Role of interleukin-6 during infection with the filarial nematode *Litomosoides sigmodontis*

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Contents

List of abbreviations

AAM	alternatively activated macrophage
APC	antigen-presenting cell also used for the commercially available fluorochrome "allophycocyanine"
BCSF	B cell stimulatory factor
BSA	bovine serum albumin
BW	body weight
CAM	classically activated macrophage
CCL	chemokine C-C motif ligand
CD	cluster of differentiation
CNTF	cilliary neurotropic factor
ConA	concanavalin A
CO_2	carbon dioxide
CTLA	cytotoxic T-lymphocyte antigen
CXCL	chemokine C-X-C motif ligand
DAMP	damage-associated molecular pattern
DC	dendritic cell
DEC	diethylcarbamazine
DNA	deoxyribonucleic acid
dpi	day(s) post infection
E/S	excretory/secretory
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FcεR	Fc-epsilon receptor
FITC	fluorescein-isothiocyanate
FMO	fluorescence minus one
FoxP3	forkhead box protein P3
FSC	forward scatter
G-CSF	granulocyte colony stimulating factor
GITR	glucocorticoid-induced TNFR family related gene
GM-CSF	granulocyte / monocyte-colony stimulating factor
gp130	glycoprotein 130 kDa
h	hour(s)
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
i.c.	intracutaneously
i.p.	intraperitoneally
i.v.	intravenously
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iT_{reg}	inducible regulatory T cells
JAK	Janus family tyrosin kinase
L1	first larval stage
L3	third larval stage
L4	fourth larval stage

LC	Langerhans cell
LF	lymphatic filariasis
LIF	leukemia inhibitory factor
LN	lymph node
LPS	lipopolysaccharide
LsAg	*L. sigmodontis* extract
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MCP	monocyte chemotactic protein
M-CSF	macrophage colony-stimulating factor
MDA	mass drug administration
Mf	microfilariae = L1
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mIL-6R	membrane-bound IL-6 receptor
min	minute(s)
MIP	macrophage inflammatory protein
mMCP	mouse mast cell protease
NO	nitric oxide
nT_{reg}	natural regulatory T cells
OD	optical density
P3C	tripalmitoyl-S-glycerylcysteine
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PFA	paraformaldehyde
PRR	pattern recognition receptor
RAG	recombination activating genes
RELM	resistin-like molecule
rIL-6	recombinant mouse IL-6
RBC	red blood cell
RNA	ribonucleic acid
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
RT	room temperature
s.c.	subcutaneously
SEM	standard error of mean
sIL-6R	soluble IL-6 receptor
SOCS	suppressor of cytokine signaling
SSC	side scatter
STAT	signal transducers and activators of transcription
TGF	tumor growth factor
Th1, Th2	helper cell response of type 1 or 2
Th17	helper cell response of type 17
TLR	Toll-like receptor
TNF	tumor necrosis factor
T_{reg}	regulatory T cells
UV	ultraviolet

VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WoLP	*Wolbachia* lipoproteins
WSP	*Wolbachia* surface protein

1. Introduction

1.1. Lymphatic filariasis

Infections with helminths, especially nematodes, still remain a public health concern worldwide. One disease that is due to nematode infection and causes high morbidity is lymphatic filariasis (LF). LF is a chronic tropical disease that affects lymph vessels and lymph nodes predominantly of the extremities (WHO 2013). Although LF is the second most common vector borne disease after malaria (Upadhyayula et al. 2012), it is considered a neglected tropical disease due to the fact that the disease can cause significant morbidity worldwide but still received limited attention from health organizations and research programs (WHO 2010). Although no direct mortality is caused by the disease, it can lead to lifelong morbidity (Upadhyayula et al. 2012). Thus, LF is for example the most frequent cause of secondary lymphedema (Pfarr et al. 2009).

1.1.1. Etiology, epidemiology and impact

LF is commonly found in tropical regions especially in Asia, Africa and South America (WHO 2012). Two thirds of the LF cases are found in India, Indonesia and Nigeria (Michael and Bundy 1997). Three species of filarial nematodes are known to cause LF in humans: *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. *W. bancrofti* causes more than 90% of total LF infections. *B. malayi* caused LF mainly occurs in Southeast Asian countries like Indonesia and Malaysia, whereas *B. timori* is only found in eastern Indonesia and Timor Leste (Taylor et al. 2010; WHO 2012).

It is estimated that 1.3 billion people in 73 countries (65% in South and Southeast Asia and 30% in Africa) live in endemic areas. Amongst them 120 million are currently infected with LF (WHO 2012). The World Health Organization (WHO) reported that LF is the second largest cause for chronic disability worldwide. Currently, 15 million people, mostly women, live with lymphedema and 25 million people suffer from urogenital swelling, mainly scrotal hydrocele (WHO 2012).

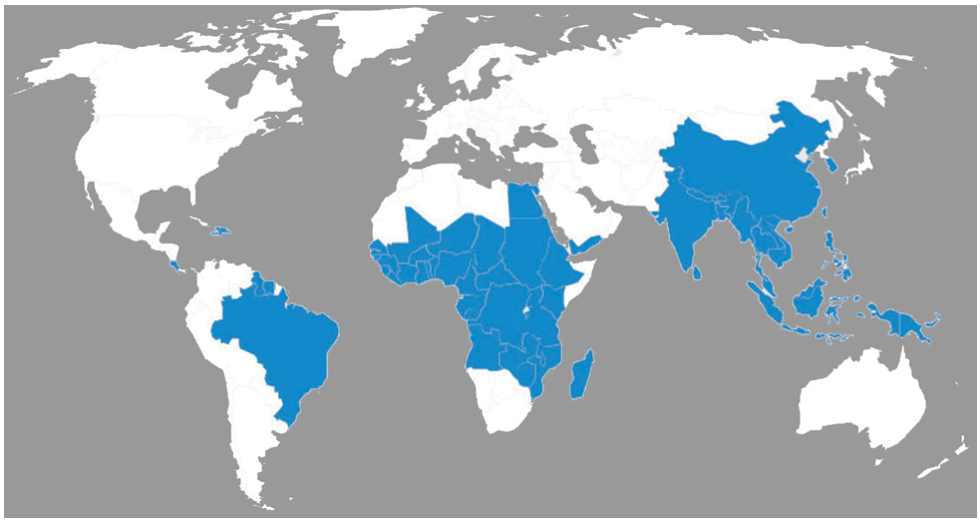


Figure 1.1. Areas endemic for lymphatic filariasis. LF is spread in the tropical regions of Asia, Africa and South America as shown in blue. Adapted from WHO 2006: http://www.bvgh.org /Portals/0/disease_maps/LF_map.gif)

The disease is transmitted by mosquitoes of the genera *Culex*, *Aedes*, *Anopheles* or *Mansonia* (Pfarr et al. 2009). In Africa, LF is mostly transmitted by *Anopheles*, which also transmits malaria (Hopkins 2013). In mosquitoes, microfilariae (larvae stage 1, L1 or Mf) shed their sheaths, penetrate into the mosquito's midgut and infiltrate the thoracic muscles of mosquitoes. After 2 molting steps in the mosquitoes, the infectious stage 3 larvae (L3) are transferred to humans by mosquito bites. In humans, L3 penetrate the skin and reside in the lymphatics. In the lymphatics the L3 molt into the larvae stage 4 (L4) and mature to adult worms. Adult worms can live for many years in the lymphatics and produce their offspring, the Mf, that have a lifespan of 3-36 months (Nutman and Weller 2005). The Mf migrate to the circulation and can be taken up by blood feeding mosquitoes and complete the parasite's life cycle (Specht and Hoerauf 2012; Taylor et al. 2010). The complete life cycle of *W. bancrofti* is shown in figure 1.2.

Although no direct mortality caused by LF has been observed, the disease has a huge economic, psychological and social impact (WHO 2013). Alone in India the economic loss caused by LF annually reaches an estimated US$ 1.5 billion due to temporary and permanent disabilities (Ramaiah et al. 2000). Those costs will further increase since the disease is chronic and lasts until the death of the patients (WHO 2013).

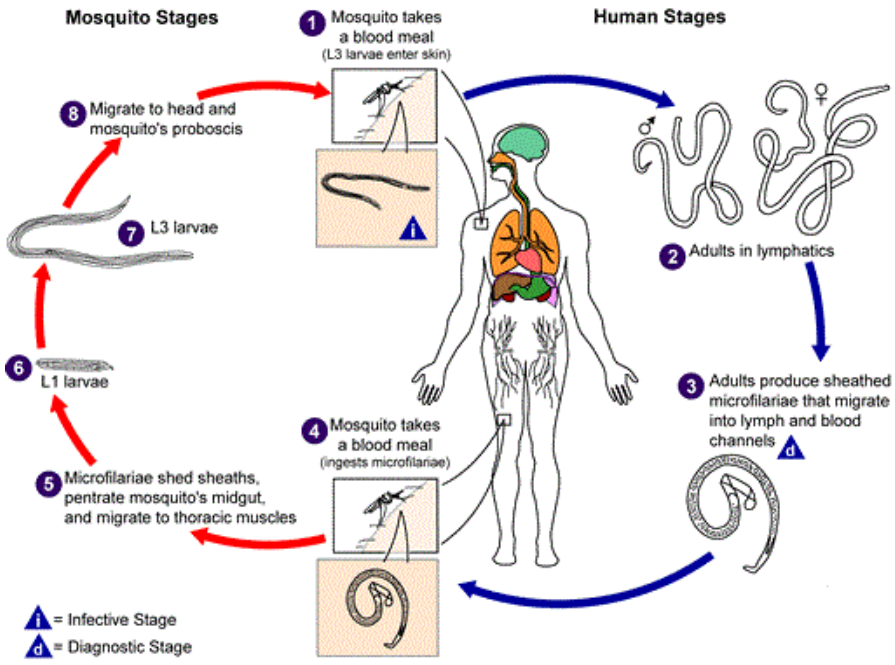


Figure 1.2. Life cycle of *W. bancrofti*. The left part of the panel shows the life cycle of *W. bancrofti* in the mosquito, whereas in the right part the development in the human is displayed. Adapted from: http://www.cdc.gov/parasites/ lymphaticfilariasis/biology_w_bancrofti.html.

1.1.2. Pathophysiology and clinical findings

Pathogenesis of the disease is divided into the asymptomatic (or subclinical), acute and chronic stages (Nutman and Weller 2005; Taylor et al. 2010). Most patients are asymptomatic and even though they are positive for Mf, no external signs or symptoms are visible (Taylor et al. 2010; WHO 2013). Additionally, damage to the lymphatic system and the kidneys as well as alterations of the immune system can be observed in patients (WHO 2013).

Acute episodes of local inflammation involve skin, lymph nodes and lymphatic vessels in the extremities, as well as breasts and genitals (WHO 2013). Most of these conditions are caused by the immune response against the parasite and result in bacterial infections due to the impairment of host immunity and lymphatic damage (WHO 2013). Acute adenolymphangitis is a common feature found in acute infection which is characterized by high fever, lymphatic inflammation (lymphangitis and lymphadenitis), and transient local edema (Nutman and Weller 2005; Taylor et al. 2010). Lymph vessel dilatation occurs when adult worms are still alive (Nutman and Weller 2005). Acute symptoms can also include dermatolymphangioadenitis. High fever, chills, myalgias and headache can accompany the acute infections (Nutman and Weller 2005; Pfarr et al. 2009). Both features

of acute conditions are mostly caused by immune responses against death worms or bacteria as secondary infection (Nutman and Weller 2005; Taylor et al. 2010).

Acute inflammatory reactions can develop into chronic lymphedema (tissue swelling) or elephantiasis (skin/tissue thickening) (Taylor et al. 2010). In these conditions, lymphedema or elephantiasis of limbs and hydrocele (fluid accumulation) are common features observed in patients (Taylor et al. 2010). Similar to acute immune responses, chronic inflammation is predominantly due to dying worms caused by antifilarial drugs or spontaneous worm death (Pfarr et al. 2009; Taylor et al. 2010).

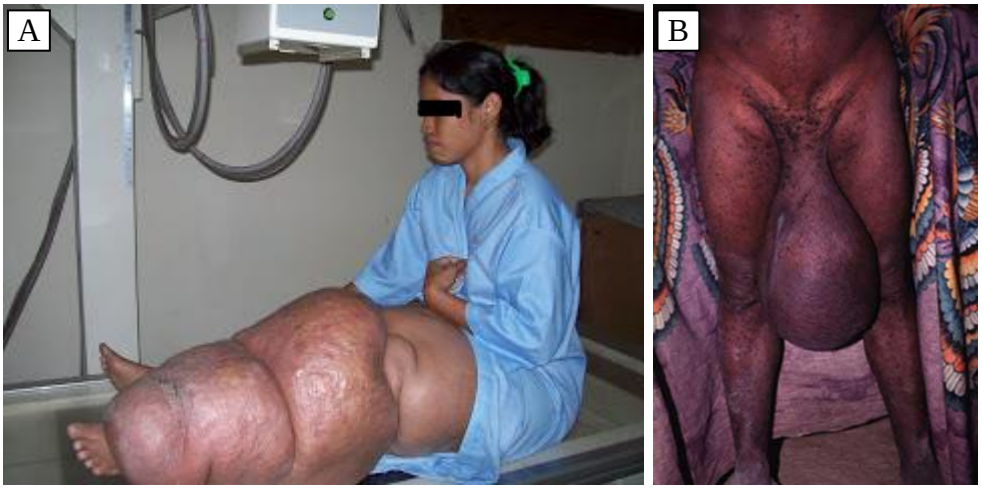


Figure 1.3. Manifestations of chronic lymphatic filariasis. Chronic manifestations of lymphatic filariasis: Chronic filarial lymphedema (elephantiasis) (A) and filarial hydrocele (B). Adapted from http://puskesmaspebayuran. blogspot.com/2010/08/filariasis.html (A) and http://www.filariasis.org/press_centre/hydrocele.html (B).

1.1.3. Diagnosis and treatment

Definitive diagnosis is made when parasites are detected, either in lymphatic fluid or in the blood (Nutman and Weller 2005; Taylor et al. 2010). Since L3 and L4 as well as adult worms reside in the lymphatic vessels or sinuses of lymph nodes, it is difficult to detect the larvae or adult worms (Nutman and Weller 2005). Alternatively, diagnosis is made by detecting Mf which circulate in the peripheral blood (Nutman and Weller 2005; Taylor et al. 2010). Periodicity of Mf in the peripheral blood during day (diurnal) or night (nocturnal) depends on the filarial species and the involved vector (e.g. nocturnal periodicity in *W. bancrofti*) (Nutman and Weller 2005; Taylor et al. 2010). Mf can also be detected in hydrocele fluid, or rarely, in other body fluids (Nutman and Weller 2005; Taylor et al. 2010). Detection of circulating worm antigen by either enzyme-linked

immunosorbent assay (ELISA) or a rapid-format immunochromatographic card test is another option to diagnose bancroftian and brugian filariasis (Rahmah et al. 1998; Taylor et al. 2010). Assays for detecting DNA of *W. bancrofti* and *B. timori* by polymerase chain reaction (PCR) have been developed (Albers 2011; Rao et al. 2006). Recently, a new method to visualize motile worms in dilated lymphatics was established by detecting worm movement by ultrasonography (Dreyer et al. 1998; Mand et al. 2005).

Diethylcarbamazine (DEC) which acts as a potent microfilaricide and weak macrofilaricide has been used as the drug of choice for LF since its discovery in 1948 (Nutman and Weller 2005; Taylor et al. 2010). In Africa where onchocerciasis is coendemic, ivermectin is used as alternative treatment as DEC causes significant adverse reactions in onchocerciasis patients (Taylor et al. 2010). Another antiparasitic drug is albendazole which has a modest macrofilaricidal property and is used in combination with DEC or ivermectin for Mass Drug Administration (MDA) program organized by the WHO since 2000 (Nutman and Weller 2005; Taylor et al. 2010).

Ongoing research is aimed to discover new and improved drugs which are more effective and cause minimal adverse reactions (Hoerauf 2008). Drugs used for ongoing MDA by the WHO such as DEC, ivermectin and albendazole are reported to have no or little effect in killing adult worms. Although the MDA program is implemented for many years, the drugs could not completely stop the transmission and alleviate pathology of the disease such as lymphedema and hydrocele (Hoerauf 2008). Therefore drugs that have adulticidal activity are required in order to reduce the pathology (Hoerauf 2008). An established alternative drug that is still being optimized for human treatment is doxycycline which targets the endosymbiotic bacterium found in the worms which is named *Wolbachia* (Hoerauf 2008) and will be discussed in the following section.

1.1.4. The worm's endosymbiont: *Wolbachia*

In recent years, it has been shown that endosymbiotic *Wolbachia* bacteria are important in the pathogenesis of filariasis (Pfarr and Hoerauf 2006; Taylor 2003). They are found in the oocytes, embryos and larval stages and in the hypodermis of male and female filarial nematodes. *Wolbachia* are essential for filarial growth, development, embryogenesis and survival (Tamarozzi et al. 2011; Taylor et al. 2010). Accordingly, *Wolbachia* are a suitable target for the development of new anti-filarial drugs (Hoerauf 2008). Treatment of

filariasis with antibiotics depleting *Wolbachia* leads to long-term sterility and eventual death of adult worms and causes significant improvements in lymphatic pathology, i.e. the severity of lymphedema and hydrocele (Debrah et al. 2006; Pfarr et al. 2009).

Besides being required by the worm for survival, *Wolbachia* bacteria have also been described to activate the host's immune system (Pfarr and Hoerauf 2006; Turner et al. 2009). This results in the production of IL-6, TNFα and IL-1β (Taylor et al. 2001). This is important in the context of classical antihelminthic treatment (e.g. DEC) where worms are killed and release *Wolbachia* (Cross et al. 2001; Taylor et al. 2001; Turner et al. 1994).

Cross and coworkers showed that administration of DEC can cause death of adult worms at a modest degree, which leads to release of *Wolbachia* (Cross et al. 2001). *Wolbachia* was previously thought to contain lipopolysaccharide (LPS) in their membranes (Cross et al. 2001; Taylor et al. 2001). This LPS then triggers inflammation via Toll-Like Receptor (TLR) 4 and leads to secretion of various cytokines, including IL-6 by macrophages and endothelial cells (Saint André et al. 2002; Taylor et al. 2001). However, studies then emphasized the role of *Wolbachia* lipoproteins (WoLP) and *Wolbachia* surface protein (WSP) in stimulating innate and adaptive immunity (including increased production of IL-6) and this mechanism occurs through TLR2/6. A role of TLR4 in *Wolbachia*-mediated immune responses in filariasis is still debatable (Brattig et al. 2004; Daehnel et al. 2007; Hise et al. 2007; Turner et al. 2009).

1.1.5. Murine model of lymphatic filariasis

Since human-pathogenic filariae such as *W. bancrofti* cannot patently infect laboratory mice, a filarial strain that can fully develop in mice is required. Similar genotype and phenotype with human filariae is beneficial (Hoffmann et al. 2000; Hörauf and Fleischer 1997). Although some *Brugia spp* can infect laboratory mice, the survival time of each stage of these worms is limited in mice (Hörauf and Fleischer 1997; Lawrence 1996). Therefore, *Brugia spp* are still not suitable for a murine LF model, notably when comparing stage-specific immune responses during the complete course of filarial infection (Hoffmann et al. 2000; Hörauf and Fleischer 1997; Lawrence 1996).

The rodent filaria *Litomosoides sigmodontis* which can infect laboratory mice has been established as a model of LF for decades (Hoffmann et al. 2000; Petit et al. 1992). It belongs to the same family as human filariae. Moreover, *L. sigmodontis* shares similar

features with human filariae such as larval migration, genomic and biochemical structure (Hoffmann et al. 2000). It shows extensive immunological cross-reactivity with *Brugia spp* and *W. bancrofti* and fully develops in BALB/c mice (Hoffmann et al. 2000; Hörauf and Fleischer 1997; Lawrence 1996). Many studies have shown the benefits of this model in filarial research by analyzing immune responses and parasitological parameters at different stages of the worm's life cycle (Hoffmann et al. 2000; Maréchal et al. 1997; Pfaff et al. 2000). In this model, mice are infected with L3 through the bite of the tropical rat mite *Ornithonyssus bacoti*. Larvae migrate to the pleural cavity where they molt twice, becoming adult worms at about 4 weeks post infection. Around 8 weeks post infection; female worms start to release Mf. The complete life cycle of *L. sigmodontis* is shown in figure 1.4.

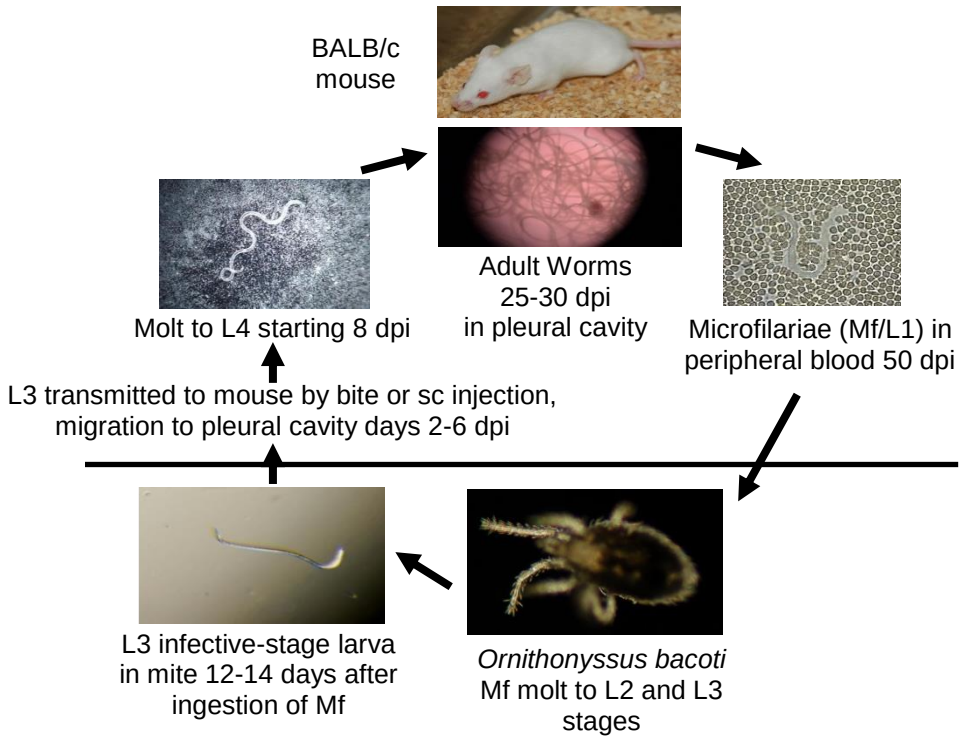


Figure 1.4. Life cycle of *L. sigmodontis*. The top panel shows life cycle of *L. sigmodontis* in permanent hosts, whereas bottom panel shows life cycle in vector. Adapted from Hübner et al. 2009.

1.2. Immune response during filariasis

The role of host immunity in reducing inflammation, number of worms, circulating Mf and in preventing disability still leaves a big question mark and requires further research (Nutman and Weller 2005; Taylor et al. 2010). Both types of human immunity, innate and adaptive immunity, are believed to take an important part in controlling or even worsening the disease (Nutman and Weller 2005; Specht and Hoerauf 2012). Local and systemic inflammation which involves various cell types and molecules has long been recognized to occur in patients with filariasis, especially in early phases of infection as well as in the chronic stage (Nutman and Weller 2005; Specht and Hoerauf 2012; Taylor et al. 2010). Inflammation which occurred in the acute phase of filarial disease is usually indistinguishable from other inflammation-related diseases causing misdiagnosis by many health professionals and leading to inappropriate treatment (Nutman and Weller 2005; WHO 2013).

Several cell types are involved in the immune response against filarial infection and are important for worm clearance. Additionally, these cells play a crucial role in disease-related pathology (Specht and Hoerauf 2012; Taylor et al. 2010). In the following section some of these cell types which are well known to be important during filarial infection, are discussed.

1.2.1. Neutrophils

Neutrophils are effector cells of the innate immune system with a short life span and are important in immunity against extracellular pathogens and during the early phase of filarial infection (Makepeace et al. 2012; Murphy 2012). They are first recruited to the site of infection during an acute phase of inflammation (Murphy 2012). Their ability to act as effectors against pathogens uses several mechanisms: phagocytic activity, release of lytic enzymes from their granules, and production of anti-microbial substances such as reactive oxygen intermediates (ROI) (Mantovani et al. 2011; Murphy 2012). Neutrophil activation which is mediated by bacteria (Mantovani et al. 2011) and release of pro-inflammatory cytokines, together with activation of other resident cells at the site of infection such as macrophages and mast cells lead to the induction of acute inflammation and recruitment of additional neutrophils and other cells i.e. monocytes and lymphocytes (Murphy 2012). During type 2 immune responses, which occur during helminth infections including LF

also leads to the migration of eosinophils and basophils to the site of infection (Makepeace et al. 2012; Murphy 2012).

Several studies have shown a role for neutrophils in filarial infection (Al-Qaoud et al. 2000; Saeftel et al. 2001, 2003). Neutrophils are important for protection (mainly by worm killing) as well as pathology of the infection (Al-Qaoud et al. 2000; Saeftel et al. 2001, 2003). Neutrophils are involved in parasite killing at least in two ways: directly by phagocytic activity or indirectly by encapsulation within granulomata (Makepeace et al. 2012; Saeftel et al. 2001). Several studies showed the importance of neutrophils in controlling adult worms by both ways which occurs during the chronic phase of *L. sigmodontis* infection (Al-Qaoud et al. 2000; Saeftel et al. 2001, 2003), and impacts larval stages, too (Porthouse et al. 2006).

