman et al., 2016). This therefore places the current findings of G. muris cyst shedding in eosinophil-deficient mice in agreement with some studies and in contradiction with others, therefore highlight the potential context dependent nature of the influence of eosinophil deficiency on parasitological parameters during gastrointestinal parasite infections.

Importantly, during infection with *G. muris* eosinophil-deficient littermates displayed a significant elevation in IgA$^+$ GC B cells compared with wild-type mice, indicative of normal IgA class switching. Interestingly, the significant increase in IgM class switching observed in the littermate mice was comparable to that in BALB/c and dblGATA-1 mice, indicating that IgM class switching is independent of potential eosinophil-mediated support. In siLP, littermate mice displayed stable IgA$^+$ cell frequencies, which were not affected neigher by eosinophil deficiency, nor by the infection. Furthermore, naïve and infected littermate mice from both phenotypes displayed notably fewer IgA$^+$ cells in siLP compared with BALB/c anddblGATA-1 mice.

Assessment of intestinal Th17 responses in naïve and infected littermate mice revealed that under homeostasis, eosinophil deficiency did not affect Th17 cell frequencies in PP and siLP. Nevertheless, the infection-induced increase in Th17 cell frequencies in siLP and the similar IL-17A responses between wild-type and eosinophil-deficient littermates indicated that eosinophil deficiency does not affect Th17 cells under homeostasis or during *G. muris* infection. Overall, the similarities in Th17 responses coincide with the very similar cyst shedding observed for the littermate mice. Furthermore, the lack of significant differences in IgA or Th17 responses under steady state or during G. muris infection observed in littermates compared with separately-kept BALB/c and dblGATA-1 mice indicate that the microbiota composition plays a role in the induction and maintenance of protective immune responses against *Giardia* and the effector mechanisms potentially responsible for this merit further investigations.

6.2.5 Eosinophil activation in response to *G. lamblia in vitro* and *in vivo*

Despite being known as important effector cells during gastrointestinal infection and inflammation, eosinophils have also been demonstrated to display elevated activation and phenotypical alterations associated with antigen processing and presentation in the context of parasite infections. For example, eosinophils challenged with *S. stercoralis* antigen display a higher activation status, as demonstrated by elevated expression levels of CD69, MHC-II, CD86, CD80 and CD29 *in vitro*, in addition to being capable of MHC-II dependent stimulation of IL-5 secretion by CD4$^+$ T-cells (Padigel et al 2006). Furthermore, eosinophils isolated from mice infected with the filarial nematode *Brugia malayi* demonstrate a shift to a hypodense phenotype and elevated MHC-II expression, considered as markers of activation (Mawhorter et al., 1993).

Considering the extracellular localization of *Giardia* trophozoites attached along the length of the proximal small intestine, parasite antigen entering the siLP via M cells potentially enters the vicinity of resident small intestinal eosinophils. Nevertheless, whether eosinophils recognize and respond to *Giardia* antigens, leading to the activation or degranulation of these granulocytes has not been explored to date. The fourth aim of this study has therefore been to assess any evidence of eosinophil activation and degranulation in response to *G. lamblia* trophozoite antigens *in vitro* and in *G. lamblia*-infected mice *in vivo* in collaboration with Christian Klotz and Anton Aebischer from the Robert Koch Institute in Berlin.

Preliminary data in this study has shown novel data from the assessment of bmEos activation in response to *in vitro* stimulation with *G. lamblia* trophozoite antigens from the human parasite strains WB6 (assemblage A) and GS (assemblage B). Namely, while bmEos challenged in vitro with G. lamblia antigens displayed mild elevations in the expression of the degranulation markerCD63, assessment of *G. lamblia* GS-infected mice 9 days post-infection revealed considerable upregulation in CD63 surface expression on eosinophils in PP and mLN, but not systemically in the spleen of infected mice. Importantly, in PP the upregulated CD63 expression was paralleled by a decrease in eosinophil granularity, as measured by their SSC values via flow cytometry.

PP are overlayed by M cells, key sites of antigen entry from the lumen under homeostasis and during infection with gastrointestinal pathogens. The significant increase in CD63 expression on eosinophils in response to *G. lamblia in vivo* and more mildly *in vitro,* suggest that bmEos potentially lack essential priming factors for their effective activation and degranulation at the molecular or at the cellular levels, such as microbiota signaling or antigen presentation and activation by professional APCs like DCs, which commonly reside in PP under steady state. Considering the novelty of the findings in this study, that small intestinal eosinophils display evidence of degranulation in response to *Giardia* infection, future studies in their direct functionality during giardiasis merit further investigations. Future work should therefore focus on histological examinations of eosinophils resident in PP and siLP during *Giardia* infection and on cell sorting of small intestinal eosinophils from naïve and *Giardia*-infected animals would allow for *ex vivo* culture stimulations with trophozoite antigens and quantification of cytokine and chemokine products released into the cell culture medium via multiplex ELISA. Last but not least, performances of trophozoite viability assays with supernatants from degranulated eosinophils would establish whether eosinophils release products with trophocidal or trophostatic properties and hence would assess whether eosinophils display direct parasite killing properties.