Porthouse and colleagues showed in Mogolian jirds that neutrophils are found 3 hours post *B. pahangi* L3 inoculation at the site of injection within the skin. This study revealed an 80% reduction of larval recovery and large numbers of neutrophil-rich inflammatory foci at this time point. They argued that this increased activation and accumulation of neutrophils was due to increased *Wolbachia* released after or prior to larval death (Porthouse et al. 2006).

The neutrophil's capability in adult worm killing *in vivo* is IFNγ dependent. IFNγ leads to an increased production of TNFα which mediates neutrophil activation (Saeftel et al. 2001, 2003). This activation may promote chemotaxis and phagocytic activities that lead to an improved worm killing by neutrophils. Accordingly, in IFNγ deficient mice, for instance, *L. sigmodontis* worm burden was increased compared to wild type mice at 80 dpi (Saeftel et al. 2001, 2003).

Furthermore, neutrophils are required for granuloma formation that also leads to parasite killing (Al-Qaoud et al. 2000). In a study using *L. sigmodontis* infection in BALB/c mice it was shown that reducing neutrophils in the granulomata by injecting anti-granulocyte colony stimulating factor (G-CSF) led to impaired worm killing in those animals although eosinophils were still present in the granulomata (Al-Qaoud et al. 2000).

1.2.2. Eosinophils

Eosinophils are granulocytes that develop in the bone marrow. They play a role as effector cells, as 'not professional' antigen-presenting cells (APC), in promoting humoral immune responses as well as in inducing pathology in the host (Rosenberg et al. 2013). Eosinophils are mainly involved in the effector mechanisms during helminth infections and allergic diseases (Rosenberg et al. 2013). The role of eosinophils in host immune responses against parasites is still under debate and probably dependent on the helminth species (Meeusen and Balic 2000). Studies showed that eosinophils do not have a role in immunity to *Schistosoma mansoni* or the induction of pathology in the host (Sher et al. 1990; Swartz et al. 2006). In contrast, depleting eosinophils in *Strongyloides stercoralis* and *Angiostrongylus cantonesis* infection leads to an increased parasite survival (Rosenberg et al. 2013). *In vitro* studies showed that eosinophils are able to kill early stages of *S. mansoni* as well as other nematodes like *Haemonchus contortus*, both in human and mouse (Meeusen and Balic 2000).

In addition, the role of eosinophils in filarial infection remains unclear as contradictory results were found in several studies. Some studies showed that IL-5 and eosinophils are rather important in worm elimination and in the protective immunity against filarial infection in vaccinated and primary infection (Le Goff et al. 2000; Martin et al. 2000b, 2000a). In these studies, eosinophil migration into subcutaneous tissue was detected to be increased in vaccinated mice prior to *L. sigmodontis* injection (Le Goff et al. 2000; Martin et al. 2000a). This led to a decreased number of worms in vaccinated mice (Le Goff et al. 2000; Martin et al. 2000a). Furthermore, these studies also elucidated the role of eosinophils during primary *L. sigmodontis* infection (Le Goff et al. 2000; Martin et al. 2000b, 2000a). They showed that eosinophilia in primary infection occurs after 3 weeks post infection therefore eosinophils are less important during the first week of primary infection as in vaccinated mice. Thus, eosinophils are implemented in the worm destruction at the chronic phase of primary infection, but not at the early time points (Le Goff et al. 2000; Martin et al. 2000b, 2000a). In contrast, to the proposed protective role of eosinophils during filarial infection, a study by Babayan and colleagues showed that IL-5 driven eosinophilia accelerates early filarial growth. This study revealed that mice which lack eosinophils showed an accelerated worm development and molting (Babayan et al. 2010).

The protective role of eosinophils against filarial infection is potentially by mediating direct or indirect worm killing induced by releasing several attractant proteins, although the exact mechanism in how eosinophils can kill the filariae is unknown (Makepeace et al. 2012; Meeusen and Balic 2000). A mechanism that has been proposed recently is the release of eosinophil granule proteins that may act as proteases such as eosinophil peroxidase (EPO) and major basic protein (MBP) (Makepeace et al. 2012; Specht et al. 2006). In a study using *L. sigmodontis* infections, worm burden in EPO- and MBP-deficient mice showed a significant increase compared to wild type mice (Specht et al. 2006).

For activation and infiltration into the site of infection, eosinophils need appropriate stimuli, including IL-5 and eotaxin chemokines (Rosenberg et al. 2013). Moreover, IL-5 is also important in development and survival of eosinophils (Rosenberg et al. 2013). It is a major cytokine that is produced by Th2 cells as effector response during filarial infection (Babayan et al. 2010).

1.2.3. Macrophages

There are two types of macrophages that are important during infection: the classically activated macrophage (CAM or M1 macrophage), is induced by IFNγ and TNFα as well as by stimulants of TNFα like LPS. The second type of macrophages is the alternatively activated macrophage (AAM or M2 macrophage) which is induced by IL-4 and IL-13 that are produced by Th2 cells, mast cells, basophils, eosinophils, natural killer T (NKT) cells, lymphocytes and macrophages (Allen and Loke 2001; Mosser 2003; Murphy 2012). CAMs are important in pro-inflammatory immune responses, whereas AAMs have anti-inflammatory properties (Allen and Loke 2001; Mosser 2003). CAMs are crucial during acute infections that require a protective type 1 immune, e.g. bacterial infections, due to their improved phagocytic activity and their ability to promote Th1 immune responses by secretion of pro-inflammatory cytokines such as TNFα, IL-6 and nitric oxide (NO) (Allen and Loke 2001; Mosser 2003). In contrast, AAMs are crucial for immune suppression and tissue repair. Moreover, they are also suggested to be important for the host's immunity against the chronic phase of helminth, fungal and viral infections, as well as in allergic and autoimmune diseases (Gordon and Martinez 2010; Mosser 2003). AAMs produce IL-10 which is crucial in immune suppression. They are also important in mediating wound

healing, angiogenesis, and extracellular matrix deposition (Gordon and Martinez 2010; Mosser 2003; Rodríguez-sosa et al. 2002).

The impact of macrophages during filariasis is generally divided into their role as effector and suppressor cells. CAMs mainly act as effector cells and AAMs have a role as suppressor cells. As effector cells, macrophages have three different roles: in parasite killing, induction of pathology, as well as natural resistance (Allen and Loke 2001). Since worms are multicellular organisms, engulfing the worm is a difficult way for the macrophage to kill the parasite. Alternatively, macrophages release toxic radicals such as reactive oxygen species (ROS) and NO derivates. The role of IFNγ-mediated-macrophage killing of worms by releasing NO has been reviewed by Allen and Loke previously (Allen and Loke 2001). *In vitro* studies showed that macrophages which were activated by IFNγ kill *B. malayi* Mf by releasing NO (Taylor et al. 1996; Thomas et al. 1997). Similarly, peritoneal macrophages kill Mf of *B. pahangi* in the presence of antimicrofilarial sera *in vitro* (Karavodin and Ash 1982; Oxenham et al. 1984). However, another study showed that IFNγ and NO are not required for immune mediated clearance of *B. malayi* Mf (Gray and Lawrence 2002). Furthermore, macrophage killing ability seems to only target larval stages and not adult stages of filariae potentially due to the antioxidant enzyme catalase that is produced by adult worms (Ou et al. 1995). During *B. pahangi* infection *in vivo*, macrophage activation was shown to be induced by L3 which then led to significantly increased phagocytic and microbicidal capacity of the macrophages (Jeffers et al. 1984).

Besides their role in larval killing, macrophage products are able to cause pathology for the host as is shown by inflammatory lesions that can be found in lymphatic vessels both in animal models and human filariasis (Rao et al. 1996). In addition, dying worms are usually surrounded by pathological granulomata which mostly consist of CAMs as a rich source of inflammatory cytokines, toxic radicals and inflammatory lipid mediators which cause tissue damage in the host (Allen and Loke 2001; Mosser 2003).

AAMs have also been demonstrated to be important in filarial disease [reviewed in (Allen and Loke 2001)]. After i.p. implantation in mice, AAMs are induced by excretory/secretory (E/S) products from live adult *B. malayi* worms (Allen and MacDonald 1998), whereas CAMs are induced by *Wolbachia* that are released by dead worms (Taylor et al. 2000, 2001). AAMs were shown to suppress proliferation of blood lymphocytes *in vitro* (Schebesch et al. 1997). Furthermore, AAMs were also shown to induce Th2 differentiation from naïve T cells in this model (Goerdt and Orfanos 1999). Later, AAMs

inhibit immunity to the disease and prevent helminth-induced pathology in the host (Allen and Loke 2001; Kreider et al. 2007; Taylor et al. 2009; van der Werf et al. 2013).

Since CAMs can mediate worm killing and induce Th1 responses that lead to pathology of the host, and AAMs may inhibit these responses, the balance between CAMs and AAMs may determine the severity of the disease (Allen and Loke 2001). This phenomenon can be seen more clearly in elephantiasis patients: while increased numbers of CAMs during LF may result in increased parasite clearance, it may also lead to higher pathology (Allen and Loke 2001). In contrast, asymptomatic Mf positive individuals have more AAMs that impair parasite clearance but lead to less pathology. However, in endemic normal a successful balance between CAMs and AAMs has been achieved that leads to the resolution of the disease (Allen and Loke 2001).

1.2.4. Mast cells

Mast cells are multifunctional granulated hematopoietic cells that reside in nearly all tissues and are typically found throughout barrier tissues such as skin and mucosa as well as in a perivascular location in the tissue. Due to these locations and their ability to rapidly release prestored inflammatory mediators, mast cells contribute to the first line of response against pathogens (Marshall 2004; Murphy 2012). Additionally, they express several cell surface receptors such as the high-affinity receptor (FcεRI) for IgE, FcγRIII for IgG, complement component receptors and various Toll-like receptors (TLRs) (Marshall 2004). This demonstrates the capability of mast cells to respond to a wide range of endogenous and exogenous stimuli such as allergens, tissue injury, viral, fungal, parasite and bacterial antigens (Marshall 2004; Murphy 2012). Cross-linking of cell surface IgE bound to FcεRI by antigen leads to activation and degranulation of mast cells that results in releasing granule proteins and secretion of lipid inflammatory mediators and cytokines and chemokines such as histamine, prostaglandins, leukotrienes, bradykinine, vascular endothelial growth factors (VEGFs) and pro-inflammatory cytokines like TNFα and IL-6, although cytokine secretion can also occur without degranulation (Marshall 2004; Murphy 2012). Histamine and leukotrienes are important for vascular permeability while leukotrienes have an additional function in eosinophil recruitment to the site of inflammation. Prostaglandins and VEGFs are further important for angiogenesis (Kunder et al. 2011; Marshall 2004).

Mast cell activation is associated with type 2 immune responses which are characterized by the presence of Th2 cells and cytokines, increased levels of IgE and eosinophilia. This type 2 immunity is mainly present in allergic diseases and helminth infections (Pennock and Grencis 2006; Voehringer 2013). In contrast to allergic diseases where mast cells only contribute to the pathologic effect to the host, they play an important role both in pathology and protective immunity against helminths (Pennock and Grencis 2006; Voehringer 2013). In nematode infection, mast cells are important in the early as well as the late phase of infection (Pennock and Grencis 2006). During the early phase of infection, mast cells are crucial for the secretion of IL-4 and IL-13 as a response to helminth-derived factors i.e. proteases or to host-derived molecules including anaphylatoxins and cytokines such as IL-18, IL-33, TSLP, IL-3 (Hepworth et al. 2012). Activation of mast cells in the early phase of infection, however, might occur independent of IgE (Hepworth et al. 2012). During the late stage of *Heligmosomoides polygyrus* infection, mast cells are important for parasite expulsion by secreting mouse mast cell proteases (mMCP)-1 which can disrupt epithelial barrier function and allow the influx of solutes and water into the lumen (Hepworth et al. 2012; Pennock and Grencis 2006). Moreover, activation and degranulation of mast cells during the late phase of infection is IgE dependent (Hepworth et al. 2012; Pennock and Grencis 2006).

In mouse models of filariasis, mast cell activation and degranulation were recently reported to be crucial during the early phase of infection (Specht et al. 2011). Bacterial components of *Wolbachia* endosymbionts, which are either derived from live or dead larvae, were capable to increase vascular permeability of the skin of *L. sigmodontis* infected mice by stimulating the TLR2 pathway. This mechanism involved CCL17 (a T cell associated chemokine) since CCL17 depletion increased mast cell activation and degranulation, and an increased worm burden in infected animals was seen as early as 10 dpi. This response, however, occurred independent of IgE (Specht et al. 2011).

1.2.5. CD4 T cell and its subsets

CD4 T cells are crucial for adaptive immune responses. Unlike CD8 T cells that are mainly important in killing pathogens, CD4 T cells are crucial for the activation of immune responses (Murphy 2012). Naïve CD4 T cells can differentiate into several effector T cells such as T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17) cells. Furthermore, CD4

T cells can also differentiate into regulatory T cells (T_{reg}) (Murphy 2012). A role for T cells in filarial infection has been known for decades. Several reports have shown the importance of T cells especially $CD4^+$ T cells in host immune responses during filariasis (Al-Qaoud et al. 1997; Hoerauf et al. 2005; Pearlman et al. 1995; van der Werf et al. 2013). In BALB/c mice lacking $CD4^+$ T cells, worm burden and microfilaremia were shown to be increased compared to wild type control mice (Al-Qaoud et al. 1997). Additionally, host immune responses against *L. sigmodontis* which are characterized by Th2 cytokines and IgE production and eosinophilia is diminished in mice depleted of $CD4^+$ T cells (Al-Qaoud et al. 1997).

1.2.5.1. T helper cells type 1 (Th1) and T helper cells type 2 (Th2)

Generally, immunity against helminths, including filarial nematode infections, is mediated by type 2 immune response, unlike bacterial infections that generate Th1 immune responses (Allen and Maizels 2011). However, during filarial infection both type 1 and type 2 are essential during filarial infection, as both type 1 and type 2 immune responses are implemented in worm elimination and development of pathology within the host (Specht and Hoerauf 2012).

Th2 differentiation from naïve T cells in filarial infection is initiated after contact with live L3 i.e. in *B. pahangi* and *L. sigmodontis* infection (Specht and Hoerauf 2012). In contrast, Th1 immunity is mainly induced by *Wolbachia* that are released by death filaria (Allen and Loke 2001; Allen and Maizels 2011). In *L. sigmodontis* infection, Th2 responses occur after ~ 12 dpi (Specht and Hoerauf 2012). After being activated, Th2 cells produce type 2 cytokines such as IL-5 and IL-4, two predominant Th2 cytokines in filarial disease. Both cytokines are important in worm elimination and in controlling patency (Allen and Maizels 2011; Specht and Hoerauf 2012). Several studies reported a role for IL-5 in controlling worm survival, both at the larval and adult stages (Martin et al. 2000b, 2000a; Volkmann et al. 2003). Additionally, IL-4 impairs the development of patent filarial i.e. in *B. malayi* and *L. sigmodontis* infection (Babu et al. 2000; Le Goff et al. 2002; Volkmann et al. 2001, 2003).

Th1 cells, which are also important effector cells, produce the type 1 cytokine IFNγ that is crucial in protective immunity against filarial parasites. Mf induce Th1 cell differentiation as a result of microfilarial degeneration and *Wolbachia* release (Allen and Maizels 2011).

Induction of Th1 responses can potentially inhibit Th2 immune responses which have been established during infection by larval and adult parasite stages to prevent worm killing and parasite transmission as well as to prolong the infection (Allen and Maizels 2011; Specht and Hoerauf 2012). However, lack of IFNγ has been shown to result in an increased worm burden during *L. sigmodontis* infection (Hoerauf et al. 2005; Saeftel et al. 2001, 2003), suggesting that pro-inflammatory immune responses are also implicated in protective immune responses against helminths. Antigen-specific pro-inflammatory Th1 immune responses are important in the pathology caused by the human filaria *B. malayi* (lymphedema). The cytokines which are involved are TNFα and IFNγ (Babu et al. 2009; Pfarr et al. 2009).

1.2.5.2. Interleukin-17 (IL-17)-producing helper T cells (Th17)

Th17 cells are a subset of effector T cells that are critical for clearing extracellular pathogens and fungi as well as the generation of multiple autoimmune diseases (Murphy 2012). Th17 cells are distinguished from other T helper cells based on their ability to produce IL-17 cytokines. IL-6 and TGFß are the main inducers of Th17 differentiation, although IL-21 is an alternative to IL-6 in inducing Th17 differentiation (Chen and Oppenheim 2009; Murphy 2012).

Besides Th1 cells, Th17 cells may be also critical during the development of pathology in human filariasis (Babu et al. 2009). A study has demonstrated that levels of *B. malayi* antigen specific Th17 pro-inflammatory cytokines such as IL-17A, IL17F, IL-21 and IL-23 are elevated in lymphedema patients (Babu et al. 2009). Th1 and Th17 cells were proposed to affect the pathology of human filariasis due to their ability to induce production of VEGF-C and –D, which are important for the development of filarial lymphedema (Babu et al. 2009; Pfarr et al. 2009).

1.2.5.3. Regulatory T cells (T_{reg})

In order to achieve a chronic infection and long term survival within the host, it is important for worms to suppress the host`s immune response. As described above, both Th1 and Th2 immune responses are implicated in protective immune responses to helminths. Additionally, inflammatory responses against the parasite also lead to the release of several proteins that result in increased tissue damage. Therefore suppressive T

cells (named regulatory T cells or T_{reg}) take part in this mechanism in order to increase the worm load and reduce tissue damage by suppressing both Th1 and Th2 immune responses (Hoerauf et al. 2005).

T_{reg} express fork head box P3 (FoxP3) as transcription marker and CD25. They also express glucocorticoid-induced TNFR family related gene (GITR) (Adalid-Peralta et al. 2011; Hoerauf et al. 2005). T_{reg} are classified into two main groups based on their origins: thymic or natural T_{reg} (nT_{reg}), which are originated from the thymus and circulate in the blood before exposure to pathogens or damage, and inducible T_{reg} (iT_{reg}) which have a regulatory function after pathogen or neoplasm exposure (Adalid-Peralta et al. 2011). The role of T_{reg} in regulating immune responses in human as well as experimental animal filarial infections has been investigated in various studies (Taylor et al. 2005; Wammes et al. 2012). In human filariasis, for instance, T_{reg} induce a regulatory environment as well as hypo-responsiveness of antigen-specific T cells and reduce Th1 and Th2 immune response (Taylor et al. 2005). Another human study with *B. malayi* infection revealed that T_{reg} can suppress proliferation of Th2 cells *in vitro* and lead to decreased production of filarial specific type 2 cytokines, especially in Mf positive individuals (Wammes et al. 2012).

Animal studies showed that removal of T_{reg} can increase the immune response against *L. sigmodontis* and prevent prolonged infection (Dittrich et al. 2008; Taylor et al. 2005, 2009). It also reduces the number of filarial parasites *in vivo* observed at 60 dpi (Taylor et al. 2009). In this report, anti-CD25 and anti-GITR (surface markers for T_{reg}) were used to inhibit T_{reg} responses. As a result of inhibition, Th1 and Th2 cells became responsive again and led to the elimination of worms in the pleural cavity (Taylor et al. 2009). Reducing the number of worms was caused by increased antigen-specific immune responses as well as a reduction of CTLA-4 expression by T_{reg} and resulted in increased IL-5 production that is crucial for worm elimination. The worms themself are also believed to be able to manipulate T_{reg} and then causes immunosuppression of the host (Taylor et al. 2005).

T_{reg} have been shown to be modulated either by L3 or adult Mf-producing female *L. sigmodontis* (Hoerauf et al. 2005; Taylor et al. 2009). Recruitment of nT_{reg} to the site of infection occurs immediately after infection and leads to increased number of T_{reg} found in the pleural cavity (Taylor et al. 2009). Additionally, increased number of nT_{reg} at the site of infection is also contributed by *in vivo* proliferation of nT_{reg}, which occurs within 7 days post infection (dpi) (Taylor et al. 2009). Moreover, iT_{reg} also contribute to the increased number of T_{reg} during the first week of infection (Taylor et al. 2009). Skewing CD4 T cell

responses towards iT_{reg} by L3 in the pleural cavity, which also occurs within 7 days, even increased T_{reg} numbers at site of infection (Hoerauf et al. 2005; Taylor et al. 2005, 2009).

1.2.6. B cells

B cells are part of the humoral immune response (Murphy 2012). The B cell population is divided into at least 5 subsets: B1, B2, regulatory, plasma and memory B cells (Montecino-Rodriguez et al. 2006; Murphy 2012). B cells are involved in humoral immune responses as they are able to produce antibodies, B1 cells produce IgM and B2 cells produce other Ig isotypes (Murphy 2012). They are also thought to be important in innate responses against viral and bacterial infection as they act as APC (Murphy 2012). B2 cells are conventional B cells that act as effector cells in adaptive immunity (Montecino-Rodriguez et al. 2006; Murphy 2012). Activated B2 cells can produce IgE in some settings after CD23 (low affinity IgE receptor) which is expressed in B2 cells, is cross linked with IgE. In *S. mansoni* infections, B2 cells are associated with the development of resistance to reinfection (Mwinzi et al. 2009).

In filariasis, B cells were shown to be important for protection against *B. malayi* since lack of B cells using different types of knockout mice such as µMT (B cell deficiency) and RAG-1 (deficient in generation of mature B and T cells) mice resulted in increased worm burdens (Babu et al. 1999). B cells protect against worms potentially by acting as early APC to initiate T cell responses (Paciorkowski et al. 2000). Additionally, B cells produce immunoglobulins that contribute to worm elimination (Babu et al. 1999; Paciorkowski et al. 2000). B1 cells have been reported to be responsible for host protection against *B. malayi* (Paciorkowski et al. 2000). A role of B cells in *B. pahangi* infection has been described previously (Gillan et al. 2005). Depletion of B cells *in vitro* and usage of µMT mice led to a reduced antigen-specific proliferation of splenocytes (Gillan et al. 2005).

The role of B cells in eliminating *L. sigmodontis* has been demonstrated in a study using BALB/c µMT mice in two different settings: vaccination with irradiated L3 and primary infection (Martin et al. 2001). The authors found a significantly decreased number of worms after challenge infection in vaccinated wild type mice at 28 dpi compared to vaccinated µMT mice (Martin et al. 2001). This was correlated with impaired degranulation of eosinophils in vaccinated µMT mice compared to wild type controls during early infections (Martin et al. 2001). The authors argued that the eosinophil-

antibody-B cell complex was diminished in vaccinated µMT mice and caused the observed reduced vaccine protection (Martin et al. 2001). In primary infection, the survival and growth of filariae was not changed in µMT mice, although the authors found that mice did not develop patent infections with microfilaraemia (Martin et al. 2001). In contrast, Al-Qaoud and colleagues reported that the lack of B1 cells in BALB/c Xid mice resulted in a higher filarial recovery rate and Mf load in the periphery compared to wild type mice (Al-Qaoud et al. 1998).

1.3. Interleukin-6

Interleukin-6 is a pleiotropic cytokine that has several effects and acts as a pro-inflammatory as well as an anti-inflammatory cytokine. It has a wide range of biological activities in inflammation, immune regulation, hematopoiesis and oncogenesis (Kishimoto 2010; Scheller et al. 2011). It was discovered in 1986 when the Kishimoto group at Osaka University Japan first cloned the complementary DNA encoding B cell stimulatory factor-2 which became later known as IL-6 (Kishimoto 1989, 2010). In the same year, IFNβ2 and another identical protein were also cloned by the Hirano group and found to be identical with IL-6 (Hirano 1998, 2010; Kishimoto 2010). Other identical proteins that have similar function with IL-6 are leukemia inhibitory factor (LIF), cilliary neurotropic factor (CNTF), IL-11 and oncostatin M. The name "interleukin-6" was officially proposed in a nomenclature meeting in New York in 1988 (Hirano 2010; Kishimoto 2010).

1.3.1. Sources

Several stimuli including ultraviolet (UV), irradiation, ROS, microbial products, viruses, or other pro-inflammatory cytokines are known to induce IL-6 production by leukocytes and non-leukocyte cells (Kishimoto 2010; Rincon and Irvin 2012). In acute infections, monocytes and CAMs are the main sources of IL-6. Immediately after TLR stimulation by specific pathogen-associated molecular patterns (PAMPs), activated monocytes and macrophages produce IL-6. The above described AAMs, however, produce negligible amounts of IL-6 *in vitro* (Lolmede et al. 2009). Other cells of the innate immune system such as dendritic cells (DCs) and neutrophils can also produce IL-6 during acute infection. DCs secrete small amounts of IL-6 in the beginning of infection as they are not yet fully activated. When encountering pathogens, DCs are fully activated and produce high amounts of IL-6 (Hirano 1998; Murphy 2012; Tanaka and Kishimoto 2012).

Other innate immune cells, eosinophils and mast cells, are also known to have the ability to produce IL-6 following their activation. IL-6 is secreted *in vitro* by mouse eosinophils following respiratory virus infection (Dyer et al. 2009; Rosenberg et al. 2013) and by human eosinophils in atopic asthma disease (Hamid et al. 1992; Lacy et al. 1998; Spencer et al. 2009). Eosinophils also express IL-6 mRNA in different diseases *in vivo* (Rothenberg and Hogan 2006). Moreover, IL-6 may be constitutively synthesized and stored in granules of unstimulated eosinophils as IL-6 positive eosinophils have been found in blood from

healthy donors although the site of IL-6 storage in eosinophils could not be determined by immunocytochemical staining (Lacy et al. 1998; Spencer et al. 2009). This preformed IL-6 in eosinophils is mobilized into budding vesicles which travel to the plasma membrane and is then released into the extracellular space. This mechanism of granule-stored cytokine secretion is named piecemeal degranulation (Melo et al. 2005; Spencer et al. 2009).

Mast cells are also able to produce IL-6 *in vitro* in response to gram-negative bacteria and their cell wall component LPS by secretion without degranulation. In contrast, peptidoglycan, a cell wall component of gram-positive bacteria induces IL-6 production from mast cells via degranulation (Leal-Berumen et al. 1994; Supajatura et al. 2002). Furthermore, IL-6 is produced by mast cells in response to IL-1ß, prostaglandin E_2 and leukotriene C_4 stimulation (Marshall 2004). Cells from the adaptive immune system such as T cells and B cells are also able to produce IL-6 following stimulation with different stimuli (Murphy 2012).

In helminths, many studies showed IL-6 production from several cell types. During infection with the helminth *Taenia crassiceps*, F4/80$^+$ peritoneal macrophages produce low levels of IL-6 during the acute phase, whereas during the chronic phase high levels of IL-6 are produced (Rodríguez-Sosa et al. 2002). In filarial disease, IL-6 is secreted by activated macrophages, DCs and endothelial cells due to *Wolbachia* release by worms dying either naturally or after anti-filarial DEC treatment that stimulates TLR2 and 6 (Daehnel et al. 2007; Hise et al. 2007; Taylor et al. 2001). Furthermore, IL-6 is produced by eosinophils and macrophages *in vitro* after stimulation with worm antigen during *L. sigmodontis* infection (Gentil et al. 2013, resubmission) as well as by CD4$^+$ and CD8$^+$ T cells in response to L3 of *B. malayi* (Babu and Nutman 2003). Another study reported that intact granulomata of *B. pahangi* were also able to spontaneously produce IL-6 *in vitro* (Rao and Klei 2006). Stimulation of the granulomata with worm antigen led to even increased IL-6 production. Although not investigated in this study, it seems that IL-6 was produced by macrophages and eosinophils as predominant cells that form the granulomata (Rao and Klei 2006).

However, IL-6 can also be secreted by non-leukocyte cells such as endothelial cells, fibroblasts, astrocytes, epithelial cells and some malignant cells (Hirano 1998). Damaged or dying cells from non-infectious inflammation, i.e. burn wounds and other injury, can also secrete IL-6 following stimulation of TLRs via damage-associated molecular patterns (DAMPs) (Tanaka and Kishimoto 2012).

1.3.2. Signaling

The pleiotropic and redundant capability of IL-6 is characterized by two functional receptors: IL-6 specific receptor (IL-6R) which has a molecular weight of 80 kDa and glycoprotein 130 kDa (gp130) as a signal transducer for cytokines related to IL-6. Furthermore, signal transduction of IL-6 via gp130 is mediated by two pathways: Janus family tyrosin kinase-signal transducers and activators of transcription (JAK-STAT) and mitogen-activated protein kinase (MAPK) pathway (Kishimoto 2010).

IL-6 signaling in the target cells occurs mainly via STAT3 and to a lesser degree via STAT1 (Kishimoto 2010). On target cells, IL-6 protein first binds to membrane-bound IL-6 (mIL-6R) receptor with a nanomolar affinity (Rose-John 2012). Several cells express mIL-6R on their surface, including neutrophils, monocytes, naive CD4 and CD8 T cells, B cells and hepatocytes (Scheller et al. 2011). This IL-6/IL-6R complex then binds to gp130 and leads to IL-6-signal transduction via JAK1 and JAK2 (Scheller et al. 2011). When excessive production of IL-6 occurs, a molecule named suppressor of cytokine signaling (SOCS) negatively regulates the signal. SOCS binds to JAKs and inhibits their activities (Kishimoto 2010; Scheller et al. 2011). Role of SOCS in IL-6 signaling, for instance, is involved in T cell differentiation. Up-regulation of SOCS1 occurs in Th2 differentiation and suppression of IFNγ signaling (Scheller et al. 2011). Furthermore, lower SOCS3 signaling is important in Th17 differentiation from naïve T cell thus inducing IL-6 production (Barnes et al. 2011; Kishimoto 2010; Scheller et al. 2011).

Previously, it was believed that membrane IL-6 receptor (mIL-6R) was the only receptor that could bind to IL-6. Recently, studies have shown that soluble IL-6 receptor (sIL-6R) which is found in body fluids such as urine and blood can also bind to IL-6 and then stimulates the target cells without requiring mIL-6R (Rose-John 2012; Scheller et al. 2011). sIL-6R is generated by two mechanisms; first by proteolytic cleavage of mIL-6R mainly after apoptosis of neutrophils and second by secretion from neutrophils and monocytes of an alternatively sliced mIL-6R product lacking transmembrane and cytosolic domains (Barnes et al. 2011; Scheller et al. 2011). Additionally, naïve and memory CD4 T cells produce sIL-6R when the T cell receptor is activated.

The sIL-6R/IL-6 complexes then bind to gp130 which activates and phosphorylates JAK and STAT3 that lead to IL-6 trans-signaling (Rose-John 2012). Since gp130 can be expressed by almost all cells and tissues, sIL-6R expands IL-6 responding cells to a

broader variety of cells such as eosinophils, endothelial cells and T cells. This trans-signaling pathway is important in pro-inflammatory responses, different from classic signaling that involves mIL-6R and is crucial to induce anti-inflammatory responses (Scheller et al. 2011).

1.3.3. Effector cells

A wide range of biological activities makes IL-6 important in various conditions including inflammatory responses, autoimmune and malignant diseases. In this part, I only focus on its role during inflammatory responses that involves several cell types.

1.3.3.1. Neutrophils

During the acute phase of infection, IL-6 is important for neutrophil mobilization from the bone marrow to the site of infection (Murphy 2012). After being activated by pathogens via pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), macrophages and DCs secrete IL-6 together with other pro-inflammatory cytokines such as TNFα and IL-1ß (Murphy 2012). These cytokines initiate the acute-phase response against pathogens by inducing neutrophil mobilization from bone marrow into the circulation and release of acute phase proteins in the liver. They also activate endothelial cells to produce chemokines e.g. IL-8 and monocyte chemotactic protein (MCP)-1 and IL-6 to attract neutrophils to the site of infection (Murphy 2012). Additionally, IL-6 trans-signaling leads to activation of endothelial cells and results in up-regulation of cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin (CD62E) on endothelial cells which then increases neutrophil adherence thus leading to increased neutrophil migration to the site of infection (Murphy 2012; Scheller et al. 2011).

At the site of infection, cleavage of mIL-6R into sIL-6R after neutrophil apoptosis leads to an increase of the IL-6 trans-signaling mechanism (Scheller et al. 2011). However, the role of IL-6 in mediating neutrophil apoptosis *in situ* is still under debate. Some studies showed that IL-6 is required to promote neutrophil apoptosis (Mcloughlin et al. 2003), while others showed anti-apoptotic activity of IL-6 on neutrophils (Colotta et al. 1992). Nevertheless, IL-6 mediates neutrophil clearance at the site of infection by inhibiting production of neutrophil attracting chemokines such as CXCL1, CXCL8/IL-8 and CX3CL1 and inducing

production of monocyte-associated chemokines such as CCL2/MCP-1, CCL8/MCP-2, CXCL5, and CXCL-6. This results in transition from neutrophils to monocytes leading to successful resolution of an inflammation (Scheller et al. 2011). Moreover, the transition between both immune responses is important in order to reduce tissue damage caused by neutrophils through releasing their proteases and ROS at the site of infection (Barnes et al. 2011; Fielding et al. 2008; Scheller et al. 2011).

1.3.3.2. Eosinophils

The capability of eosinophils to produce IL-6 has been mentioned in several studies in addition to previous descriptions above (Dyer et al. 2009; Hamid et al. 1992; Lacy et al. 1998; Spencer et al. 2009). Cytokine release, including IL-6, is also a sign for eosinophil activation. Nevertheless, few studies investigated the role of IL-6 in eosinophil activation or cytokine release as a result of its activation (Dyer et al. 2009, 2010). However, one study showed that IL-6 is able to activate mouse eosinophils cultured from bone marrow progenitor cells (Dyer et al. 2010). Following stimulation of recombinant mouse IL-6 (rIL-6), the eosinophils produced significant amounts of cytokines including IFNγ, IL-1ß, IL-9, IL-12, MCP-1/CCL2 and TNFα (Dyer et al. 2010). This data suggests that either eosinophils express mIL-6R which is not suggested by any review related to eosinophils (Dombrowicz and Capron 2001; Rosenberg et al. 2013; Rothenberg and Hogan 2006) or activation of eosinophils is mIL-6R independent and occurs primarily by the trans-signaling mechanism that involves sIL-6R (Scheller et al. 2011).

1.3.3.3. Monocytes and macrophages

As described above, IL-6 trans-signaling plays an important role in transition between innate immunity, which is dominated by neutrophils, to adaptive immunity by inducing monocyte associated chemokines which lead to monocyte migration to the site of infection. Furthermore, IL-6 trans-signaling is also crucial to increase the expression of macrophage colony-stimulating factor (M-CSF) receptor on monocytes which skew their differentiation towards macrophages (Scheller et al. 2011).

In chronic inflammation, the role of IL-6 in mediating macrophage activation especially AAM activation is still not elucidated yet. Some studies showed that IL-6 induces macrophage activation and differentiation into AAMs as was shown by a study in human

prostate cancer (Roca et al. 2009). Similarly, it was shown that IL-6 is important to promote survival of $CD11b^+$ peripheral blood mononuclear cells (PBMCs) and induce AAM polarization (Roca et al. 2009). In contrast, another study using a mouse model of spinal cord injury suggested that blockade of IL-6 signaling (potentially classic-signaling) leads to inhibition of CAMs and induces AAM polarization (Guerrero et al. 2012).

1.3.3.4. Mast cells

It is unknown whether mast cells express mIL-6R on their surface. However, several reports showed that IL-6 is important for mast cell development and survival (Cruse et al. 2008; Saito et al. 1996). In a study by Saito and colleagues, IL-6 has been shown to support mast cell development in the presence of prostaglandin E_2 (Saito et al. 1996). They showed that IL-6 induced mast cell growth after culturing umbilical cord blood mononuclear cells and was also able to enhance mast cell growth from purified $CD34^+$ (a cell surface glycoprotein which is important in cell to cell adhesion) cells (Saito et al. 1996). Furthermore, Cruse and colleagues showed that the survival of cultured human lung mast cells was reduced after addition of anti-IL-6 (Cruse et al. 2008). Moreover, production of IL-6 in this culture was significantly increased in the presence of IgE. This data suggested that survival of mast cells was mediated by IL-6 in an autocrine manner (Cruse et al. 2008).

1.3.3.5. T cells

As previously described, IL-6 induces transition from innate immunity to adaptive immunity by inducing migration of monocytes and lymphocytes (Scheller et al. 2011). To induce T cell infiltration to the site of infection, IL-6 induces, via trans-signaling, up-regulation of T cell attracting molecules including CCL17 i.e. by peritoneal cells (McLoughlin et al. 2005). McLoughlin and colleagues showed that *Staphylococcus epidermidis* infection led to decreased T cell migration into the peritoneal space of $IL\text{-}6^{-/-}$ mice. This was due to a decreased production of T cell attracting molecules in the peritoneal lavage (McLoughlin et al. 2005). Additionally, IL-6 also increases the production of adhesion molecules like CD62L (L-selectin) on the T cell surface that enhances the capability of T cells to trespass onto endothelial junctions (Scheller et al. 2011). Furthermore, IL-6 is responsible for T cell survival and proliferation. IL-6 helps T

cells to escape from apoptosis in an anti-apoptotic STAT3-dependent pathway (Scheller et al. 2011).

IL-6 is involved in T cell differentiation of naïve T cells into Th1, Th2, T_{reg} and IL-17-producing Th17 cells. Via trans-signaling, IL-6 skews the Th1/Th2 balance towards Th2 (Dienz and Rincon 2009; Scheller et al. 2011). Th17 is induced by IL-6 together with TGFβ, whereas IL-6 negatively regulates TGFβ induced T_{reg} differentiation. This significant function of IL-6 during T cell differentiation increases the spectrum of IL-6 to several diseases including autoimmunity, cancer and parasite infection (Kimura and Kishimoto 2010; Kishimoto 2010). In an animal model of systemic lupus erythematosus, an autoimmune disease, IL-6 has been described to inhibit the generation of T_{reg} (Tackey et al. 2004).

1.3.3.6. B cells

In acute infections, infiltration of B cells into the site of infection is also mediated by IL-6 through a mechanism similar to those attracting T cells (Scheller et al. 2011). Other functions of IL-6 involve B cell differentiation and survival (Hirano 1998). Several studies have shown that IL-6 promotes B cell differentiation into plasma cells that enhance antibody production (Kishimoto 2010; Scheller et al. 2011; Yoshizaki et al. 1984). Recently, several studies reported that IL-6 is not a single player in this mechanism which also involves the cytokine IL-21 (Dienz et al. 2009; Gottenberg et al. 2012; Karnowski et al. 2012). Since IL-6 induces IL-21 production by $CD4^+$ T cells, the role of IL-6 in plasma cell differentiation and antibody secretion becomes significant (Barnes et al. 2011; Dienz and Rincon 2009). However, Eto and colleagues showed that plasma cell numbers were only modestly reduced in IL-$6^{-/-}$ compared to C57BL/6 wild-type mice; but absence of IL-21 significantly reduced their numbers (Eto et al. 2011). Moreover, they could show that drastic reduction of plasma cell frequencies occurred in the absence of both cytokines. However, IL-6 is as important in antibody production as IL-21 as in the absence of IL-6 antibody production is as reduced as in IL-21-deficient mice (Eto et al. 2011). Furthermore, IL-6 is not only important in plasma cell differentiation but also in its survival as an *in vitro* study showed that IL-6 which was released by eosinophils is important to support plasma cell survival (Chu et al. 2011). Another effect of IL-6 on B cells includes the regulation of B cell immune responses. In an animal model of systemic

lupus erythematosus, IL-6 has been described to induce B cell hyperactivity (Tackey et al. 2004).

1.3.4. Interleukin-6 deficient mice

Manfred Kopf and Georges Kohler (Max Planck Institut für Immunbiologie, Freiburg Germany) developed an interleukin-6 gene targeted mutation in the mouse genome in 1994 (Kopf et al. 1994). They generated IL-6 knockout (IL-$6^{-/-}$) mice by disrupting the IL-6 gene in the first coding exon (exon 2) and inserted a *neo* cassette into the target vector. IL-$6^{-/-}$ mice were first developed on C57BL/6 background which was then followed by developing IL-6 deficiency in other strains of mice including BALB/c (Kopf et al. 1994). In the same year, Valeria Polil and co-workers from the Department of Genetics and Development at the Columbia University, New York, USA also developed IL-$6^{-/-}$ mice by replacing a 2.1 kb fragment with the new cassette [MC1-Neo poly$(A)^{+}$] (Polil et al. 1994). The 2.1 kb fragment contains the proximal promoter region and the first three exons of the gene (Polil et al. 1994).

Using these IL-$6^{-/-}$ mice, Kopf and colleagues then investigated immune responses in IL-6 deficiency (Kopf et al. 1994) . They found a reduced bactericidal ability of macrophages in IL-$6^{-/-}$ compared to wild type mice which is probably due to a reduced NO intermediates released, although they could not find decreased nitrite released in IL-$6^{-/-}$mice after macrophages stimulation *in vitro* (Kopf et al. 1994). Furthermore, they also showed that thymocytes and peripheral T cells were reduced in IL-$6^{-/-}$ mice suggesting that IL-6 is essential for T cell proliferation, whereas B220, CD23, IgM and IgD expression of bone marrow and splenic B cells were normal compared to wild type mice (Kopf et al. 1994).

Nowadays, there are many studies using IL-$6^{-/-}$ mice to investigate the role of IL-6 in various diseases and pathologies (Jones et al. 2011; McLoughlin et al. 2005; Ohshima et al. 1998; Wang et al. 2000). IL-$6^{-/-}$ mice have been shown to be protected against many autoimmune diseases such as antigen and collagen-induced arthritis, experimental autoimmune encephalomyelitis and multicentric Castleman disease, a lymphoproliferative disorder affecting the lymph nodes and related tissues (Jones et al. 2011; Ohshima et al. 1998). In antigen-induced arthritis, lymph nodes of C57BL/6 IL-$6^{-/-}$ has been shown to produce more Th2 (IL-4 and IL-10) *in vitro* than IL-$6^{+/+}$ mice, thus preventing the Th1-associated disease (Ohshima et al. 1998). IL-$6^{-/-}$ mice were also used to investigate chronic

inflammatory and allergic diseases like asthma as well as metabolic diseases like diabetes mellitus (Chida et al. 2006; Qiu et al. 2004; Wang et al. 2000). Increased eosinophilia, lung Th2 cytokines (IL-4, IL-5 and IL-13) and eotaxins in IL-6$^{-/-}$ mice were found in the ovalbumin model of asthma, suggesting a protective role of IL-6 against allergies (Wang et al. 2000).

Moreover, studies to investigate the role of IL-6 in several infectious diseases caused by bacteria, fungi, viruses and helminths also used IL-6$^{-/-}$ mice (Jones et al. 2011; Scheller et al. 2011). In a model of staphylococcal microbial keratitis, for instance, a more severe disease with increased bacterial burden and PMNs was found in IL-6$^{-/-}$ compared to wild type controls (Hume et al. 2006). Furthermore, in a fungal antigen-exposure model of allergic airway inflammation, reduced IgG1 levels and increased IgE levels were found in IL-6$^{-/-}$ mice (Neveu et al. 2009). In viral infections with vesicular stomatitis virus and vaccinia virus, 5-10 fold reduced levels of IgG were found in IL-6$^{-/-}$ mice (Kopf et al. 1994).

1.3.5. Interleukin-6 and filariasis

Previous studies have shown increased pro-inflammatory cytokines during the acute and chronic phase of filariasis, both in human patients and in murine models of LF (Babu and Nutman 2003; Rao and Klei 2006; Satapathy et al. 2006; Taylor et al. 2001; Turner et al. 1994). This pro-inflammatory response is mainly mediated by *Wolbachia*, but excretory-secretory products of filariae can also induce production of pro-inflammatory cytokines (Babu and Nutman 2003; Rao and Klei 2006; Satapathy et al. 2006; Taylor et al. 2001). Among the cytokines that are involved in *Wolbachia*-induced inflammation, IL-6 has been identified to be involved in the pathogenesis of filariasis (Taylor et al. 2001; Turner et al. 1994). Increased levels of IL-6 were found in filariasis patients after administration of DEC during the chronic phase of the disease, most likely induced by the release of *Wolbachia* (Taylor et al. 2001; Turner et al. 1994). In a rodent model of filariasis using gerbils infected with *B. pahangi*, IL-6 was elevated in the blood as well as at the site of infection (Porthouse et al. 2006; Rao and Klei 2006). IL-6 mRNA peaked at all infection sites in gerbils 3 hours after intracutaneous inoculation with L3 of *B. pahangi* and also in the spleen after 28 dpi (Porthouse et al. 2006).

As previously described, IL-6 is important during the pathogenesis of LF. IL-6 induces a pro-inflammatory response during acute and chronic infection LF (Babu and Nutman 2003; Cross et al. 2001; Taylor et al. 2001). This response is mainly mediated by *Wolbachia* that are released by dying worms (Taylor et al. 2001). IL-6 maybe also induces pathology by inducing several proteins such as VEGFs (Pfarr et al. 2009). Besides its role during pathogenesis of LF, it has been shown that IL-6 is also involved in adverse reactions caused by anti-filarial drugs (Cross et al. 2001; Turner et al. 1994). Additionally, a study by our group previously showed that eosinophils and macrophages from Eotaxin-1 deficient (Eotaxin-$1^{-/-}$) mice have a reduced propensity to produce IL-6 *in vitro*. While increased worm burden was found in those mice at later time points of infection, it was proposed that increased worm burden in Eotaxin-$1^{-/-}$ mice may be due to decreased IL-6 production and reduced local immune responses in these mice (Gentil et al. 2013, resubmission). Although IL-6 has been suggested to play several roles during LF infection, however, the precise role of IL-6 has not been identified.

1.4. Aims of study

Lymphatic filariasis remains a public health problem worldwide. It has a huge economic, social and psychological impact on the patients. Pro-inflammatory immune responses characterized by increased pro-inflammatory cytokines including IL-6 are found in the pathogenesis of acute as well as chronic stages of the disease. Moreover, IL-6 was also increased during antifilarial treatment with DEC due to the release of *Wolbachia* from dying worms. Several studies have suggested a role for IL-6 in LF both in human and mouse models of disease. The main aim of this study was to investigate whether IL-6 contributes to protective immune responses during the rodent *L. sigmodontis* model.

The first aim of this thesis was to investigate whether IL-6 affects the number of adult worms and Mf during acute and chronic infection. As it has been previously described that IL-6 is involved in numerous immunological aspects, the impact of neutrophils, eosinophils, macrophages, CD4 T cells, B cells and mast cells during *L. sigmodontis* infection in IL-$6^{-/-}$ and BALB/c controls was further planned to be analyzed. This should include the investigation of neutrophil and macrophage numbers as well as their activation in *L. sigmodontis* infected IL-$6^{-/-}$ and control mice. The role of IL-6 in eosinophil-mediated worm clearance was another aspect that should be addressed in this study by investigating the number of eosinophils and their activation as well as the effect of eosinophil depletion in IL-$6^{-/-}$ mice during *L. sigmodontis* infection. Furthermore, IL-6 deficiency affecting B cell numbers and antibody production as well as T_{reg} and Th17 differentiation may present additional responses determining immunity to *L. sigmodontis* infection and were therefore aimed to be investigated. Protective immune responses at the site of infection (skin) and modulation of the vascular permeability that may facilitate the migration of the larvae to the pleural cavity, the site where the adult worms reside, were further intended to be investigated in order to determine the role of IL-6 in early immunity.

In summary, the aim of this thesis was to determine the impact of IL-6 on *L. sigmodontis* infection and the induction of protective immune responses.

2. Material and methods

2.1. Mice

BALB/c and C57BL/6 wild type mice which were purchased from Janvier (Janvier, La Genest St. Isle, France) or bred in the animal facility of the Institute of Medical Microbiology Immunology and Parasitology (IMMIP) at the University Hospital Bonn, Germany were used in this study. To investigate the role of IL-6 in a mouse model of lymphatic filariasis, IL-6 deficient (IL-$6^{-/-}$) mice on a BALB/c background were used together with immunocompetent BALB/c mice as control. IL-$6^{-/-}$ mice were a gift by Manfred Kopf (Zürich, Switzerland) and bred in the animal facility of the IMMIP. Female and male mice of 6-9 weeks of age were used in this study and animals were kept under specific pathogen-free conditions, according to the animal protocols. All animal protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Recklinghausen, Germany).

2.2. The life cycle of *Litomosoides sigmodontis*

The life cycle of *Litomosoides sigmodontis* was maintained in cotton rats (*Sigmodon hispidus*). *Ornithonyssus bacoti* mites were allowed to feed on infected cotton rats and the transferred larvae molted from L1 to L3 within 12 days. L3 then migrated to the salivary glands of the *O. bacoti* mites and were transmitted to other cotton rats to maintain the life cycle or to mice during experimental infections.

2.3. Experimental *L. sigmodontis* infection

Mice were naturally infected by the bite of infected *O. bacoti* mites that contain L3 in their salivary glands. While the mites suck the blood of mice, L3 are transferred into the mouse skin and the life cycle continues. Alternatively, mice were infected by injecting 40 L3 subcutaneous. The L3 were derived by dissection from infective mites. Within 3-8 days post infection (dpi), L3 enter the pleural cavity and then one week later they become L4 (approximately 8-14 dpi) and reach adulthood in two more weeks (around 30 dpi). Adult female worms start to release Mf at around 56 dpi.

Based on the *L. sigmodontis* life cycle, infections were terminated and the mice were euthanized at different time points post infection: 14-15 (when worms are in the pleural

cavity and molted to L4), 30 (worms are molting into adult worms), 60 (beginning of Mf release) and 90 (chronic phase and dying of worms) dpi. To determine immune responses that involve the very early time points when worms reach the pleural cavity, mice were also euthanized at 7 dpi.

To generate a time course of infection, susceptible BALB/c and resistant C57BL/6 mice were infected. The mice were euthanized every week at 1, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70 and 77 dpi.

L. sigmodontis extract (LsAg) was obtained from adult worms derived from the pleural cavity of chronically infected cotton rats. Worms were homogenized in RPMI medium (PAA, Cölbe, Germany) using a 15 ml glass homogenizer. LsAg protein concentration was then measured using the Advanced Protein Assay (Cytoskeleton, Denver, USA) and stored at -80 °C at a concentration of 1 mg/ml.

2.4. Euthanasia of mice

As described above, after different time points post infection, experiments were performed. Mice were anesthetized using isoflurane (Abbot, Wiesbaden, Germany). Blood was then taken from the retro-orbital vein using micro-haematocrit capillaries (Brand, Wertheim, Germany) and transferred into disodium ethylenediaminetetraacetic acid (EDTA)-containing tubes (Kabe Labortechnik, Nümbrect-Elsenroth, Germany) and clear tubes (Eppendorf, Hamburg, Germany). Up to 200 µl of fresh blood was added into EDTA-containing tubes for blood leukocyte analysis, and the remaining blood was collected for serum analysis. For experiments which were terminated later than 60 dpi, 10 µl of blood was added into 300 µl Hinkelmann's solution (0.5 % [wt/vol] eosin Y, 0.5 % [wt/vol] phenol (both Merck, Darmstadt, Germany) and 0.185 % [vol/vol] formaldehyde [Sigma-Aldrich, Munich, Germany] in distilled water) for microfilarial examination.