7. SUMMARY

Title: Host determinants of immunity to the gastrointestinal protozoan parasite *Giardia muris*

Background: Giardiasis remains among the most common parasitic causes of food- and water-borne diarrhoea disease worldwide and often presents with diverse clinical manifestations ranging from self-resolved asymptomatic infection to acute diarrhoea, nausea, vomiting and malabsorption. Host protective immunity to the parasite relies preferentially on intestinal IgA production and on pro-inflammatory Th17 responses. However, whether additional immune mechanisms like regulatory T cells influence the efficiency of Th17 responses during giardiasis remains unexplored. Furthermore, small intestinal eosinophils are known to provide support for IgA class switching in PP and IgA^+ plasma cells in siLP and in addition, they have been shown to constrain intestinal Th17 responses. Whether eosinophils are an essential component of host immunity in terms of support for IgA and constrains for Th17 responses has also not been investigated so far. Therefore this study focused on investigating intestinal Treg/Th17 balance in inbred mouse strains with known differences in susceptibility to *G. muris* and in their propensity for Th17 responses. In addition, eosinophil-deficient dblGATA-1 mice were investigated for their induction and maintenance of IgA and Th17 responses during the acute and chronic stages of infection.

Aims:

1. To ascertain whether inbred mouse strains of different genetic backgrounds, known to display differences in their control of *Giardia* infection.

2. To establish whether *Giardia* infection leads to differences in Treg responses or an imbalance in Treg/Th17 ratios in these hosts and how that correlates with efficient control of infection.

3. To determine whether small intestinal eosinophils play a role in the induction and maintenance of intestinal IgA and Th17 responses during *G. muris* infection.

4. To establish whether murine eosinophils can directly recognise and respond to *Giardia* trophozoite antigens

Results: It was found that while acute *G. muris* infection does not lead to a significant upregulation in IL-17A secreting cells in PP or siLP in either mouse strain, it does induce higher proliferation, metabolic activity and retention of Th17 cells in C57BL/6 mice, but not in BALB/c. BALB/c mice were found to harbour constitutively lower frequencies of Th17 cells and IL-$17A^+$ T cells, however they display significantly higher frequencies of a subset of RORγt^+ Tregs than C57BL/6 mice and this coincides with their higher cyst shedding rates and lower Th17 cell activity, and resulted in a shift in RORγt^+ Treg:Th17 ratios in response to infection. Furthermore, C57BL/6 mice displayed a trend for unregulated IgA-producing cells in siLP in response to infection and harboured significantly higher serum IgA and IgG2b antibodies than BALB/c mice. Furthermore, eosinophil-deficient dblGATA-1 mice mice and littermate control mice infected with *G. muris* showed no decreases in their propensity for IgA class switching in PP or Th17 cell responses during the chronic stages of infection.

Conclusions: This work demonstrates that poor control of *G. muris* infection in inbred mice coincides with low Th17 proliferation, metabolic activity and IL-17A production, as well as with constitutively higher frequencies of RORγt^+ Tregs and with a significant shift in RORγt^+ Treg:Th17 ratios, suggesting that Tregs potentially play an important role in the maintenance of host protective immunity during giardiasis. Furthermore, results here indicate that small intestinal eosinophils are potentially redundant with respect to their involvement in the induction and maintenance of intestinal IgA and Th17 responses during giardiasis.

8. ZUSAMMENFASSUNG

Titel: Immunitätsfaktoren des Wirtes vom gastrointestinalen protozoischen Parasiten *Giardia muris*