Mice were then killed using an overdose of isoflurane anaesthesia and were fixed on a small Styrofoam board. The skin and peritoneum were carefully dissected and the spleen was removed and transferred into sterile phosphate-buffered saline (PBS) (PAA). The pleural cavity was then opened and washed with 10 ml of PBS using sterile plastic Pasteur pipettes (Ratiolab, Dreieich, Germany) for determining worms, cytokine and chemokine concentration as well as leukocyte composition in the pleural cavity. For cytokine analysis, the first 1 ml of pleural wash was collected in separate tubes. To separate worms, the

pleural wash was filtered through 41 µm gaze (Buekmann, Moenchengladbach, Germany). Worms were then stored in 2 ml of 4 % formaldehyde in distilled water for later analysis. For monitoring Mf in the pleural cavity, 100 µl from the 1st ml pleural lavage was added into 300 µl of Hinkelmann's solution and treated as described for peripheral blood Mf.

2.5. Pleura and blood cell preparation

The first 1 ml of pleural lavage was centrifuged at 350 X *g* at 4 °C for 5 minutes (min) and supernatants were stored at -20 °C for cytokine analysis. The cell pellet was combined with the remaining 9 ml pleural wash and centrifuged at 350 X *g* at 4 °C for 5 min. Cells were resuspended into 500 µl of 1x red blood cell (RBC) lysis buffer (eBioscience, Frankfurt, Germany) at room temperature (RT) for 4 min and washed with 10 ml of cold PBS. For analysis of blood cells, erythrocytes were lysed twice with 1 ml of 1x RBC lysis buffer. Leukocytes were then counted using an automated cell counter CASY® (Innovatis, Bielefeld, Germany) and kept on ice for flow cytometric staining after filtering through 41 µm gaze.

2.6. Fluorescence-activated cell sorting (FACS)

$5x10^5$ blood or pleura cells were added into 5 ml FACS tubes (Becton Dickinson, New Jersey, USA) and then washed with FACS buffer (1 % fetal bovine serum [FBS] [PAA] and 5 mM EDTA [Carl Roth, Karlsruhe, Germany] in PBS). Cells were incubated for 20 min on ice with 1 µg/ml rat IgG or anti-CD16/32 (BD Pharmingen, Heidelberg, Germany) for 15 min in 50 µl FACS buffer to block unspecific antibody binding. To examine leukocyte composition in blood and pleura cells, FITC NIMP/R14 and PE-Cy7 Ly-6G (Gr1) were used to stain neutrophils, PE Siglec F and APC F4/80 for eosinophils and macrophages, PE CD19 for B cell and PECy5.5 CD4 for CD4 T cell staining (all eBioscience, except for PE Siglec F that was from BD Pharmingen). For identifying B2 cells, FITC CD23 was used together with PE CD19 (both eBioscience). FITC IgE was used together with PE c-Kit (CD117) (both eBioscience) to stain mast cells. Cells were incubated with antibodies for 30 min on ice in the dark and then washed once using FACS buffer before acquiring data on the BD FACS Canto 1 (BD Biosciences, Heidelberg, Germany). 50,000 events were recorded per tube and data was analyzed using the FACS Diva software (BD Biosciences). Neutrophils, eosinophils, macrophages, mast cells, B

cells, B2 cells, T cells and their subsets from both pleural cavity and blood were gated by first gating total live leukocytes by side scatter (SSC) and forward scatter (FSC) as shown in figure 2.1.

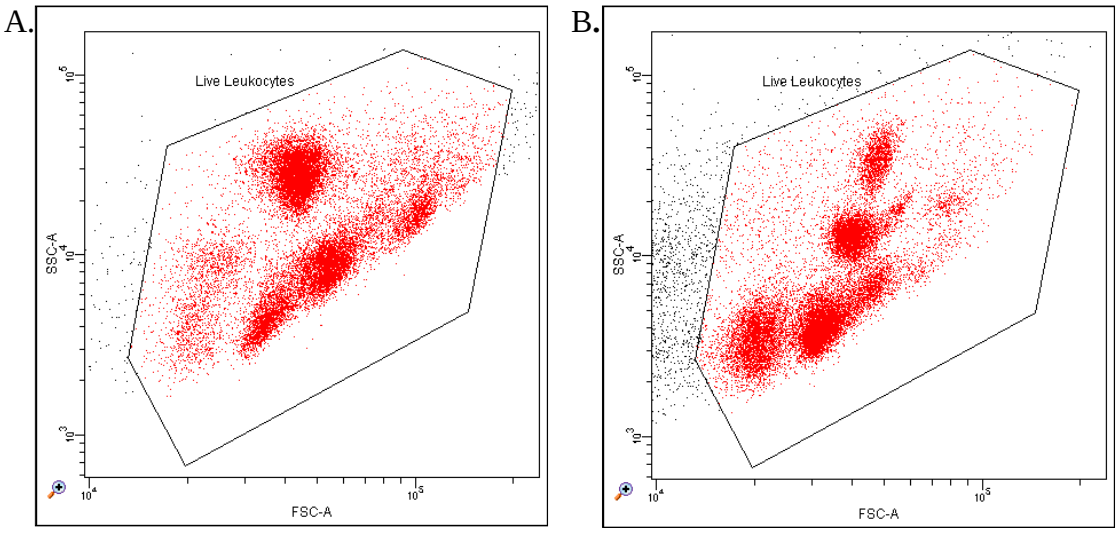


Figure 2.1. Gating strategy for leukocytes from the pleural cavity and blood. Total live leukocytes from pleural cavity (A) and blood (B) were gated by SSC and FSC.

To determine the number of alternatively activated macrophages (AAMs), $1x10^6$ pleura cells were added into 5 ml FACS tubes and washed once with 1 ml of PBS/1 % bovine serum albumin (BSA) (PAA) followed by a centrifugation step at 400 X *g* for 6 min at 4 °C. After all supernatant was discarded, 300 µl of 1x fixation/permeabilization buffer (eBioscience) was added to the cells and the cells were incubated for a minimum of 1 hour (h) at 4 °C. Cells were then washed and blocked with 1 µg/ml rat IgG in 1 ml of PBS/1 % BSA at 4 °C overnight. The following day, cells were washed, all supernatant discarded and 300 µl of 1x permeabilization buffer (eBioscience) were added. The cells were then incubated for 20 min at RT. After another washing step, rabbit anti-murine RELMα antibody (Peprotech, New Jersey, USA) in 100 µl of 1x permeabilization buffer was added to the cells and incubated for 1 h at 4 °C. Cells were washed and stained for AAMs by adding Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen, Darmstadt, Germany), PE Siglec F and APC F4/80 followed by incubating for 30 min at 4 °C in the dark. Cells were washed once before acquiring data on the BD FACS Canto 1. 50,000 events were recorded per tube and data was analyzed using the FACS Diva software. AAMs were gated by first gating total leukocytes by SSC and FSC as shown in figure 2.1.

2.7. Spleen cell preparation and culture

Spleens were meshed on metal sieves using the plunger of a 5 ml syringe (BD, Heidelberg, Germany) followed by adding 3 ml of cold PBS and then filtered using a 100 µm metal sieve. Splenocytes were transferred into 15 ml falcon tubes (Greiner Bio-One, Frickenhausen, Germany) and centrifuged at 350 X *g* at 4 °C for 5 min. The supernatant was discarded and cells were resuspended in 1 ml of 1x RBC lysis buffer as described before. Splenocytes were counted using an automated cell counter after washing once with sterile PBS.

Using splenocyte medium (10 % FBS and 1 % Penicillin/Streptomycin in high glucose DMEM [all PAA]), $1x10^6$ cells were plated in 96 well round-bottom plates (Greiner Bio-One) and stimulated at 37 °C, 5 % CO_2 for 72 h with medium, LsAg at a concentration of 25 µg/ml, recombinant mouse IL-6 (rIL-6) (PeproTech) at a concentration of 5 ng/ml, LsAg together with rIL-6 and 2.5 µg/ml concanavalin-A (ConA) (Sigma-Aldrich) as positive control. After incubation, plates were centrifuged at 350 X *g* at 4 °C for 5 min, and supernatant was transferred into new plates and stored at -20 °C for cytokine analysis.

2.8. Splenocytes intracellular staining

$2x10^6$ spleen cells in 1 ml of splenocyte medium were plated into 12-well plates (Greiner Bio-One). The cells were stimulated with LsAg. For positive control, 2 µg/ml of anti-CD28 (eBioscience) was added into wells which had been coated with 5 µg/ml of anti-CD3 (eBioscience) in PBS at 4 °C overnight. The cells were then incubated at 37 °C, 5 % CO_2 for 2 h. To inhibit protein secretion, 2 µg/ml of GolgiStop™ (BD Biosciences) which contains monensin was added into each well, followed by additional 4 h of incubation. After a total of 6 h incubation time, the cells were transferred into 15 ml falcon tubes and centrifuged in 2 ml of PBS at 350 X *g* at 4 °C for 10 min. The cells were fixed and permeabilized using 500 µl of freshly prepared 1x fixation/permeabilization buffer at 4 °C after all supernatant had been discarded overnight.

The following day, the fixed and permeabilized cells were washed with 2 ml of PBS/1 % BSA and 1x permeabilization buffer was added to the cells followed by centrifugation at 780 X *g* at 4 °C for 10 min. After discarding the supernatant, splenocytes were incubated with rat IgG in a concentration of 1 µg/ml in 1 ml of PBS/1 % BSA for 1 h to prevent

unspecific antibody binding. Cells were washed by centrifugation at 350 X *g* at 4 °C for 5 min and surface stained with PerCPCy5.5 CD4 and APC CD25, and intracellularly stained with FITC FoxP3, PE IL-17A and APC IL-6 (all eBioscience). Data was acquired using a BD FACS Canto 1 and analyzed using FACS Diva software after first gating on splenocytes as shown in figure 2.2 followed by gating of $CD4^+$ T cells then $CD4^+CD25^+FoxP3^+$ for regulatory T cells (T_{reg}), $CD4^+IL\text{-}17A^+$ for IL-17-producing Th17 cells and $CD4^+IL\text{-}6^+$ for IL-6-producing T cells.

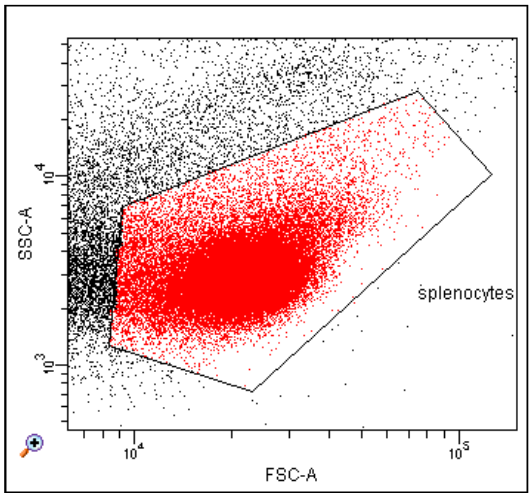


Figure 2.2. Gating strategy for splenocytes. Splenocytes were gated by SSC and FCS

2.9. Worm and microfilaria counting

L3 and L4 were counted using a binocular microscope at 16x or 40x magnification. Adult worms were counted using light microscopy and differentiated into male and female worms.

Mf in Hinkelmann's solution were centrifuged for 5 min at 250 X *g* and the supernatant was removed leaving only about 20 µl liquid for counting the worms using light microscopy at 40x or 100x magnification.

2.10. Enzyme-linked immunosorbent assay (ELISA)

IL-6, Eotaxin-1, Eotaxin-2, RANTES, sIL-6R, IL-17, TNFα, MIP-2, CCL17 (all DuoSet R&D Systems, Wiesbaden, Germany), IL-5, IgE (both BD Pharmingen) and IFNγ (eBioscience) concentrations were measured by two-sided cytokine ELISA according to the manufacturers' direction. ELISA plates (Greiner Bio-One) were coated with primary

antibodies in PBS or coating buffer (Na_2HPO_4 0.1 M [Merck] in distilled aqua pH 9.0) overnight. Plates were then blocked with PBS/1 % BSA for a minimum of 2 h at RT and washed three times with PBS/0.05 % Tween20® (Sigma-Aldrich). 50 µl of sample were added for a minimum of 2 h at RT or at 4 °C overnight. The detection antibody was added for 2 h. Following 20 min incubation with horseradish peroxidase (HRP)-streptavidin (R&D Systems) and tetramethylene benzidine (TMB) (Carl Roth), the color reaction was stopped with 1 M H_2SO_4 (Merck) and optical density (OD) was measured at 450 nm using a plate reader SpectraMAX 340 Pc (Molecular Devices, Ismaning, Germany). The data was analyzed with Softmax Pro software (Molecular Devices). The limit of detection were 4 pg/ml (IFNγ), 5.8 pg/ml (IgE), 7.8 pg/ml (Eotaxin-1, Eotaxin-2, TNFα, sIL-6R, MIP-2), 12 pg/ml (IL-5), 15.6 pg/ml (IL-6, IL-17) and 31.2 pg/ml (RANTES, CCL17)

For *L. sigmodontis*-specific IgG1 and IgG2a ELISA, polysorb ELISA plates (Nunc, Roskilde, Denmark) were first coated with 10 µg/ml of *L. sigmodontis* antigen extract in PBS at pH 9 overnight. Plates were then blocked with PBS/1 % BSA for a minimum of 2 h at RT and washed three times with PBS/0.05 % Tween20® (Sigma-Aldrich). A series of 1:100 to 1:100,000 serum dilutions were added for a minimum of 2 h at RT or at 4 °C overnight following by adding secondary antibodies (BD Pharmingen) for 2 h at RT. The plates were then treated as described for the cytokine ELISA.

2.11. Neutrophil purification and stimulation

To induce neutrophil migration into the peritoneal cavity, mice were injected intraperitoneally with casein solution. To prepare 9 % w/v of casein solution, 2.25 gram of casein powder (Carl Roth) was combined with 20 ml of pure water and 1 ml of 1 M NaOH (Carl Roth) and then mixed well for 1 h at 70 °C. After casein dissolved, 2.5 ml of $MgCl_2$ 5 mM (Sigma Aldrich) and 2.25 ml of $CaCl_2$ 10 mM (Carl Roth) were added into the solution.

Mice were injected with 1 ml of casein solution 18 and 2 h before euthanasia and then killed using an overdose of isoflurane. Abdominal skin was carefully dissected and the peritoneum was exposed. 10 ml of sterile cold PBS was carefully injected into the peritoneum and gently agitated during injection. The peritoneal wash was withdrawn slowly and transferred into sterile 50 ml falcon tubes (Greiner Bio-One). The peritoneal wash from the same strain of mice was pooled into one tube and then centrifuged at 350 X

g at 4 °C for 5 min. Supernatant was discarded and RBC lysis buffer was added to remove erythrocytes as described above.

Neutrophils were also generated from bone marrow. Femurs and tibias were dissected and transferred into sterile PBS. After muscles and ligament were dissected from the bones, both ends of the bone were cut off. The bones were set into 0.6 ml tubes (Eppendorf) with a small hole at the bottom. After setting this tube into a larger tube (1.5 ml), the tubes were centrifuged at 2000 X *g* for 30 seconds and bone marrow was flushed into the larger tube. Bone marrow was then resuspended with 100 µl PBS. Bone marrow from the same strain was pooled in 50 ml conical tubes and 10 ml of PBS was added followed by centrifugation at 350 X *g* at 4 °C for 10 min. After discarding the supernatant, erythrocytes were lysed with 3 ml of RBC lysis buffer as described previously and bone marrow was washed with 20 ml of PBS by centrifugation at 300 X *g* at 4 °C for 10 min.

Finally, neutrophils were also purified from the peripheral blood. 200 µl fresh blood which was acquired from the retro-orbital vein as described above was resuspended twice with 1 ml of 1x RBC lysis buffer to remove erythrocytes. After washing with sterile PBS, cells were then counted using an automated cell counter.

Cells from peritoneal lavage, bone marrow or blood were then transferred into a 15 ml falcon tube after adding 4 ml of sterile PBS. Using 19 G lumbar puncture needles, 3 ml of 53.4 % of Percoll (Sigma-Aldrich) in PBS was carefully underlayered into the tube followed by underlayering of 62.3 % and 71.7 %. The tubes were centrifuged at 500 X *g* at 15 °C for 20 min. The cells from the second layer from the top containing primarily neutrophils were carefully aspirated using an 18G needle (BD) and transferred into a new tube. Neutrophils were washed twice with 10 ml cold sterile PBS. To determine the purity of neutrophils, FACS analysis was carried out as described above. The purity was between 90-95 %.

$1x10^5$ neutrophils were stimulated at 37 °C, 5 % CO_2 for 18 h with medium (10 % FBS and 1 % Penicillin/Streptomycin in RPMI), LsAg, rIL-6, LsAg together with rIL-6 and 500 ng/ml tripalmitoyl-*S*-glycerylcysteine (P3C) (InvivoGen, Toulouse, France), for positive control. After 18 h incubation, plates were centrifuged and supernatants were stored at -20 °C for cytokine analysis.

2.12. Macrophages purification and stimulation

Macrophage accumulation in the peritoneal cavity was elicited by intraperitoneal injection with 2 ml of sterile thioglycollate solution (Heipha, Eppelheim, Germany) 4 days prior to sacrificing. Mice were killed with an overdose of isofluran anesthesia and abdominal skin was carefully dissected. Cells were harvested from the peritoneal cavity by injecting 10 ml of sterile PBS as described for neutrophil purification. Peritoneal wash from the same mouse strain were collected in 50 ml conical tubes and washed twice with 10 ml of sterile cold PBS by centrifuging at 300 X *g* at 4 °C for 5 min. If necessary, erythrocytes were lysed with 1 ml of 1x RBC lysis buffer.

$1x10^5$ cells in 100 µl of medium (10 % FBS and 1 % Penicillin/Streptomycin in RPMI) were plated into 96-wells flat-bottom plates (Greiner Bio-One). The cells were then rested at 37 °C, 5 % CO_2 overnight. The supernatant was removed and adherent cells were used for further experiments. $1x10^5$ macrophages were restimulated at 37 °C, 5 % CO_2 for 18 h with LsAg, rIL-6, rIL-6 together with LsAg and P3C.

2.13. Eosinophil purification and stimulation

Mice were infected with *L. sigmodontis* as described previously. At 15 dpi, mice were euthanized and the pleural cavity was washed with 10 ml sterile cold PBS. Cells were washed with PBS by centrifugation at 350 X *g* at 4 °C for 10 min and the supernatant was discarded. Erythrocytes were lysed with 1x RBC lysis buffer as described above followed by washing with 10 ml of sterile cold PBS. Eosinophils were purified by negative selection using magnetic beads of the MACS® cell separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) per manufacturer's instruction. Briefly, up to 10^7 cells were resuspended into 3 ml of MACS® buffer and washed by centrifugation at 300 X *g* for 10 min. After all supernatant was discarded completely, 90 µl of MACS buffer were added to 10^7 total cells and then cells were labeled with 10 µl each of anti-MHCII, anti-CD45R, and anti-CD90.2 microbeads. After incubation at 4 °C for 15 min and washing with 1-2 ml of MACS buffer per 10^7 total cells. LD columns were applied to the magnetic field followed by rinsing the columns with 3 ml of MACS buffer. The cells were then transferred into the LD columns. Eosinophils were collected as unlabelled cells that pass through and then washed once with sterile cold PBS. Purity of eosinophils was determined by flow

cytometry and generally reached a purity of 70-80 %. Purified eosinophils were then stimulated with LsAg, rIL-6, LsAg with IL-6, and P3C.

2.14. Interleukin-6 intracellular staining

One million of pleural leukocytes or purified eosinophils were incubated in 500 µl of medium (10 % FBS and 1 % Penicillin/Streptomycin in RPMI) in 24-well plates (Greiner Bio One) and stimulated with either LsAg, 500 ng/ml of Lipopolysaccharide (LPS) (InvivoGen, Toulouse, France) or medium alone. In order to inhibit protein transport into the extracellular compartment, GolgiPlug™ (BD Biosciences) which contains Brefeldin A solution was added into the culture followed by incubation at 37 °C, 5 % CO_2 for 5 h. Cells were then transferred into FACS tubes and 2 ml of FACS Buffer was added, followed by centrifugation at 350 X *g* for 5 min at 4 °C. Cells were incubated with rat IgG for 15 min to reduce unspecific binding. Eosinophils and macrophages were stained with PE Siglec-F and APC F4/80 for 30 min. The cells were then washed with 2 ml of FACS buffer by centrifugation at 350 X *g* at 4 °C and fixed with 500 µl of 2 % paraformaldehyde (PFA) solution (PFA [Merck] in FACS buffer) followed by incubation for 7 min on ice. Cells were then washed once with 500 µl FACS buffer by centrifugation at 790 X *g* for 5 min. After supernatant was discarded, 120 µl of saponin buffer (0.5 % Saponin [Sigma Aldrich] in PBS/2 % BSA) was added to the cells for 15 min at RT. IL-6 and isotype control for intracellular staining were then carried out by adding FITC IL-6 and isotype antibodies in 90 µl of saponin buffer for 30 min at RT. Data was acquired using a BD FACS Canto 1 machine and analyzed using FACS Diva software after first gating on Siglec-F^+F4/80^{low}IL-6^+ and Siglec-F^{low}F4/80^+IL-6^+ cells for IL-6-producing eosinophils and macrophages respectively.

2.15. Dendritic Cell (DC) generation and stimulation

Bone marrow from murine femurs and tibias was prepared as described for the generation of bone marrow neutrophils. Cells were counted and resuspended into 5 ml of DC growth medium (10 % FBS, 1 % sodium pyruvate, 1 % 4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid [HEPES], 1 % L-Glutamin, 1 % penicillin/streptomycin [all PAA] and 0.1 % 2-mercaptoethanol in RPMI). $2.5x10^6$ of cells in medium containing 20 ng/ml of GM-CSF (PeproTech) were plated into 6-wells plates and incubated at 37 °C, 5 % CO_2. After 72 h, 2

ml of medium with GM-CSF was added followed by changing the medium 48 h later. After 7 days incubation time, loosely adherent cells were harvested and washed once using PBS. $1x10^6$ of cells were stimulated with LsAg, rIL-6 and LPS for positive control at 37 °C, 5 % CO_2 for 18 h. FACS analysis was conducted to determine purity of DC. The purity was between 80-90 %.

2.16. Phagocytosis assay

Cells from the pleural wash of *L. sigmodontis* infected mice were acquired at 15 dpi as described previously. $2x10^6$ cells were incubated with $30x10^6$ of Fluoresbite Plain YG 1.0 micron microspheres (Polysciences, Eppelheim, Germany) at 37 °C for 1 h. As negative control, cells were stored at 4 °C for 1 h. Phagocytosis of macrophages and eosinophils was determined using FACS. Cells were stained with PE Siglec-F and APC F4/80 for eosinophils and macrophages as described above. Data was acquired using FACS Diva software and the percentages of phagocytosed eosinophils and macrophages were calculated as percent of total eosinophils and macrophages.

2.17. Vascular permeability assay

An *in vivo* vascular permeability assay was conducted to examine the vascular permeability in either the skin at the lumbar area or in the ears. For measuring vascular permeability in the skin of mice, at first, the injected lumbar area was shaved a week before the injection in order to reduce skin irritation of the mice that could change the result. Mice were then injected subcutaneously with 10 µg of LsAg in 100 µl of sterile PBS or PBS alone. 6 h later, mice were injected intravenously with 30 mg/kg body weight (BW) of Evans Blue (Sigma Aldricht) in 100 µl of NaCl 0.9 % (Fresenius Kabi, Bad Homburg, Germany). After 1 h, mice were killed by an overdose of isoflurane anesthesia followed by cutting off the skin at the injected areas approximately 1 cm in diameter. The skin was weighed and transferred into 3 ml of >99 % formamide (Sigma Aldricht).

Alternatively, 10 µg each of PBS, LsAg or anti-mouse FcεRIα (MAR-1) (eBiosciences) were injected intracutaneously (i.c.) in the ears of mice. After 3 min, 30 mg/kg BW of Evans Blue in 200 µl of NaCl 0.9 % were injected i.v.. 10 min later, the complete ears were cut off and transferred into 500 µl of >99 % formamide. Evans Blue leakage into the

tissues both in the skin or ears were quantified after 48-72 h of incubation at 56 °C by determining optical density on a plate reader at 620 nm and normalized to the weight of the tissue.

2.18. Mast cells stabilizing assay

To stabilize mast cells, mice were injected intraperitoneally with 8 mg/kg BW of sodium cromoglycate (hereafter cromolyn) (Sigma Aldricht) in 100 µl of sterile NaCl 0.9 % or NaCl 0.9 % alone daily starting 6 days prior to infection until euthanasia. Mice were euthanized at 14 dpi and parasitological as well as immunological changes were examined as described previously.

2.19. Histamine neutralizing assay

Histamine receptor-1 and -2 antagonists' mepyramine and cimetidine respectively (both Sigma Aldrich) were used to block histamine activity in infected mice. 20 mg/kg BW of mepyramine and 40 mg/kg BW of cimetidine in 100 µl of PBS or PBS alone were injected intraperitoneally at 1 h prior and 2 h after *L. sigmodontis* infection, followed daily at 1 until 5 dpi. Worm burden, leukocyte composition in pleural cavity and blood and other immunological changes were determined after 14 dpi.

2.20. IL-5 depletion *in vivo*

Anti-IL-5 (TRFK5) was used to deplete IL-5 *in vivo*. TRFK5 and the isotype control antibody TRFK4 or rat IgG1 were either purified in our laboratory or purchased. TRFK5 and TRFK4 were acquired from hybridoma cells (Lopez, Strath, & Sanderson, 1984) which were cultured with hybridoma medium (10 % FBS, 1 % sodium pyruvate, 1 % L-Glutamin, 1 % penicillin/streptomycin and 1.9 % 2-mercaptoethanol in RPMI). The antibodies were harvested every other day (TRFK5) and every 3 days (TRFK4). Supernatant was then purified using a protein purifier ÄKTAprime™ plus (GE Healthcare, Munich, Germany) and dialyzed using a protein membrane Slide-A-Lyzer® 3.5 dialysis cassette (Thermo Scientific, Rockford, USA). The purity of antibodies was then examined with dotblot, westernblot, Comassie staining and ELISA.

Using antibodies which were purified in our lab, mice were injected intraperitoneally with 100 µg of TRFK5 or TRFK4 in 300 µl of PBS 5 dpi until euthanasia at 14 dpi.

Alternatively, mice were injected with 50 µg of commercial antibodies TRFK5 or isotype control rat IgG1 (BioXcell, New Hampshire, USA) in 100 µl of sterile PBS at 1 day prior infection and followed at 3, 7, 10, 13 dpi, and euthanized at 14 dpi.

IL-5 depletion at a later time point of infection was also conducted to investigate the effect of IL-5 between day 14 and day 28 post infection. Commercial antibodies TRFK5 or rat IgG1 were injected i.p. at 12, 17, 23 and 28 dpi. Mice were then sacrificed at 30 dpi. Worm burdens as well as immunological changes were determined as previously described.

2.21 Statistics

Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, California, USA), using the Mann Whitney t-test (u test) for non-parametric data. All data are shown as median except for *in vitro* stimulation and time course data that are shown as means ± standard error of mean (SEM). P values ≤ 0.05 were considered as significant.

2.22. Funding

My study was funded by the Government of Aceh (Indonesia) in collaboration with the German Academic Exchange Service (DAAD) and intramural grant by the University Hospital Bonn. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

3. Results

3.1. Comparison of susceptible BALB/c and resistant C57BL/6 mice during *Litomosoides sigmodontis* infection

To determine the progress of filarial infection and examine parasitological and immunological variables during infection, susceptible BALB/c and resistant C57BL/6 wild type mice were infected with *L. sigmodontis* and euthanized every week starting at 1 day post infection (dpi), followed at 1 week until 11 weeks post infection. Worms in the pleural cavity were counted starting at 2 weeks post infection. As previously reported by several studies (Babayan et al. 2003; Le Goff et al. 2002), BALB/c mice had a higher worm burden compared to C57BL/6 wild type mice throughout the infection (Figure 3.1.1). Total leukocytes in the pleural cavity of infected mice were counted to determine whether total cells correlated with the worm burden at those time points. However, there was no correlation between worm burden and total cell numbers at the site of infection (figure 3.1.1).

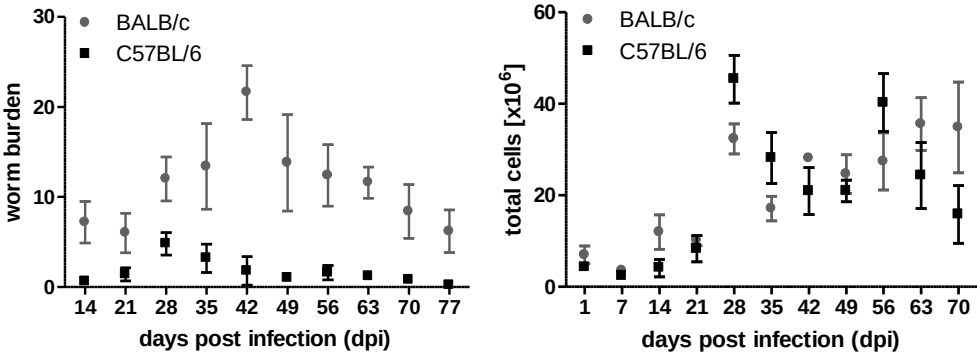


Figure 3.1.1. Increased worm burden and total pleural cavity cell numbers in *L. sigmodontis* infected susceptible BALB/c mice compared to resistant C57BL/6 mice. *L. sigmodontis* worm burden (left) and total cells derived from the pleural cavity (right) of susceptible BALB/c and resistant C57BL/6 mice. (Time course experiments were done with 5 mice per group. The experiment was conducted once. Data is shown as means ± SEM).

Next, cellular composition in the pleural cavity was investigated for both types of mice by flow cytometry. Interestingly, relative and absolute number of neutrophils in BALB/c mice showed a significant increase after 4 weeks post infection, and reached its peak at 8 weeks post infection. In contrast, neutrophil numbers in C57BL/6 mice were maintained at low levels during the infection (figure 3.1.2 A). Parallel with neutrophil numbers, eosinophil numbers in BALB/c mice became prominent after 4 weeks post infection; although there was no clear peak in eosinophil numbers as with neutrophils. Eosinophils in C57BL/6 mice

also tended to increase after 4 weeks post infection; although the numbers were lower than in BALB/c mice (figure 3.1.2 B). Relative and absolute numbers of macrophages were higher in C57BL/6 mice than in BALB/c mice, and their numbers were maintained at high levels throughout the infection in both C57BL/6 and BALB/c mice (figure 3.1.2 C). No difference of both T cell and B cell or total lymphocytes were found in BALB/c compared to C57BL/6 mice during *L. sigmodontis* infection (figure 3.1.2 D, E and F).

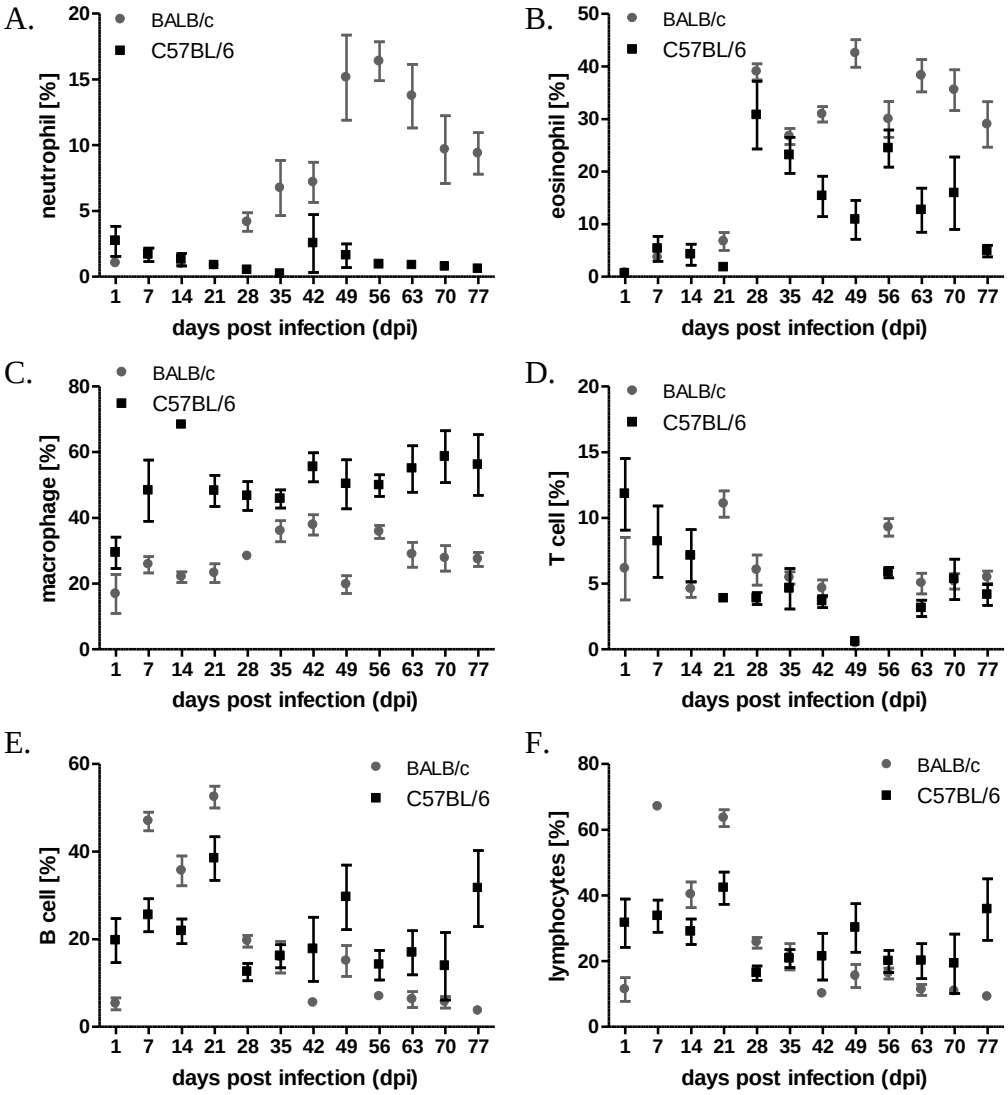


Figure 3.1.2. Increased frequencies of neutrophils and eosinophils during the chronic phase of *L. sigmodontis* infection in susceptible BALB/c mice. Percentage of leukocytes in the pleura cavity of *L. sigmodontis* infected BALB/c and C57BL/6 mice at different time points post infection. Frequencies of neutrophils (A), eosinophils (B), macrophages (C), T cells (D), B cells (E) and total lymphocytes (F) were measured by FACS and are indicated as percentages of living cells. (Time course experiments were conducted with 5 mice per group. The experiment was carried out once. Data is shown as means ± SEM)

3.2. IL-6$^{-/-}$ mice have a higher worm burden than BALB/c controls at 7 and 15 days post infection

Previous studies have shown increased pro-inflammatory cytokine levels like IL-6 in acute and chronic phases of filariasis, both in patients and in murine models of lymphatic filariasis (Babu and Nutman 2003; Rao and Klei 2006; Satapathy et al. 2006; Taylor et al. 2001; Turner et al. 1994). To investigate the role of IL-6 in murine filarial infection, IL-6$^{-/-}$ mice were used on a BALB/c background. BALB/c and IL-6$^{-/-}$ mice were naturally infected with *L. sigmodontis*. Based on the life cycle of *L. sigmodontis* in mice, the infection was terminated at three different time points: 15, 30 and 60 dpi. Interestingly, increased worm burden occurred at 15 dpi, but not at 30 and 60 dpi (figure 3.2 A, B, and C respectively). Worm burden at a late time point of infection (90 dpi) was lower compared to earlier time points and revealed no significant difference in worm numbers between BALB/c and IL-6$^{-/-}$ mice (figure 3.2 D).

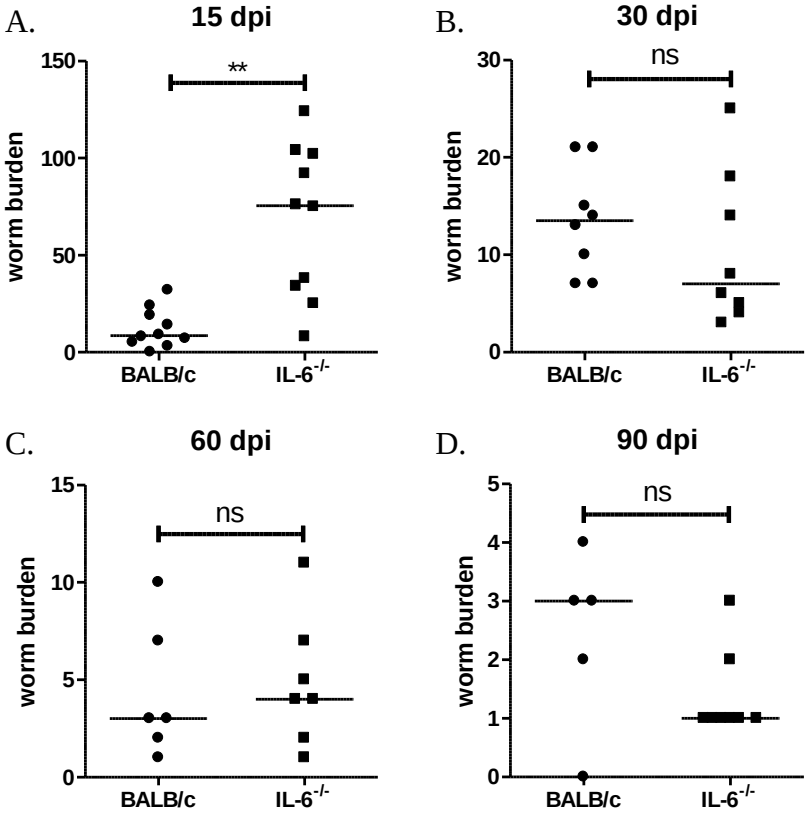


Figure 3.2.1. Increased numbers of *L. sigmodontis* worms in the pleural cavity of IL-6$^{-/-}$ mice at early time points of infection. Mice were infected with *L. sigmodontis* for 15 (A), 30 (B), 60 (C) and 90 (D) days. Worm burden in the pleural cavity was determined by light microscopy. (Data shown is representative of a minimum of 3 experiments, except for 90 dpi, which was conducted once, with a minimum of 5 mice per group. Data is shown as median. ** indicates $p < 0.01$, ns: not significant).

Given that worm burdens were increased early during infection in IL-6$^{-/-}$ mice, a following experiment examined whether increased worm burden in IL-6$^{-/-}$ mice occurs before 15 dpi. The time span between 5 and 10 dpi was investigated as during those time points larvae migrate into pleural cavity. Seven dpi when most larvae reached the pleural cavity was investigated twice. Interestingly, there was already an increase of worm burden in IL-6$^{-/-}$ mice during larval migration (figure 3.2.2, left) especially at 7 dpi (figure 3.2.2, right). Increased worm burden in IL-6$^{-/-}$ mice occurred as early as 5 dpi when worms start to arrive in the pleural cavity.

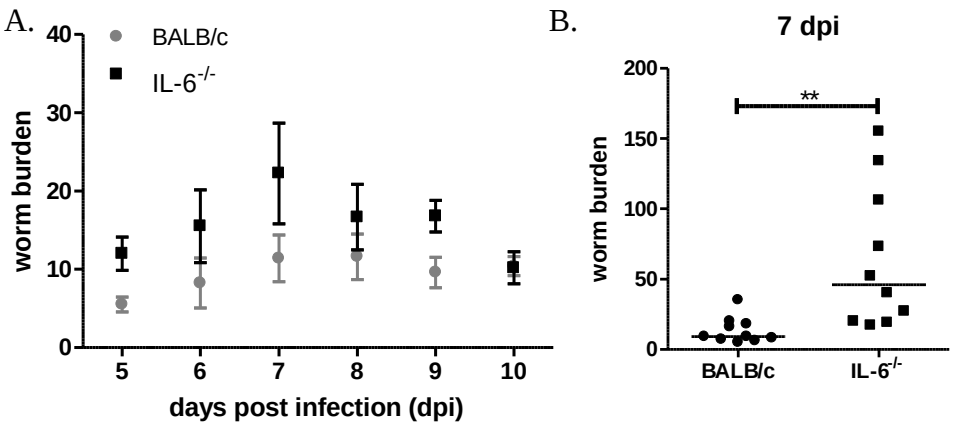


Figure 3.2.2. IL-6$^{-/-}$ mice have an increased worm burden at the time point they reach the pleural cavity. *L. sigmodontis* worm burden of BALB/c and IL-6$^{-/-}$ mice during larval migration into the pleural cavity at 5 until 10 days post infection (dpi) (A). The worm burden at 7 dpi (B). (The time course experiment shown in [A] was conducted once. [B] Is representative of two experiments, with minimum of 5 mice per group. Data is shown as means ± SEM [time course] and median [7 dpi] ** indicates $p < 0.01$)

3.3. No difference in microfilaria levels between BALB/c and IL-6$^{-/-}$ mice

To determine the role of IL-6 on the microfilarial burden during *L. sigmodontis* infection, circulating Mf and Mf levels in the pleural cavity were counted 60 and 90 dpi. No difference in Mf levels was found between both strains of mice neither at 60 dpi (figure 3.3 A) nor 90 dpi (figure 3.3 B) both in the blood and pleural cavity.

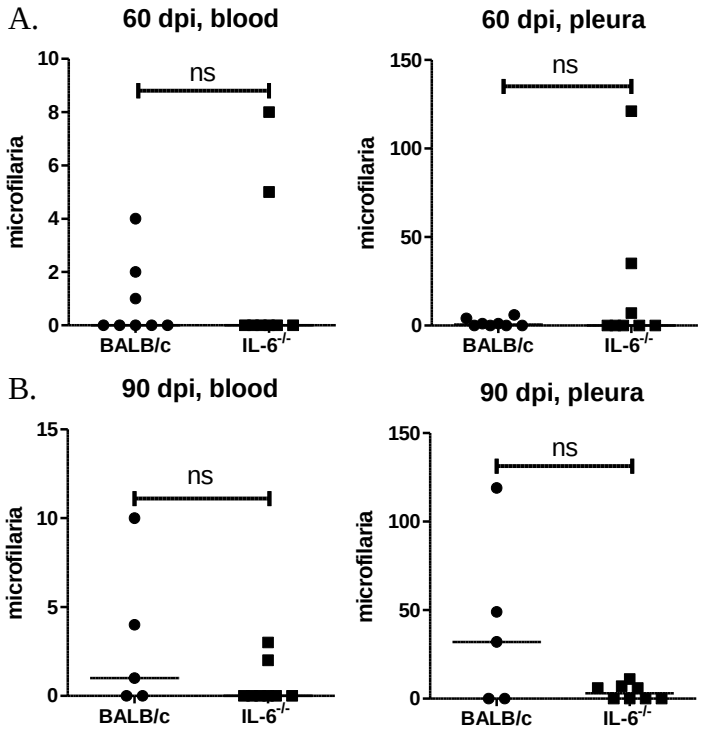
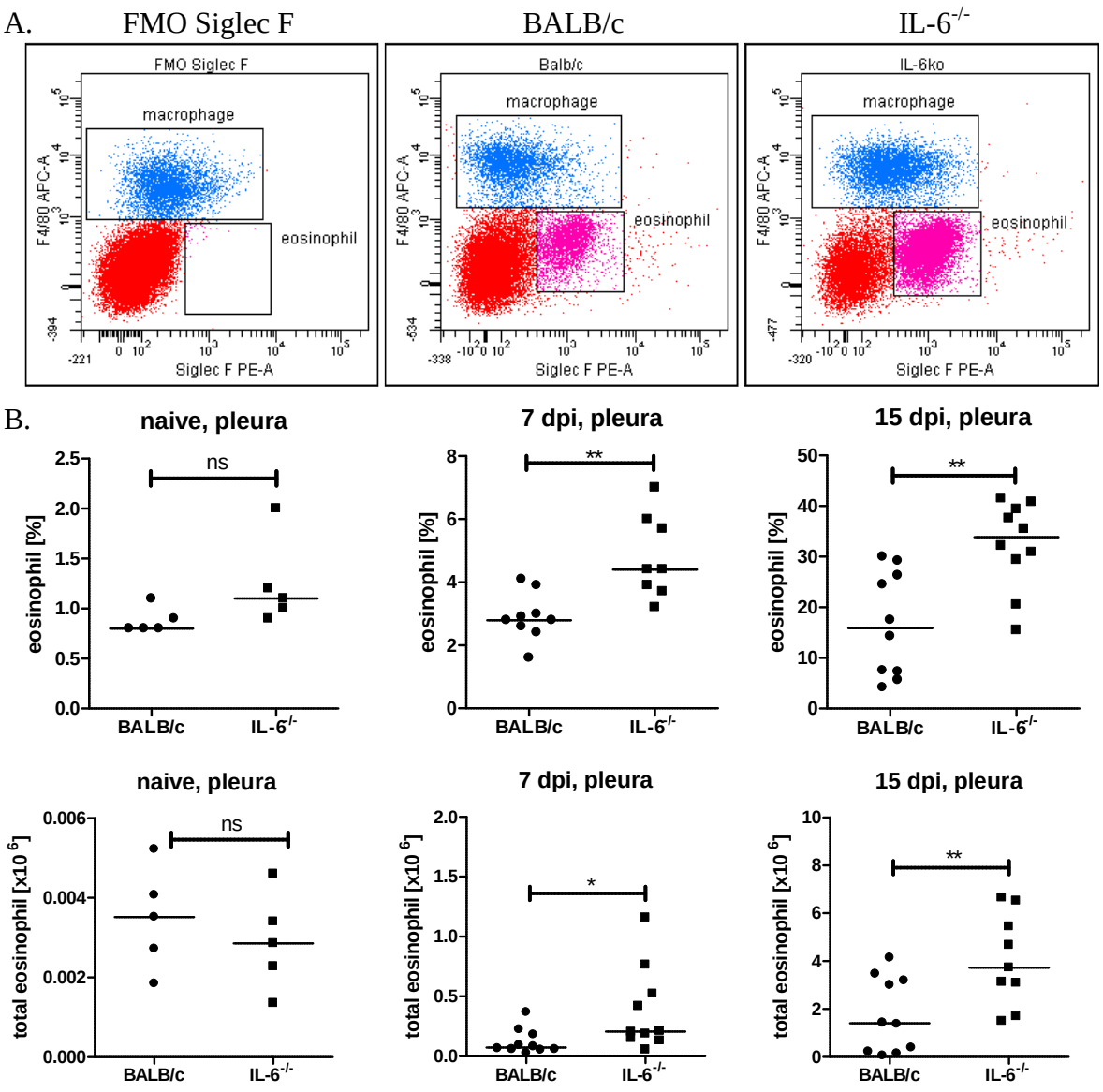


Figure 3.3. Microfilaria load is comparable between BALB/c and IL-6$^{-/-}$ mice. Mice were infected with *L. sigmodontis*. After 60 dpi (A) and 90 dpi (B), Mf in the peripheral blood (left) and in the pleural cavity (right) were counted using light microscopy. (Data shown as representative of a minimum of 2 experiments except for 90 dpi, which was conducted only once, with minimum of 5 mice per group. Data is shown as median).

3.4. Increased number of eosinophils in the pleural cavity, but not in the blood, correlates with increased worm burden in IL-6$^{-/-}$ mice

Eosinophilia is a hallmark of helminth infections including lymphatic filariasis. Eosinophils have been reported to play a significant role in protective immunity during filarial infection potentially by secreting their granule contents (Makepeace et al. 2012). To determine the role of IL-6 in eosinophilia during filarial infection, the eosinophil population was investigated by FACS at different time points post infection in both

BALB/c and IL-6$^{-/-}$ mice. Eosinophils were gated as SiglecF^{+}F4/80^{low} cells and differentiated from macrophages which were defined as SiglecF^{low}F4/80^{high} cells (figure 3.4.1 A). Uninfected mice were found to have low numbers of eosinophils (approximately 1 % of total leukocytes found in the pleural cavity). Number of eosinophils started to rise at 7 dpi with significantly higher levels at 15 dpi. Interestingly, significantly increased eosinophil numbers (relative and absolute) were found in IL-6$^{-/-}$ mice compared to BALB/c mice at 7 and 15 dpi (figure 3.4.1 B). However, this increase was not observed at later time points when there was no difference in worm burden (data not shown). Increased number of eosinophil correlated with increased worm burden in IL-6$^{-/-}$ mice at early time points post infection (figure 3.4.1 C).



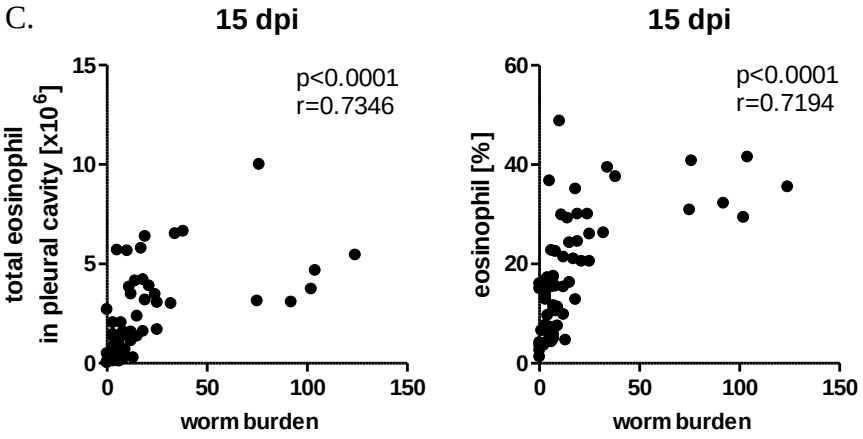


Figure 3.4.1. Increased pleural eosinophilia in IL-6$^{-/-}$ mice at early time points post *L. sigmodontis* infection. Eosinophil population was determined with FACS as Siglec-F^{+}F4/80^{low} cells and differentiated with macrophages that were defined as SiglecF^{low}F4/80^{high}. Eosinophils are absent in the fluorescence minus one (FMO) Siglec F staining (left). Gating of eosinophils and macrophages in BALB/c mice (middle) and IL-6$^{-/-}$ mice (right) at 15 dpi (A). Relative (top row) as well as absolute (bottom row) number of eosinophils in both types of mice at different time points; 0 (left columns), 7 (middle columns), and 15 (right columns) dpi are shown (B). Correlation between worm burden and absolute (left) and relative (right) number of eosinophils in the pleural cavity were determined at 15 dpi (C). (Representative of a minimum of 2 experiments, with a minimum of 5 mice per group. Data is shown as median. * indicates $p < 0.05$, ** < 0.01, ns: not significant).

To determine if the increased number of eosinophils in IL-6$^{-/-}$ mice was due to increased active migration to the site of infection or due to an overall increased eosinophil number in IL-6$^{-/-}$ mice, the eosinophil population was investigated in the blood of naive and infected animals. It is well known that there are few eosinophils in the circulation, less than 5% of total circulating leukocytes in humans (Behm and Ovington 2000). No difference in circulating eosinophil numbers was found in BALB/c compared to IL-6$^{-/-}$ mice at 7 and 15 dpi (Figure 3.4.2). The percentage of eosinophils was not different at either time point suggesting that the increased eosinophil numbers found at the site of infection of IL-6$^{-/-}$ mice was due to active migration of eosinophils into the pleural cavity.