Hintergrund: Giardiasis zählt zu den weltweit meist verbreiteten Gründen von Diarrhöerkrankungen, die durch Nahrungsaufnahme oder Trinkwasser übertragen werden. Sie weist verschiedene klinische Erscheinungsformen auf, die von der selbstheilenden asymptomatischen Infektion bis zu Infektionen mit schwerwiegendem Brechdurchfall reichen. Die Immunreaktion des Wirtes auf den Parasiten besteht vorrangig aus einer intestinalen IgA Produktion und einer proinflammatorischen Th17 Antwort. Ob zusätzliche Immunmechanismen, wie regulatorische T Zellen, die Effizient der Th17 Antwort während der Giardiasis beeinflussen ist unerforscht. Zusätzlich ist bekannt, dass Eosinophile des Dünndarms den IgA Isotypen Wechsel in PP und IgA^+ Plasmazellen der siLP unterstützen und es wurde gezeigt, dass Sie die intestinale Th17 Antwort hemmen. Ob Eosinophile notwendige unterstützende Immunkomponenten des Wirtes bei der IgA Induktion und der Hemmung der Th17 Antwort sind, wurde bisher ebenfalls nicht untersucht. Aus diesem Grund liegt der Fokus dieser Arbeit auf der Analyse des intestinalen Treg/Th17 Gleichgewichts in Inzucht Mausstämmen mit abweichender Empfänglichkeit für *G. muris* Infektionen und unterschiedlicher Ausprägung der Th17 Antwort. Zusätzlich wurden Eosinophildefiziente dblGATA-1 Mäuse auf deren IgA Induktion und Aufrechterhaltung und auf die Th17 Antwort während der akuten und chronischen Phase der Infektion untersucht.

Ziele:

1. Untersuchung, ob inzucht Mausstämme mit unterschiedlichem genetischen Hintergrund verschiedene Ausprägungen bei der Kontrolle von *Giardia* Infektionen zeigen.

2. Feststellung, ob *Giardia* Infektionen zu Unterschieden in der Treg Antwort oder einem Ungleichgewicht des Treg/TH17 Verhältnisses in den verschiedenen Wirten führen und wie das mit der wirksamen Kontrolle der Infektion zusammenhängt.

3. Bestimmung, ob die Eosinophilen des Dünndarms eine Rolle bei der Induktion und Aufrechterhaltung von intestinalem IgA und der Th17 Antwort während der *G. muris* Infektion spielen.

4. Nachweis, ob murine Eosinophile Antigen aus *Giardia* Trophozoiten direkt erkennen und darauf reagieren können.

Ergebnisse: Es wurde herausgefunden, dass es während der akuten *G. muris* Infektion in keinem der Mausstämme zu einer signifikanten Vermehrung von IL-17A sekretierenden Zellen in den PP oder den siLP kommt. Jedoch wird im Gegensatz zu BALB/c Mäusen in C57BL/6 Mäusen eine höhere Proliferation, metabolische Aktivität und Retention von Th17 Zellen induziert. BALB/c Mäuse haben konstitutiv eine geringere Anzahl von Th17 Zellen und $IL\text{-}17A^+$ T Zellen, dahingegen zeigen sie eine signifikant größere $ROR\gamma t^+$ Tregs Population als C57BL/6 Mäuse, was mit der höheren Rate in der Zysten Ausscheidung und einer geringeren Th17 Zellaktivität korreliert. Als Antwort auf die Infektion führte dies zu einer Veränderung des $ROR\gamma t^+$ Treg/Th17 Verhältnisses. Zusätzlich zeigte sich bei C57BL/6 Mäusen; als Antwort auf die Infektion, eine Zunahme der IgA produzierenden Zellen in den siLP und sie weisen signifikant größere Mengen IgA und IgG2b Antikörper im Serum auf, als die BALB/c Mäuse. Des Weiteren zeigen die Eosinophildefizienten dblGATA-1 Mäuse und ihre Wurfgeschwister als Kontrolle, während der chronischen Phase der *G. muris* Infektion keine Abnahme in ihrer Neigung zum IgA Isotypen Wechsel in den PP oder Th17 Zellen.

Fazit: Diese Arbeit zeigt, dass eine schwache Kontrolle der *G .muris* Infektion in inzucht Mausstämmen mit einer geringen Th17 Zellproliferation, metabolitischen Aktivität und IL-17A

Produktion einhergeht. Zusammen mit einer konstitutiv höheren RORγt$^+$ Treg Population und einer Verschiebung des RORγt$^+$ Treg/Th17 Verhältnisses führt das zu der Annahme, dass Tregs potentiell eine wichtige Rolle in der Erhaltung des Immunschutzes des Wirtes während der Giardiasis spielen. Zusätzlich zeigen die Ergebnisse, dass die Eosinophilen des Dünndarms möglicherweise überflüssig bei der Induktion und Aufrechterhaltung des IgA Spiegels und der Th17 Antwort während der Giadiasis sind.

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9.1 Publication List

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11. DECLARATION

I hereby declare that this thesis has been written by me in its entirety, using no other resources other than those stated above. All the work presented here has been performed by me in collaboration with those people mentioned in the text and in the acknowledgements. I declare that there are no existing conflicts of interest or financial conflicts associated with this project.

Berlin, 11.11.2019

Ivet A. Yordanova