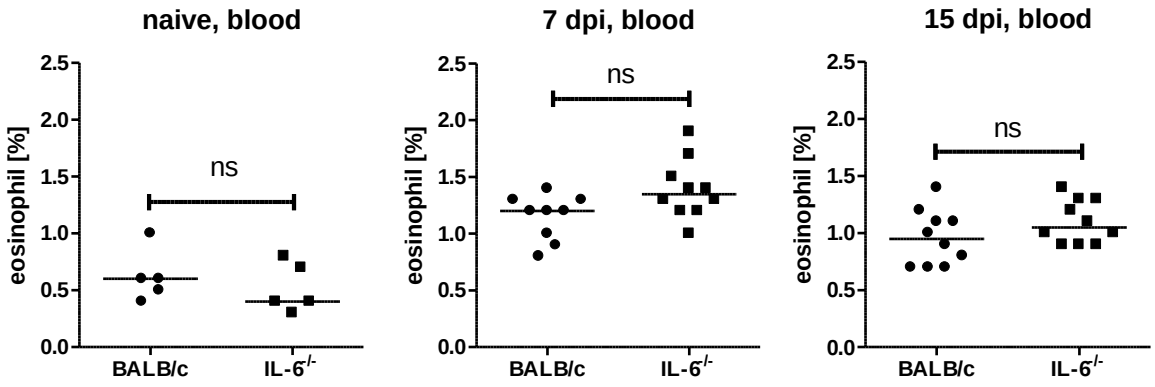


Figure 3.4.2. IL-6 deficiency does not alter blood eosinophilia during *L. sigmodontis* infection. Blood eosinophilia which was determined using FACS, in naive (left), 7 dpi (middle) and 15 dpi (right). (Representative of a minimum of 2 experiments, with a minimum 5 mice per group. Data is shown as median. ns: not significant).

3.5. Increased pleural eosinophilia in IL-6$^{-/-}$ mice is associated with increased IL-5 levels

Increased eosinophil numbers which were found in the pleural cavity of IL-6$^{-/-}$ mice is potentially due to an increased migration or survival of eosinophils. Activation and migration of eosinophils involve several chemokines and cytokines. Eotaxin-1, Eotaxin-2 [reviewed in (Bandeira-Melo et al. 2001; Rosenberg et al. 2007)] and RANTES [reviewed in (Appay and Rowland-Jones 2001; Levy 2009)] are well known as eosinophil attracting chemokines. The levels of these chemokines were measured in the serum as well as in the pleural lavage of naïve and infected animals. Low levels of Eotaxin-1 were found in the pleural lavage of infected animals at 7 (figure 3.5.1 A) and 15 dpi (figure 3.5.1 B). Eotaxin-1 levels did not differ in IL-6$^{-/-}$ mice compared to BALB/c mice at 7 and 15 dpi, neither in the pleural lavage nor in the serum. There was also no difference in the levels of Eotaxin-2 and RANTES, both in the pleural lavage and serum for both types of mice.

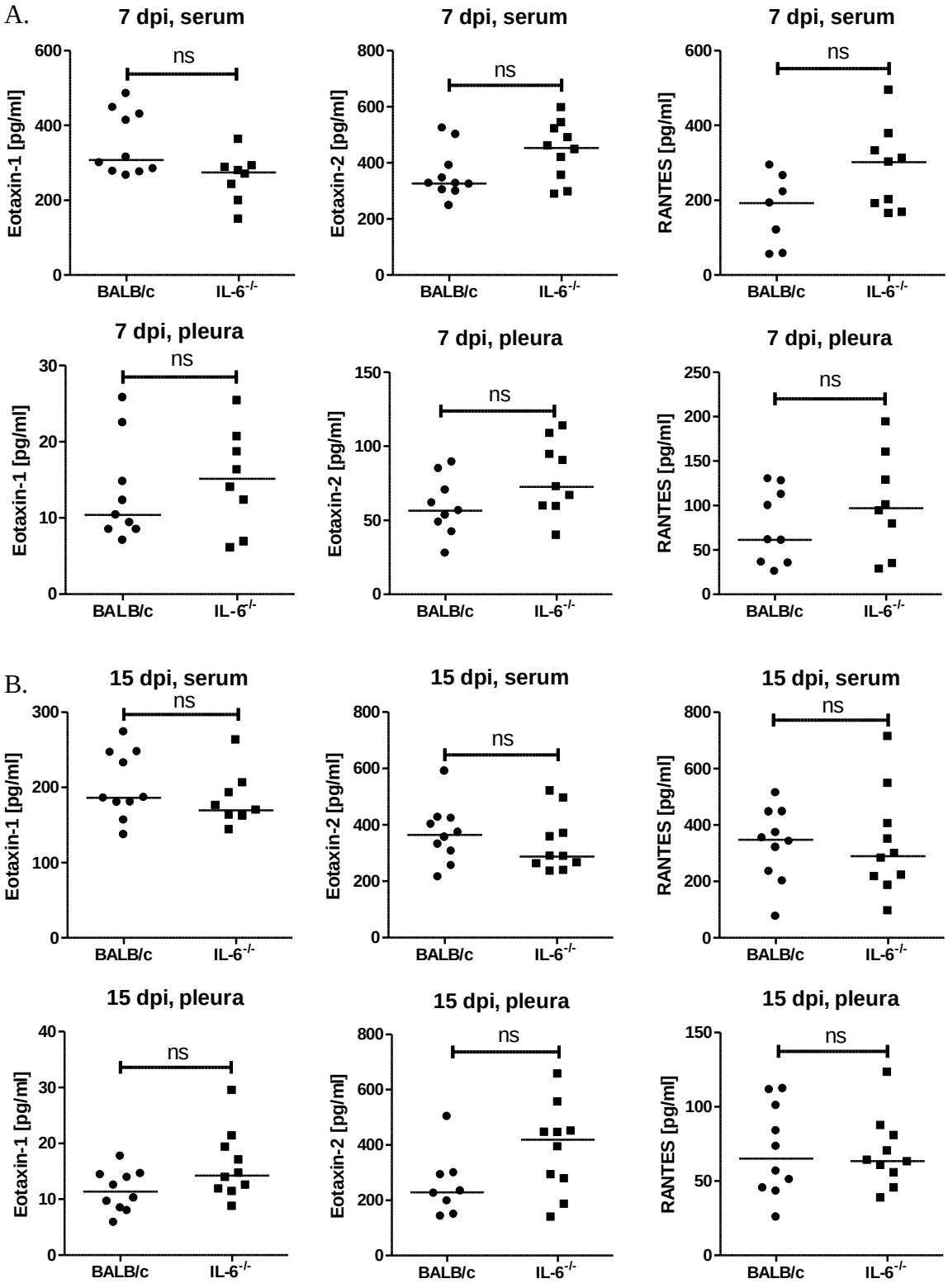


Figure 3.5.1. Similar concentrations of eosinophil-associated chemokines between *L. sigmodontis* infected BALB/c and IL-6$^{-/-}$ mice. Levels of eosinophil associated chemokines (Eotaxin-1, Eotaxin-2, and RANTES) measured by ELISA in serum (top) and pleural cavity (bottom) of 7 (A) and 15 days (B) *L. sigmodontis* infected animals. (Representative of a minimum of 2 experiments, with a minimum 8 mice per group. Data is shown as median. ns: not significant).

IL-5 is important in eosinophilia [reviewed in (Takatsu and Nakajima 2008)]. To determine levels of IL-5 in IL-6$^{-/-}$ mice, IL-5 production was investigated *in vivo* and *in vitro* after splenocyte stimulation with LsAg as well as co-stimulation with LsAg and rIL-6 in IL-6$^{-/-}$ mice at 7 and 15 dpi. IL-5 secretion *in vivo* (in serum and pleural cavity) during *L. sigmodontis* infection in BALB/c as well as in IL-6$^{-/-}$ mice was not detectable (below 15.6 pg/ml) (data not shown). Interestingly, increased IL-5 secretion by splenocytes from IL-6$^{-/-}$ mice were observed *in vitro*. Production of IL-5 was increased in IL-6$^{-/-}$ mice in the supernatant of cultured splenocytes after stimulation with LsAg, at 7 and 15 dpi (figure 3.5.2). Increased IL-5 production was also found at 30 dpi (data not shown) but not in naïve mice, or after 60 dpi (figure 3.5.2 and data not shown). However, co-stimulation with rIL-6 did not have a synergistic effect on cytokine production.

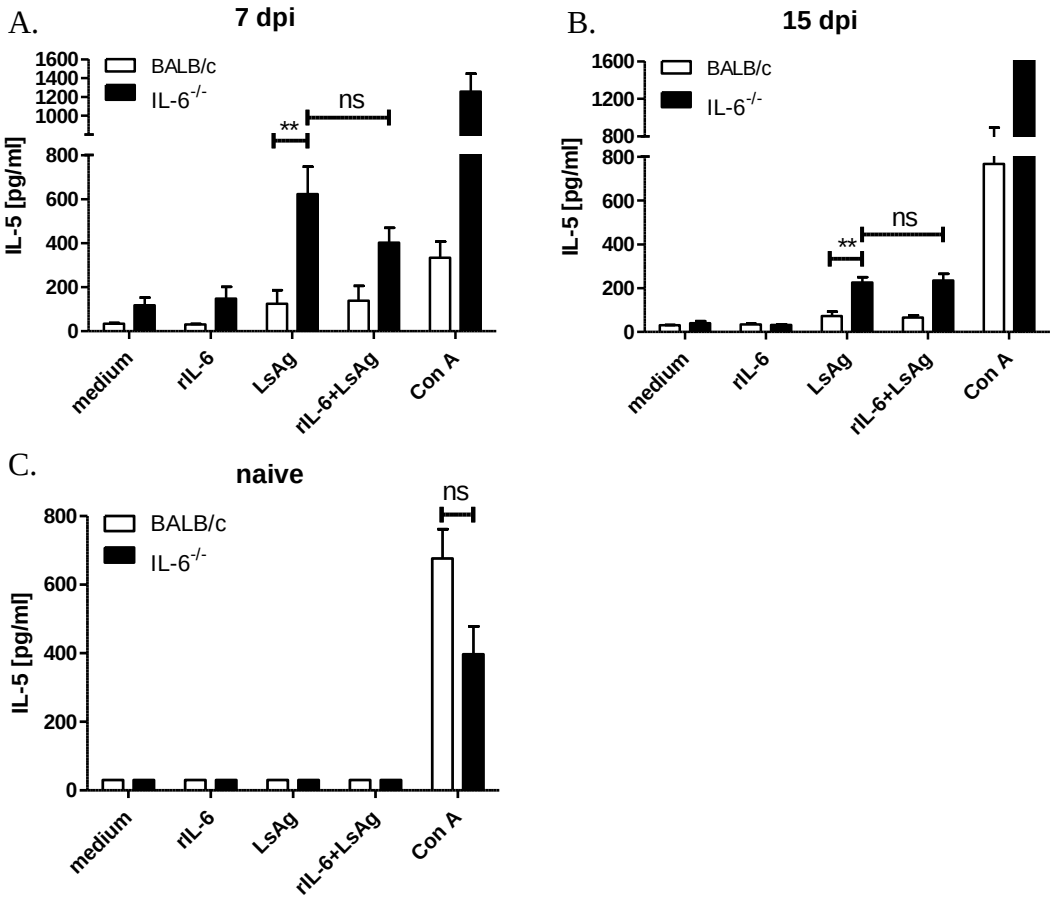


Figure 3.5.2. Increased *in vitro* IL-5 production by LsAg-stimulated splenocytes from IL-6$^{-/-}$ mice. Animals were infected with *L. sigmodontis*. After 7 dpi (A) and 15 dpi (B), spleen cells were isolated and restimulated with different stimuli for 72 hours. Spleen cells from naïve mice were also restimulated with rIL-6, LsAg, rIL-6+LsAg and ConA (C). IL-5 secretion into the supernatant was measured by ELISA. (Representative of a minimum of 2 experiments, with a minimum of 9 mice per group. Each stimulation was conducted in triplicate. Data is shown as means ± SEM. ** $p<0.01$ ns: not significant).

3.6. Depletion of IL-5 does not affect the worm burden in IL-6$^{-/-}$ mice

To investigate whether increased levels of IL-5 *in vitro* were relevant *in vivo* and resulted in the increased worm burden observed in IL-6$^{-/-}$ mice, IL-5 signaling was inhibited by injecting anti-IL-5 antibodies (TRFK5) *in vivo*. Experiments were conducted in two different settings. In the first setting, purified antibodies were generated in our lab. Mice were injected with 300 µg TRFK5 or isotype control antibody (TRFK4) in 100 µl sterile PBS i.p. daily starting at 5 dpi until the day of sacrifice (14 dpi). The second setting used commercial antibodies. The mice were injected with 50 µg TRFK5 or isotype control antibody (rat IgG1) in 100 µl sterile PBS 24 hours prior to and at 3, 7, 10, 13 dpi. However, *in vivo* administration of TRFK5 by both settings did not reduce the worm burden in IL-6$^{-/-}$ mice at 14 dpi. The isotype treated control groups showed the expected increase in worm numbers in IL-6$^{-/-}$ mice (figure 3.6.1 A). Inhibition of IL-5 was successful as drastically reduced eosinophil numbers were found in the pleural cavity after TRFK5 injection, both in IL-6$^{-/-}$ and BALB/c mice (figure 3.6.1 B).

As there was a trend to increased worm burden in IL-6$^{-/-}$ mice after IL-5 depletion, it was hypothesized that increased IL-5 levels in IL-6$^{-/-}$ mice may affect the worm burden at later time points of infection, as similar worm burden were observed between both BALB/c and IL-6$^{-/-}$ mice at 30, 60 and 90 dpi. Therefore, anti-IL-5 was injected starting at 12 dpi and followed at 17, 23 and 28 dpi and mice were analyzed for worm burden at 30 dpi. As expected, no different worm burden was found between isotype treated BALB/c and IL-6$^{-/-}$ mice at 30 dpi. However, IL-5 depletion did not increase the worm burden in IL-6$^{-/-}$ mice compared to isotype treated BALB/c controls (figure 3.6.2 A) suggesting that IL-5 and eosinophils are not mediating the worm clearance in IL-6$^{-/-}$ mice at that time point of infection.

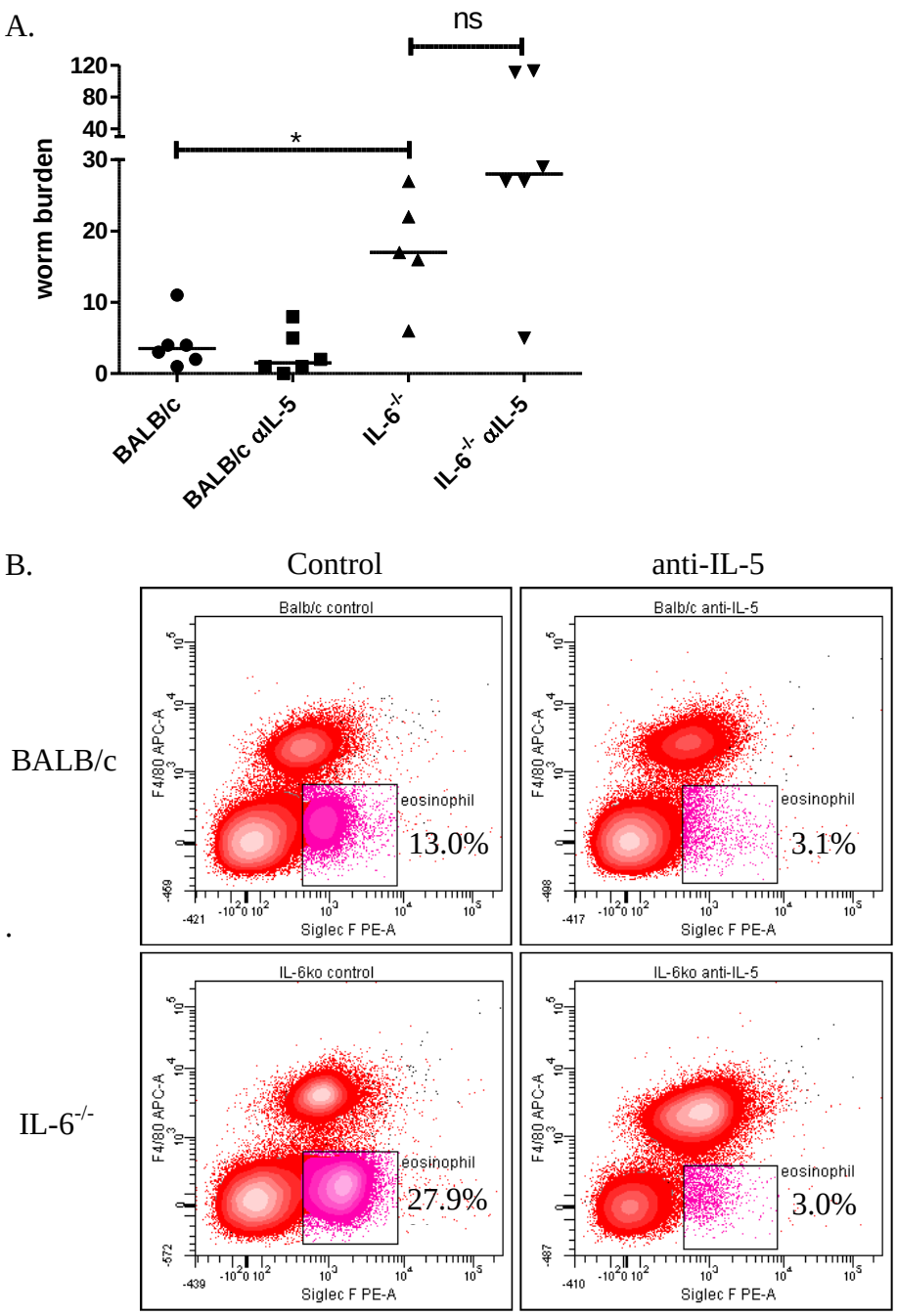
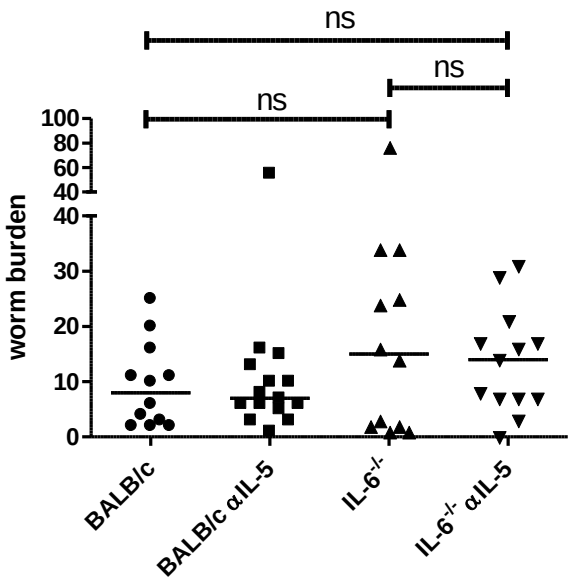


Figure 3.6.1. Administration of anti-IL-5 does not reduce the worm burden in IL-6$^{-/-}$ mice at early time points of infection. Mice were injected with anti-IL-5 (TRFK5) and isotype control (rat IgG1 or TRFK4) starting at 5 dpi until sacrificing, or at day 1 prior to and day 3, 7, 10 and 13 after infection. Worm burden at 14 dpi was determined by light microscopy (A). Frequency of eosinophils in the pleural cavity of BALB/c (top row) and IL-6$^{-/-}$ mice (bottom row), in control mice (left column) and after anti-IL-5 injection (right column) determined by flow cytometry (B). (Representative of 3 experiments using different settings with a minimum 5 mice per group. Data is shown as median. * $p < 0.05$ ns: not significant).

A.



B.

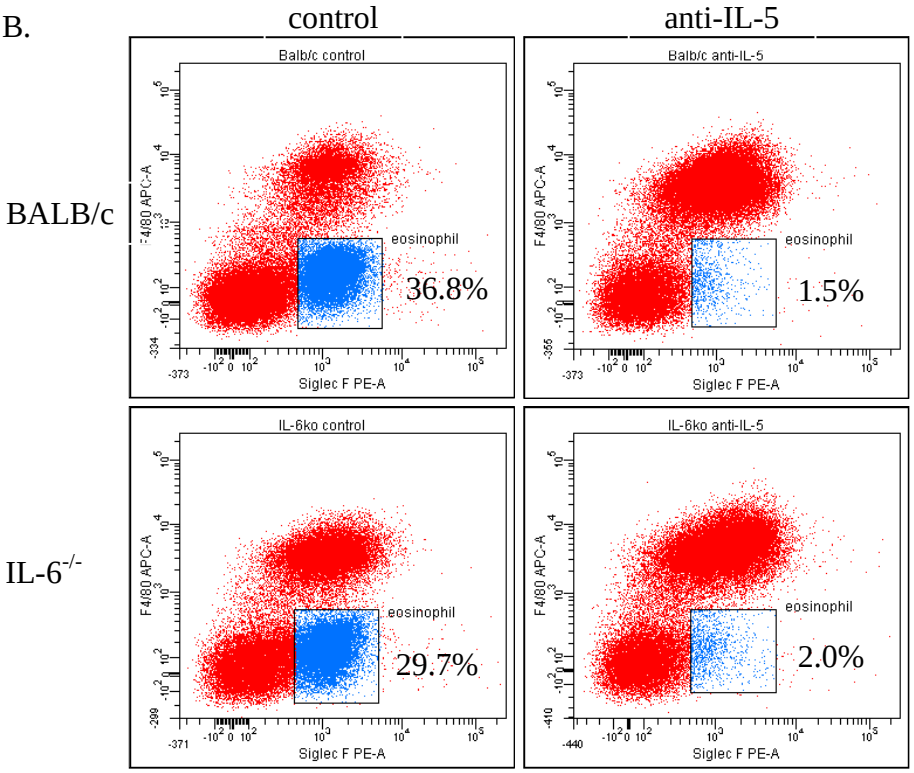


Figure 3.6.2. Depletion of IL-5 does not maintain increased worm numbers in IL-6$^{-/-}$ mice at 30 dpi. Mice were injected with anti-IL-5 (TRFK5) and isotype control (rat IgG1) at 12, 17, 23, and 28 dpi. Worm burden at 30 dpi was determined by light microscopy (A). Eosinophil population in the pleural cavity of BALB/c (top row) and IL-6$^{-/-}$ mice (bottom row), in control mice (left column) and after anti-IL-5 injection (right column) as determined by flow cytometry (B). (Representative of 1 experiment with a minimum of 12 mice per group. Data is shown as median. ns: not significant).

3.7. Increased IFNγ production in IL-6$^{-/-}$ mice 15 days post *L. sigmodontis* infection

Saeftel and colleagues have shown that IFNγ is important for the encapsulation of adult worms by increasing neutrophil activation and granuloma formation during chronic infection with *L. sigmodontis* (Saeftel et al. 2001)**.**

To investigate the role of type 1 cytokines, mainly IFNγ, IFNγ was measured in the supernatant of cultured splenocytes. Interestingly, increased amounts of parasite-specific IFNγ were found in IL-6$^{-/-}$ mice compared to BALB/c mice at 15 dpi (figure 3.8). However, this increase was not found at later time points of infection (data not shown). Levels of *in vivo* IFNγ in serum and pleural lavage during *L. sigmodontis* infection were below the limit of detection (data not shown).

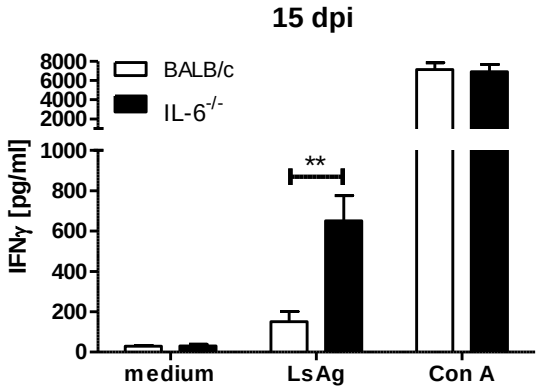


Figure 3.7. Increased in vitro IFNγ production by splenocytes of IL-6$^{-/-}$ mice 15 days post *L. sigmodontis* infection. Splenocytes from *L. sigmodontis* infected BALB/c and IL-6$^{-/-}$ mice at 15 dpi were removed and restimulated with medium, LsAg and ConA for 72 hours. IFNγ in the supernatant was measured by ELISA. (Representative of a minimum of 2 experiments with 6 mice per group. Each stimulation was conducted in triplicate. Data is shown as means ± SEM. ** $p<0.01$).

3.8. Neutrophil migration and activation is independent of IL-6

It has been described that IL-6 is involved in neutrophil migration from the bone marrow to the peripheral blood (Murphy 2012). Therefore, neutrophil numbers were determined in the peripheral blood and pleural cavity of naive mice and mice infected with *L. sigmondontis* for 7 and 15 days, as well as at later time points. FACS plots for the determination of neutrophil numbers are shown in figure 3.8.1 A. Neutrophils were found in low numbers in the pleura cavity before infection (figure 3.8.1 B, left columns) and few days after infection. This is consistent with previous data showing that neutrophil numbers started to increase after 4 weeks post infection and peaked at ~2 months post infection (figure 3.1.2 A). However, no differences in neutrophil frequencies were found in blood as well as in the pleural cavity between BALB/c and IL-$6^{-/-}$ mice before infection and at 7 dpi (figure 3.8.1 B, C and D). Neutrophil numbers in the peripheral blood at 15 dpi were significantly increased in IL-$6^{-/-}$ mice compared to BALB/c controls, although no differences were observed in the pleural cavity. The number of neutrophils at later time points (30, 60 and 90 dpi) was not different for either type of mouse, neither in blood nor in the pleural lavage (data not shown).

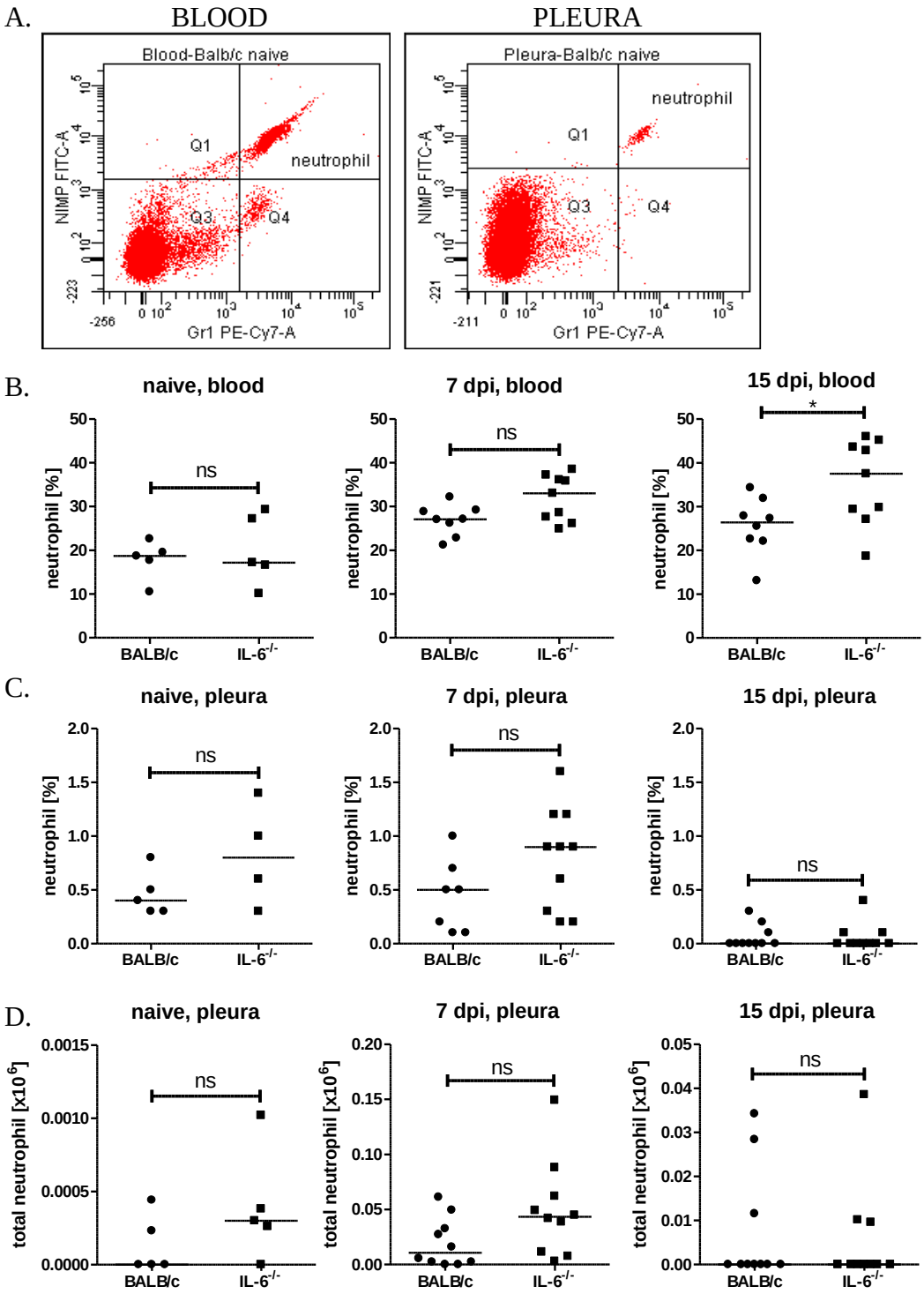


Figure 3.8.1. Pleural cavity neutrophil numbers are comparable between BALB/c and IL-6$^{-/-}$ mice during *L. sigmodontis* infection. Gating strategy for neutrophils in the blood (left) and pleural cavity (right). Neutrophils were determined as Gr1^{+}NIMP^{high} cells (A). Frequency of neutrophils in the blood of naïve (left) and 7 (middle) and 15 dpi (right) infected BALB/c and IL-6$^{-/-}$ mice (B). Relative (C) and absolute (D) numbers of neutrophils in the pleural cavity of naive mice (left), 7 (middle) and 15 dpi (right). (Representative of 2 experiments with a minimum of 5 mice per group. Data is shown as median. * $p < 0.05$, ns: not significant).

Furthermore, neutrophil activation was analyzed by measuring neutrophil-activating cytokines released *in vivo* and *in vitro*. Levels of the neutrophil activation-associated cytokines TNFα and MIP-2 were not different in the pleural cavity of BALB/c and IL-6$^{-/-}$ mice (data not shown). Neutrophil activation as determined by TNFα and MIP-2 production *in vitro* after neutrophil stimulation was also comparable in both groups of mice. Interestingly, stimulation with LsAg led to increased IL-6 secretion by neutrophils in the supernatant (figure 3.8.2).

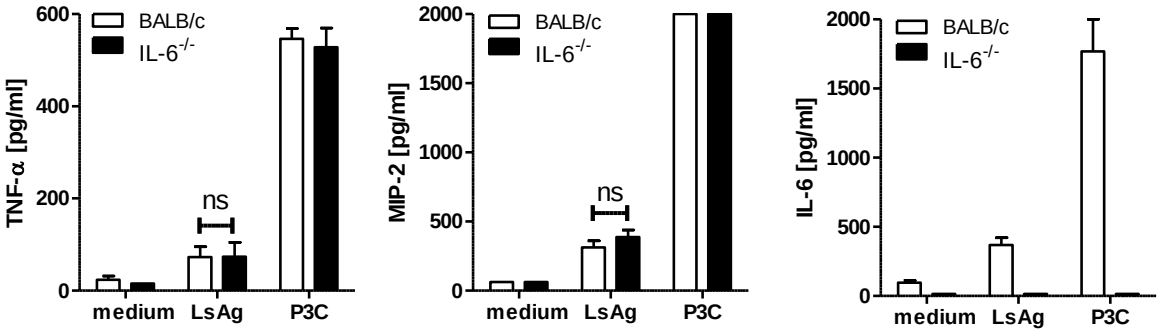


Figure 3.8.2. Levels of neutrophil activation associated cytokines are similar between BALB/c and IL-6$^{-/-}$ mice. Neutrophils from the peritoneal lavage of naive mice were enriched with casein solution. Neutrophils were then purified by gradient density using Percoll and stimulated for 18 hours with medium, LsAg and Pam3CSK. Secretion of TNFα (left), MIP-2 (middle) and IL-6 (right) in the supernantant was measured by ELISA. (Representative of 2 experiments with a minimum of 3 mice per group. Data is shown as means ± SEM. ns: not significant).

3.9. Increased levels of soluble IL-6 receptor in BALB/c mice after *L. sigmodontis* infection.

Soluble IL-6 receptor (sIL-6R), but not membrane bound IL-6R (mIL-6R), is important in the pro-inflammatory response against pathogens. Since not every leukocyte expresses mIL-6R, sIL-6R expands the function of IL-6 to other cells which do not express mIL-6R, such as eosinophils (Scheller et al. 2011).

To determine the role of sIL-6R in IL-6 deficiency during *L. sigmodontis* infection, sIL-6R was measured by ELISA in serum and in the pleural lavage of naive and infected mice as well as *in vitro* after splenocyte and neutrophil stimulation. No significant different levels of sIL-6R were found in the serum or the pleural lavage of infected mice at 7 and 15 dpi between BALB/c and IL-6$^{-/-}$ mice (figure 3.9 A). Moreover, levels of sIL-6R in the supernatant of LsAg stimulated splenocytes and neutrophils were not different between BALB/c and IL-6$^{-/-}$ mice, either (figure 3.9 B), although levels of sIL-6R in IL-6$^{-/-}$ mice

were significantly increased after splenocyte stimulation with ConA (figure 3.9 B, left). However, *L. sigmodontis* infection increased sIL-6R levels in BALB/c mice *in vivo* but not *in vitro* as less sIL-6R secretion was measured in naïve BALB/c mice (data not shown). Additionally, no different production of sIL-6R was observed after LsAg stimulation compared to unstimulated cells, both in splenocyte (figure 3.9.B left) and neutrophil cultures (figure 3.9 B right).

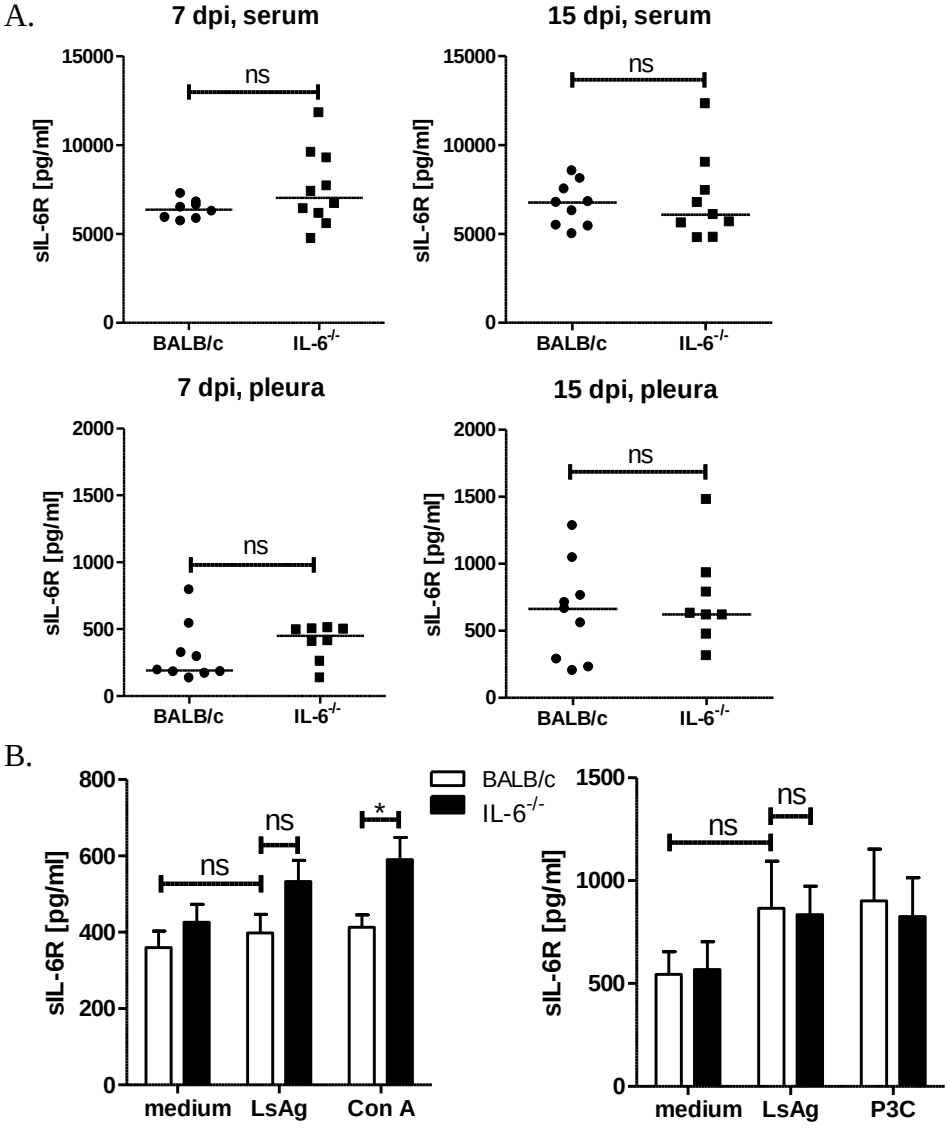


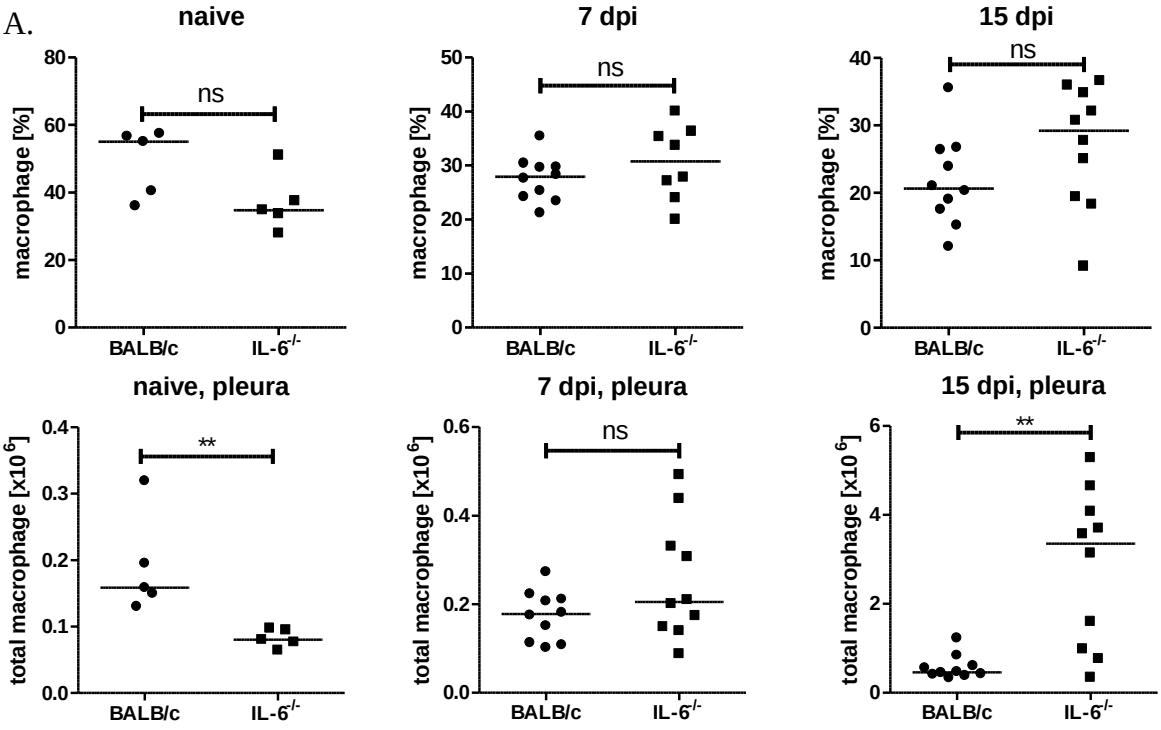
Figure 3.9. Levels of sIL-6R are not different between BALB/c and IL-6$^{-/-}$ mice. Level of soluble interleukin-6 receptor (sIL-6R) measured in serum (top row) and pleura (bottom row) in BALB/c and IL-6$^{-/-}$ mice at 7 (left) and 15 (right) dpi (A). sIL-6R levels measured in the supernatant after splenocyte culture for 72 hours (left) and neutrophil stimulation for 18 hours (right) (B). (Representative of a minimum of two experiments with a minimum 6 mice per group. Data is shown as median [sIL-6R level *in vivo*] and means ± SEM [sIL-6R level *in vitro*]. ns: not significant).

3.10. Macrophage numbers and activation are comparable between BALB/c and IL-6$^{-/-}$ mice

Macrophages are one of the main sources of IL-6 in the early response against pathogens. Additionally, IL-6 is important to switch from neutrophil to monocyte infiltration into the site of infection (Scheller et al. 2011; Zhang et al. 2013).

To investigate the role of IL-6 in macrophage-associated protection against *L. sigmodontis* early after infection, numbers of macrophages at the site of infection of naïve and infected mice were determined. The absolute number of macrophages was significantly reduced in IL-6$^{-/-}$ mice compared to BALB/c controls in the pleural cavity in the absence of infection. Macrophages composed around half of the total leukocyte numbers that reside in the pleural cavity in naïve BALB/c mice. However, no difference in either relative or absolute macrophage numbers was found between IL-6$^{-/-}$ and BALB/c mice at 7 dpi. Total but not relative numbers of macrophages in the pleural cavity was significantly increased in IL-6$^{-/-}$ mice at 15 dpi (figure 3.10 A). Additionally, numbers of AAMs (F4/80^{high}RELMα^{high} cells) and Mean Fluorescence Intensity (MFI) of RELMα from macrophages were not different between BALB/c and IL-6$^{-/-}$ mice at 30 dpi (figure 3.10 B and C), although the percentage of AAMs from total macrophages was significantly increased in IL-6$^{-/-}$ mice.

A.



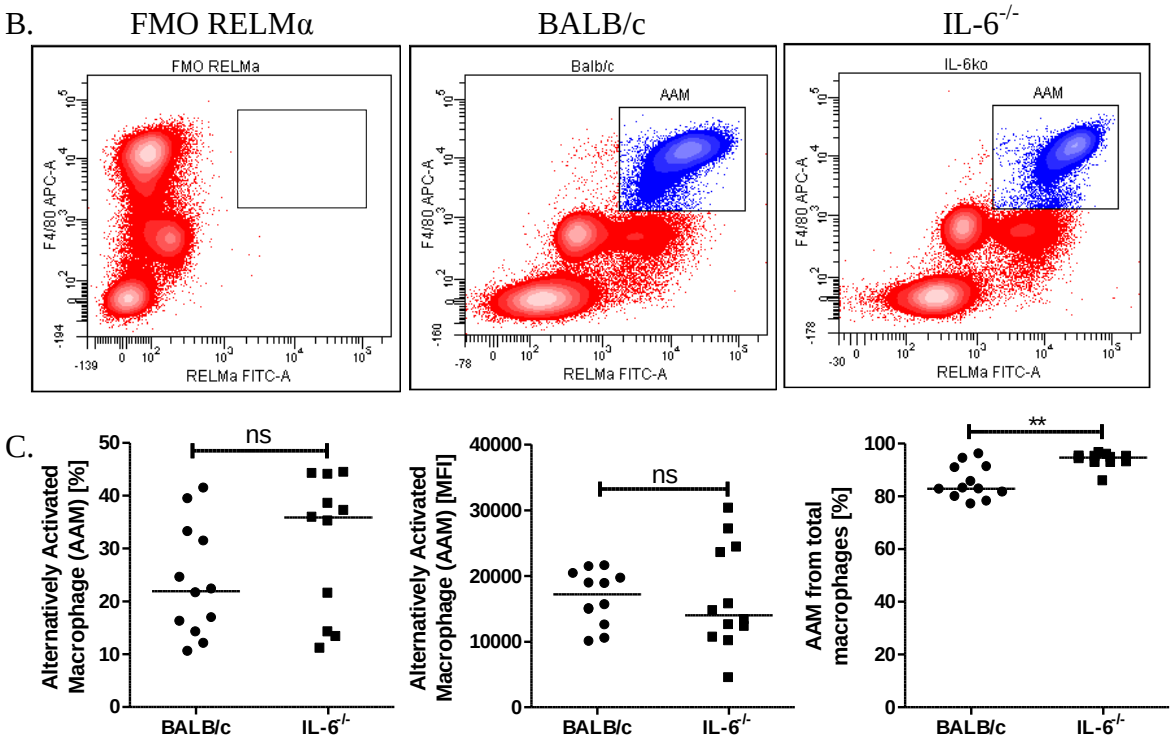


Figure 3.10.1. Numbers of macrophages are not different between BALB/c and IL-6$^{-/-}$ mice. Relative (top row) and absolute (bottom row) numbers of macrophages which were determined in the pleural cavity of naïve (left), 7 (middle) and 15 days (right) *L. sigmodontis* infected BALB/c and IL-6$^{-/-}$ mice (A). AAMs in BALB/c (middle) and IL-6$^{-/-}$ (right) mice at 30 dpi were determined in the pleural cavity using FACS by firstly gating on F4/80^{high}RELMα^{high} using florescence minus one (FMO) approach (left) (B). Frequency (left) and MFI (middle) of AAMs as well as and percentage of AAMs from total pleural macrophages (right) at 30 dpi were determined with FACS (C). (Representative of 2 experiments with minimum of 5 mice per group. Data is shown as median, ns: not significant, ** $p < 0.01$).

In order to investigate whether IL-6 deficiency affected macrophage activation, macrophages were purified from the peritoneal cavity after thioglycollate enrichment and stimulated with different stimuli. No difference in TNFα (figure 3.10.2 A) and MIP-2 (figure 3.10.2 B) production after stimulation with LsAg was found in IL-6$^{-/-}$ and BALB/c mice. As expected, IL-6 production by macrophages following stimulation with LsAg and P3C was determined in BALB/c but not IL-6$^{-/-}$ mice (figure 3.10.2 C).

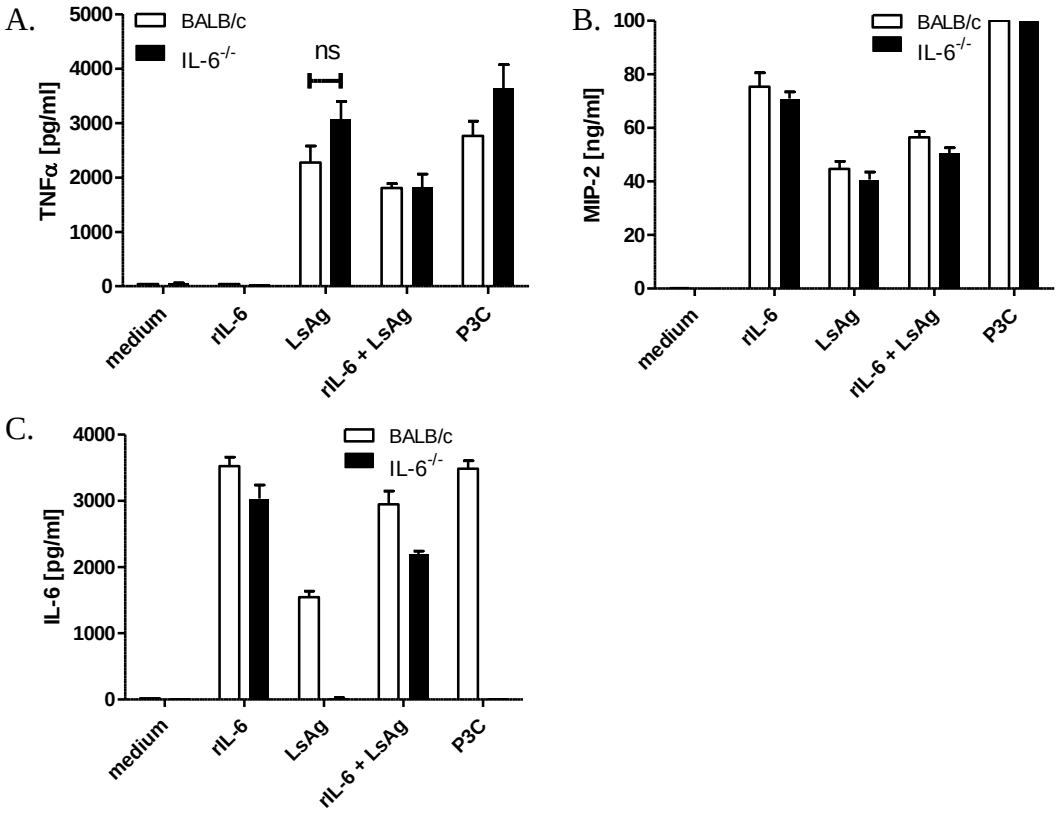


Figure 3.10.2. Concentrations of macrophage activation-associated cytokines are not different between BALB/c and IL-6$^{-/-}$ mice. Macrophages in the peritoneal lavage were enriched by thioglycollate injection. Macrophages were then purified and stimulated with different stimuli. TNFα (A), MIP-2 (B) and IL-6 (C) secretion in the supernatant was measured by ELISA. (Representative of 2 experiments with minimum of 5 mice per group. Data is shown as means ± SEM [cytokines]. ns: not significant).

3.11. Decreased relative, but not absolute numbers of B cells in IL-6$^{-/-}$ mice at early time points of *L. sigmodontis* infection

IL-6 was first described as a B cell stimulatory factor (BCSF) which was originally identified as a B cell differentiation factor. IL-6 supports the development and maturation of B cells (Ishihara and Hirano 2002; Scheller et al. 2011). In addition, although B cells are not important for worm survival and growth in *L. sigmodontis* infection, it was reported that lack of B-cells prevents the development of filarial patency in primary infection (Martin et al. 2001).

To investigate the role of IL-6 on B cells during *L. sigmodontis* infection, B cell populations were determined in the blood as well as in the pleural cavity by flow cytometry. Significantly decreased relative but not absolute numbers of total B cells in IL-6$^{-/-}$ mice were observed at 7 and 15 dpi, both in the circulation (figure 3.11 A) and in the pleural cavity (figure 3.11 B and C). However, no significant differences in B cell numbers between both groups occurred in naive mice (figure 3.11 A, B and C, left row), as well as at 30, 60 and 90 dpi (data not shown).

3.12. Decreased percentage of B2 cells in IL-6$^{-/-}$ mice at early time points of infection

Since B2 cells (CD19^{+}CD23^{+} B cells) are important for the occurrence of microfilaraemia in *L. sigmodontis* infection (Martin et al. 2001), it was investigated whether the B2 cell subset was normal in IL-6$^{-/-}$ mice. The relative number of B2 cells was significantly decreased in the peripheral blood (figure 3.12 A) and the pleural cavity (figure 3.12 B) of IL-6$^{-/-}$ mice at 7 (top rows) and 15 (bottom rows) dpi but not at later time points (data not shown). However, absolute numbers of B2 cells in the pleural cavity were not different between BALB/c and IL-6$^{-/-}$ mice at 7 and 15 dpi (figure 3.12 C).

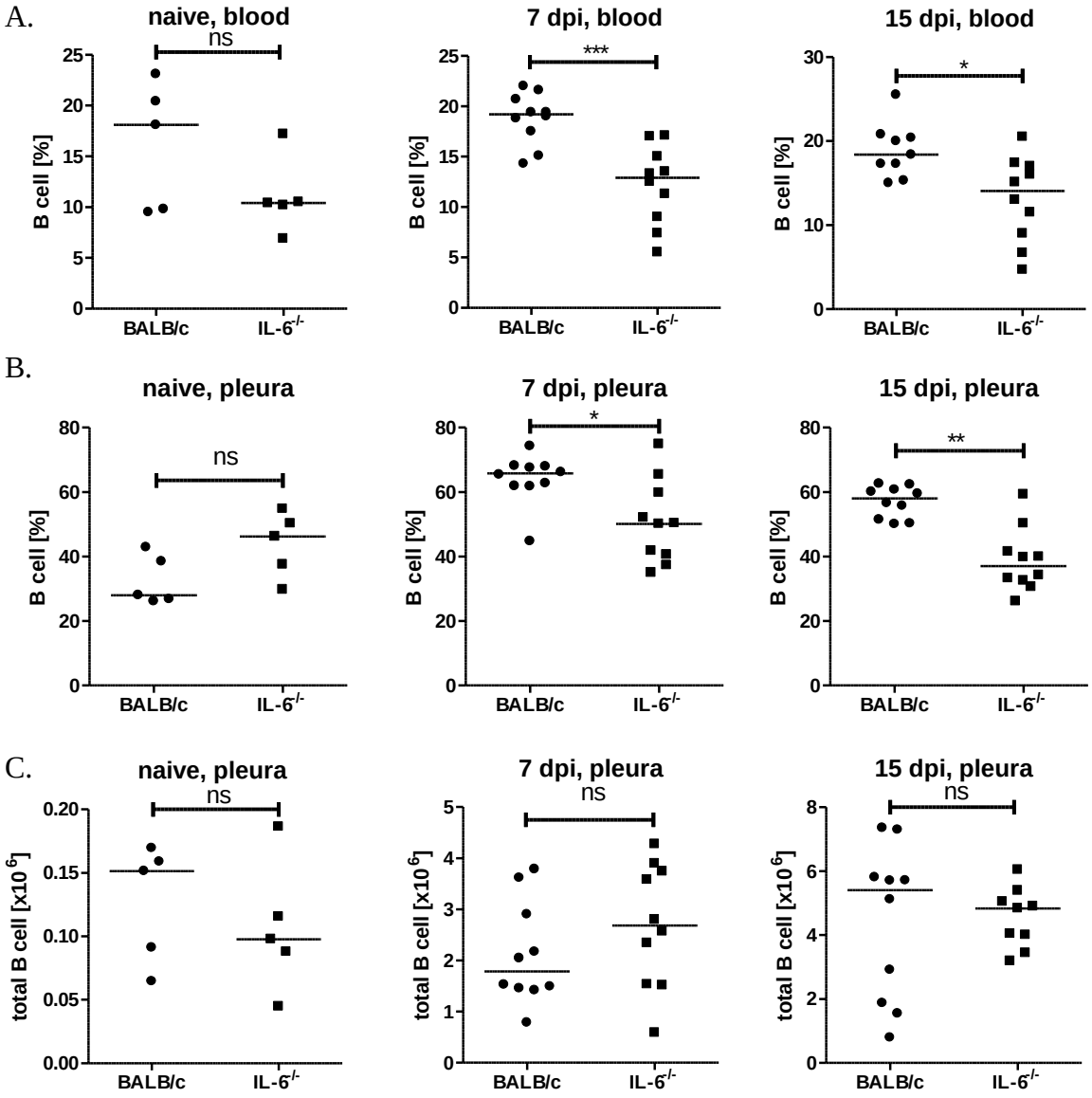


Figure 3.11. Reduced B cell frequencies in the blood and pleural cavity of IL-6$^{-/-}$ mice at early time points of infection. Frequency of B cells in the peripheral blood (A) as well as in the pleural cavity (B) of naïve (left), 7 (middle), and 15 (right) days infection of *L. sigmodontis* infected mice. Total number of B cells in the pleural cavity was also calculated (C). (Representative of a minimum of 2 experiments with a minimum of 5 mice. Data is shown as median * $p<0.05$, ** $p<0.01$, *** $p<0.001$ ns: not significant).

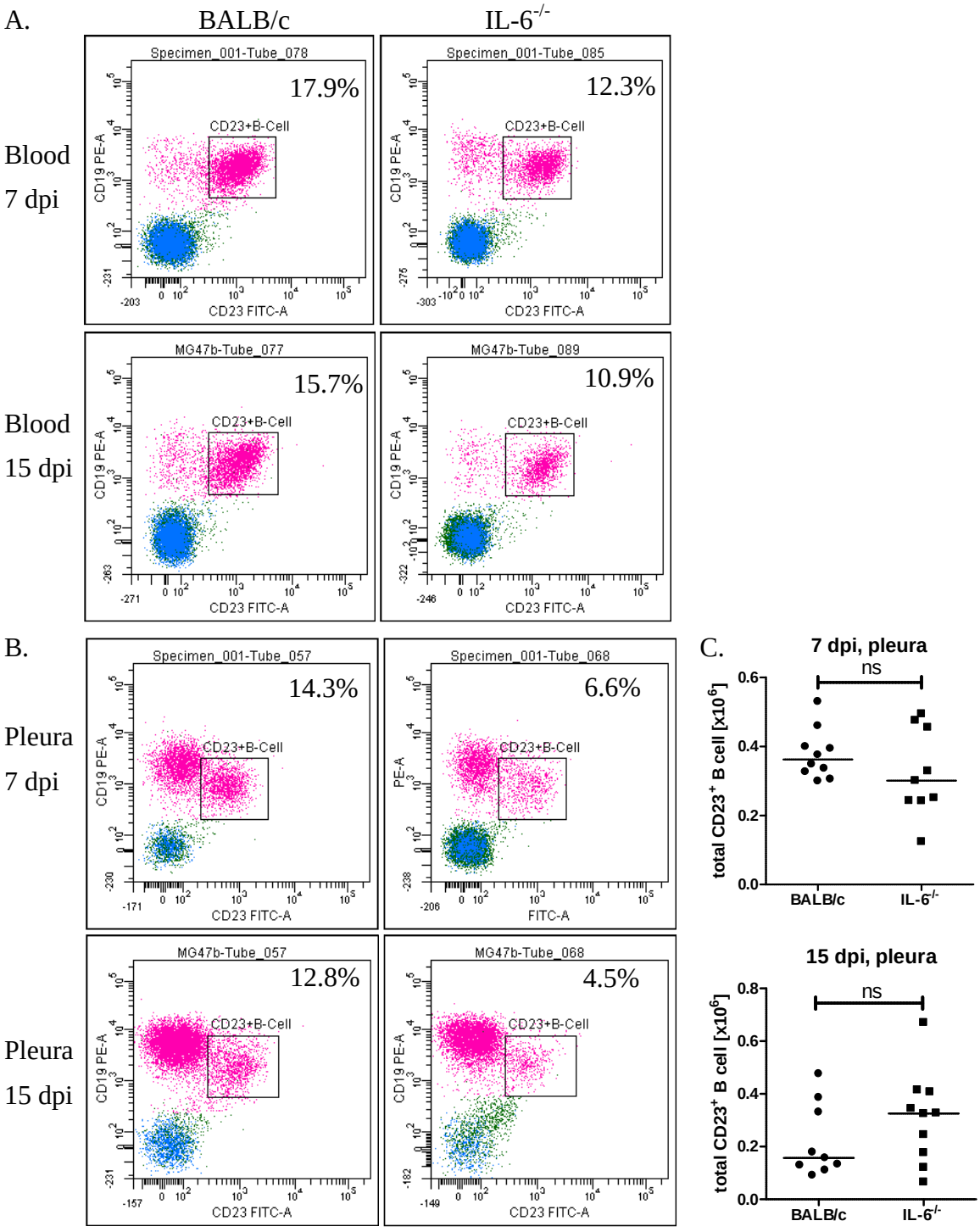


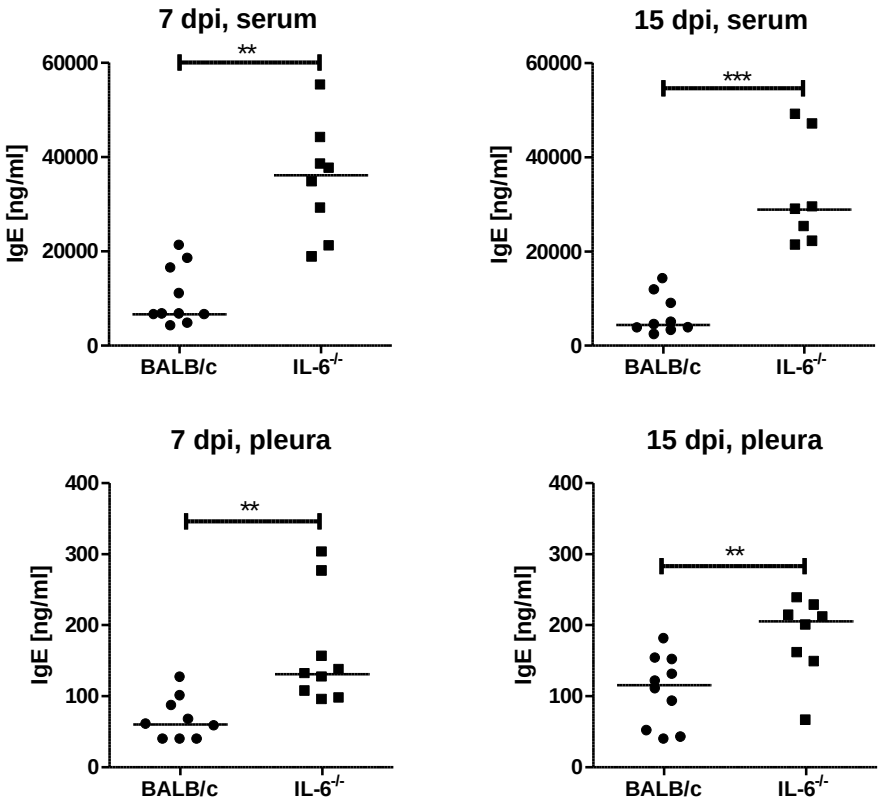
Figure 3.12. Decreased percentage of B2 cells in IL-6$^{-/-}$ at early time points of *L. sigmodontis* infection. Frequencies of B2 cells (CD19^{+}CD23^{+} B cells) of BALB/c (left) and IL-6$^{-/-}$ (right) mice was determined by FACS in blood (A) and pleural cavity (B) at 7 (top rows) and 15 (bottom rows) dpi. The graphs show the absolute number of B2 cells in the pleural cavity (C). (Representative of a minimum of two experiments with at least 9 mice per group. Data is shown as median, ns: not significant).

3.13. Increased IgE production in IL-6$^{-/-}$ mice

Increased IgE secretion is found during helminth infections as a result of increased type 2 immune responses. In *L. sigmodontis*-infected BALB/c mice, polyclonal IgE was detected in plasma as early as 2 weeks post infection and became strongly elevated by 6 weeks until 10 weeks post infection (Torrero et al. 2010).

In this study, IgE levels were increased both systemically and in the pleural cavity at 7 and 15 dpi (figure 3.13 A). These increases mirror the augmented worm burden at those time points, although B cell and B2 cell frequencies were reduced in IL-6$^{-/-}$ mice early during infection. Increased frequencies of IgE positive cells, probably basophils, were also found in IL-6$^{-/-}$ mice at 15 dpi (figure 3.13 B) suggesting that an increase in the overall secretion of IgE occurred in IL-6$^{-/-}$ mice at early time points post infection.

A.



B.

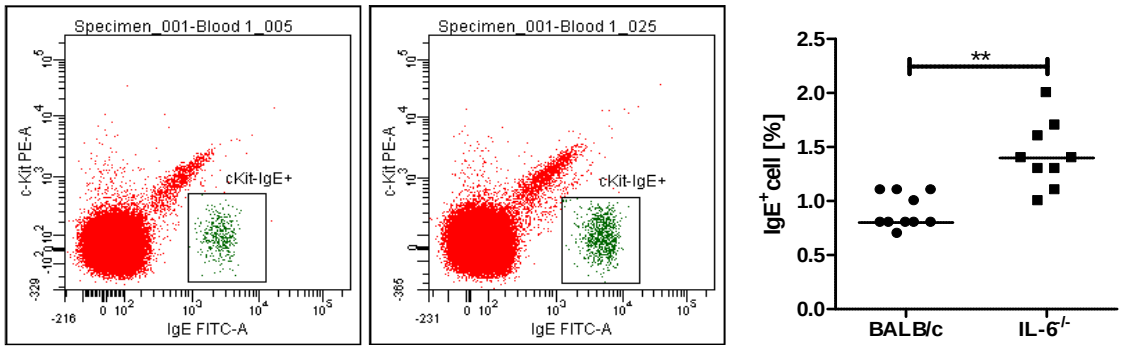


Figure 3.13. Increased production of IgE in IL-6$^{-/-}$ mice at early time points of *L. sigmodontis* infection. Concentration of total IgE in the serum (top) and pleural lavage (bottom) of infected BALB/c and IL-6$^{-/-}$ mice at 7 (left) and 15 (right) days post infection (A). IgE^{+} cells were identified by FACS in the blood of 15 days infected BALB/c (left) and IL-6$^{-/-}$ (middle) mice, and their percentages (right) (B). (Representative of a minimum of 2 experiments with a minimum of 8 mice per group. Data is shown as median. ** p<0.01 ***p<0.001).

3.14. IgG production is independent of IL-6

IL-6 is known to promote B cell differentiation into plasma cells (Kishimoto 1989). Additionally, IL-6 is reported to be crucial for promoting IgG1 production by B cells by inducing IL-21 production (Neveu et al. 2009). Therefore, IgG1 and IgG2a production were investigated in the serum of mice at 15, 30 and 60 days post infection. No difference in the production of IgG was found at any of those time points between the two groups (figure 3.14, middle and right columns).

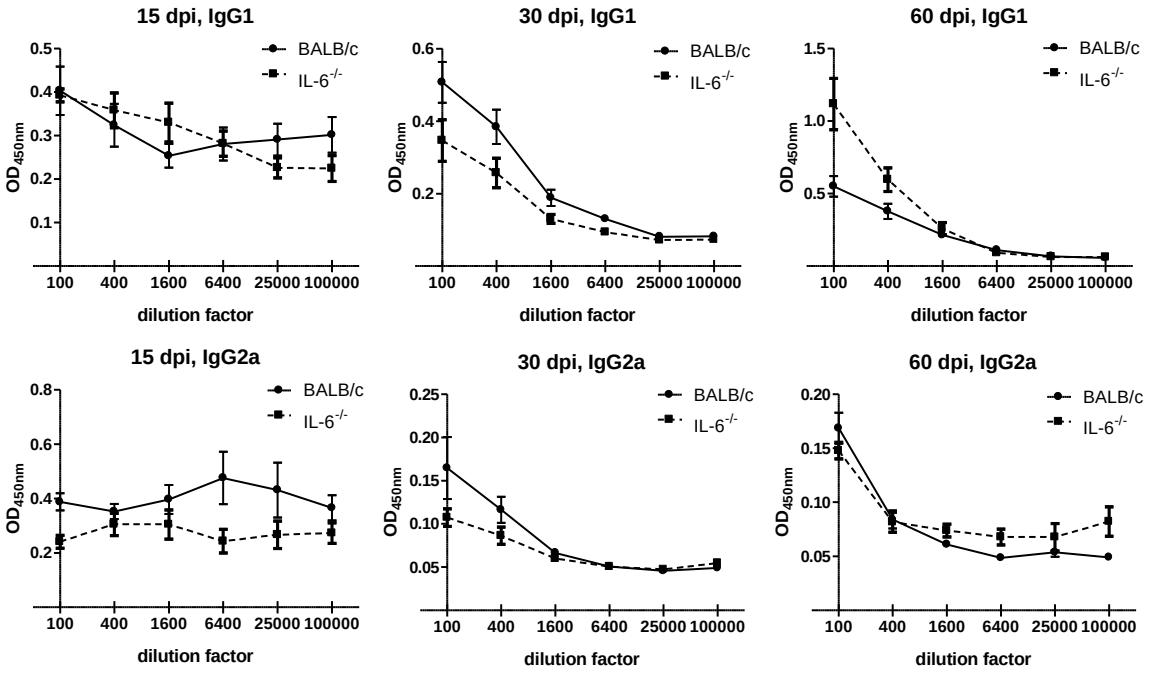


Figure 3.14. IL-6 deficiency does not lead to a decreased IgG production during *L. sigmodontis* infection. Levels of *L. sigmodontis* specific IgG1 (top row) and IgG2a (bottom row) in the serum of infected animals at 15 (left column), 30 (middle column) and 60 (right column) dpi were measured by ELISA. (Representative of two experiments with a minimum of 6 mice per group).

3.15. IL-6 deficiency does not affect CD4 T cell numbers and frequencies in the pleural cavity during *L. sigmodontis* infection

IL-6 is important for lymphocyte infiltration into the infected tissues by increasing the expression of cell adhesion molecules (Scheller et al. 2011). Additionally, CD4 T cells are crucial for *L. sigmodontis* protection as the depletion of CD4 T cells with anti-CD4 antibodies in BALB/c mice led to an increased worm burden and microfilaremia 8 weeks post infection (Al-Qaoud et al. 1997).

The number of CD4 T cells was investigated prior to infection, 7 and 15 dpi as well as during chronic infection. Absolute, but not relative numbers of CD4 T cells in the pleural cavity were decreased in naive IL-6$^{-/-}$ mice (figure 3.15 B, left row). No difference in CD4 T cell numbers were found at 7 and 15 dpi in the blood (figure 3.15 A) as well as in pleural cavity (figure 3.15 B) between BALB/c and IL-6$^{-/-}$ mice. CD4 T cell numbers were also not different at later time points of infection (data not shown).

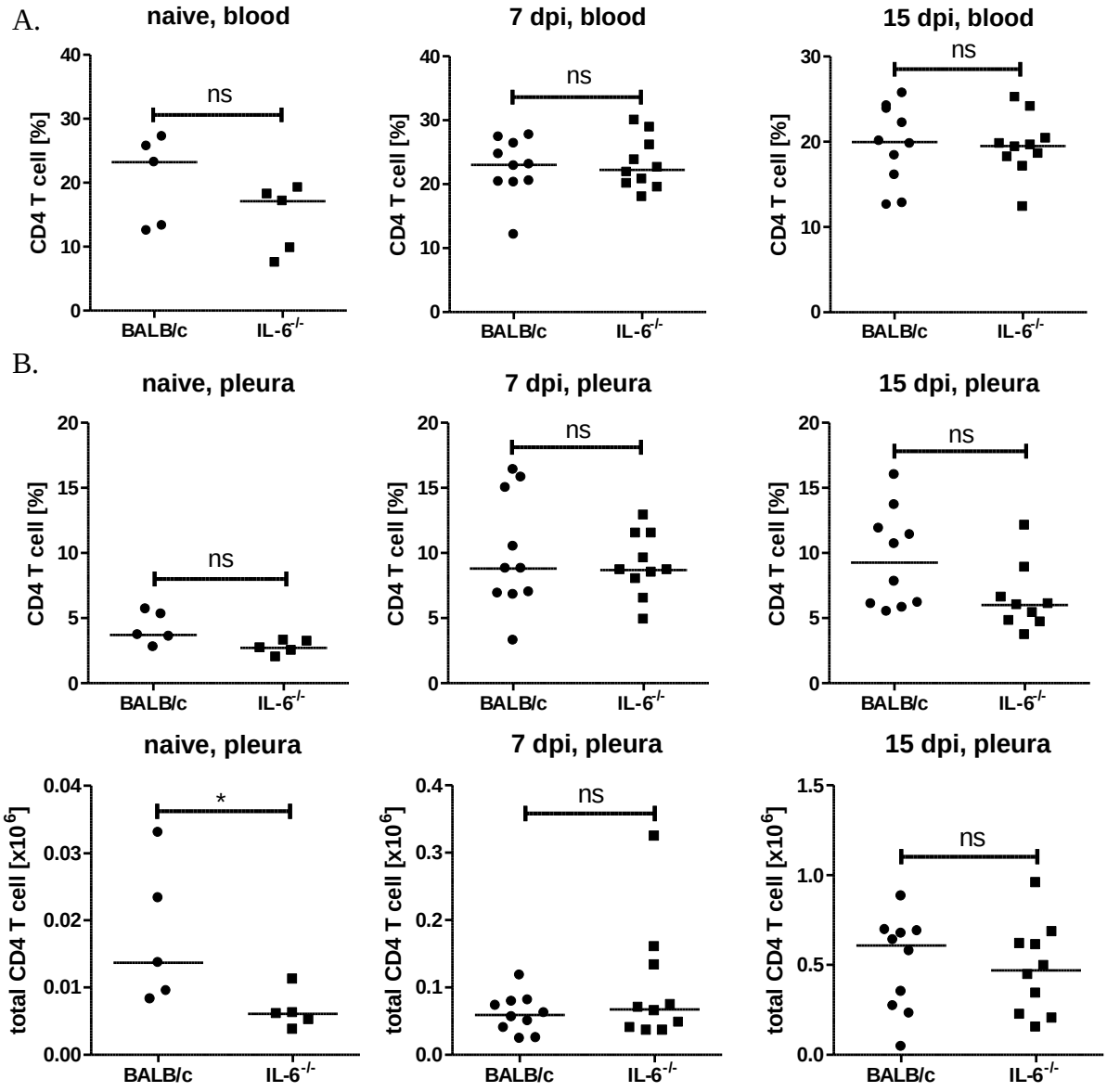


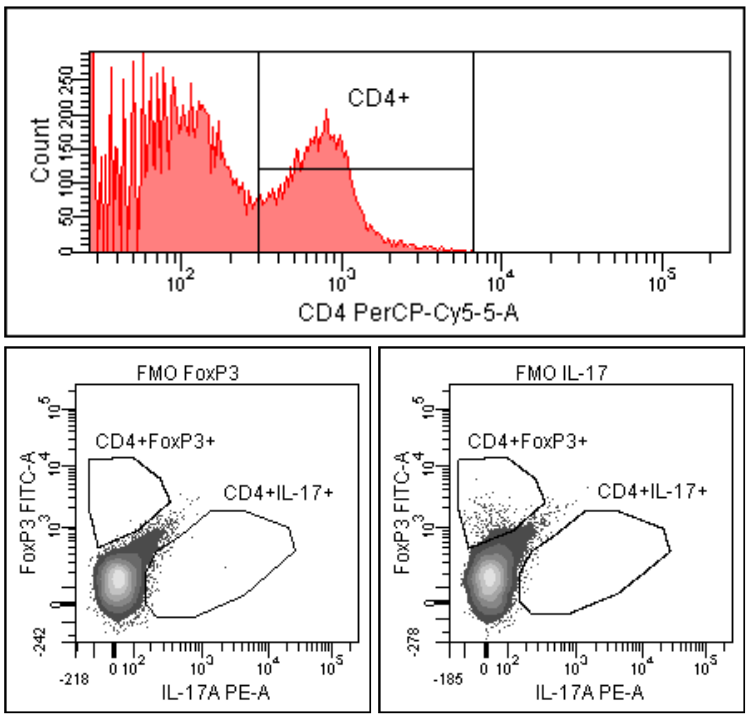
Figure 3.15. CD4 T cell numbers and frequencies are similar between BALB/c and IL-6$^{-/-}$ mice. BALB/c and IL-6$^{-/-}$ mice were infected with *L. sigmodontis*. Percentage of CD4 T cells in the blood (A) and pleural cavity (B, top row) of naive (left), and 7 (middle) and 15 (right) days infected animals were analyzed using FACS. Total CD4 T cells are also given (B, bottom row). (Representative of a minimum of 2 experiments with a minimum of 5 mice per group. Data is shown as median. * $p<0.05$ ns: not significant).

3.16. IL-6 deficiency does not affect the number of regulatory T cells (T_{reg}) and IL-17 production during *L. sigmodontis* infection

Several studies have demonstrated the importance of IL-6 for CD4 T cell differentiation (Dienz and Rincon 2009; Scheller et al. 2011). IL-6 is involved in the differentiation of T_{reg} and Th17 cells (Kimura and Kishimoto 2010), which were both shown to be important for filarial immunity (Specht and Hoerauf 2012). T_{reg} can suppress host protective immune responses during *L. sigmodontis* infection and lead to an increased worm burden during chronic infection (Taylor et al. 2005).

The numbers of T_{reg} and Th17 cells in spleens of infected mice were determined by FACS after 6 hours of *in vitro* culture with LsAg and anti-CD3/CD28 as a positive control. Due to the low number of $CD4^+IL\text{-}17^+$ T cells which were detected by FACS, IL-17 levels were measured in the supernatant after splenocyte culture for 3 days. T_{reg} were defined by FACS as $CD4^+FoxP3^+$ cells (figure 3.16 A). Interestingly, there was no difference in T_{reg} (figure 3.16 B and C) frequencies and IL-17 levels (figure 3.16 D) in LsAg-stimulated splenocytes of BALB/c and IL-$6^{-/-}$ mice at 7 and 15 dpi. Levels of IL-17 in the pleural lavage of infected mice were below the limit of detection (15.6 pg/ml; data not shown).

A.



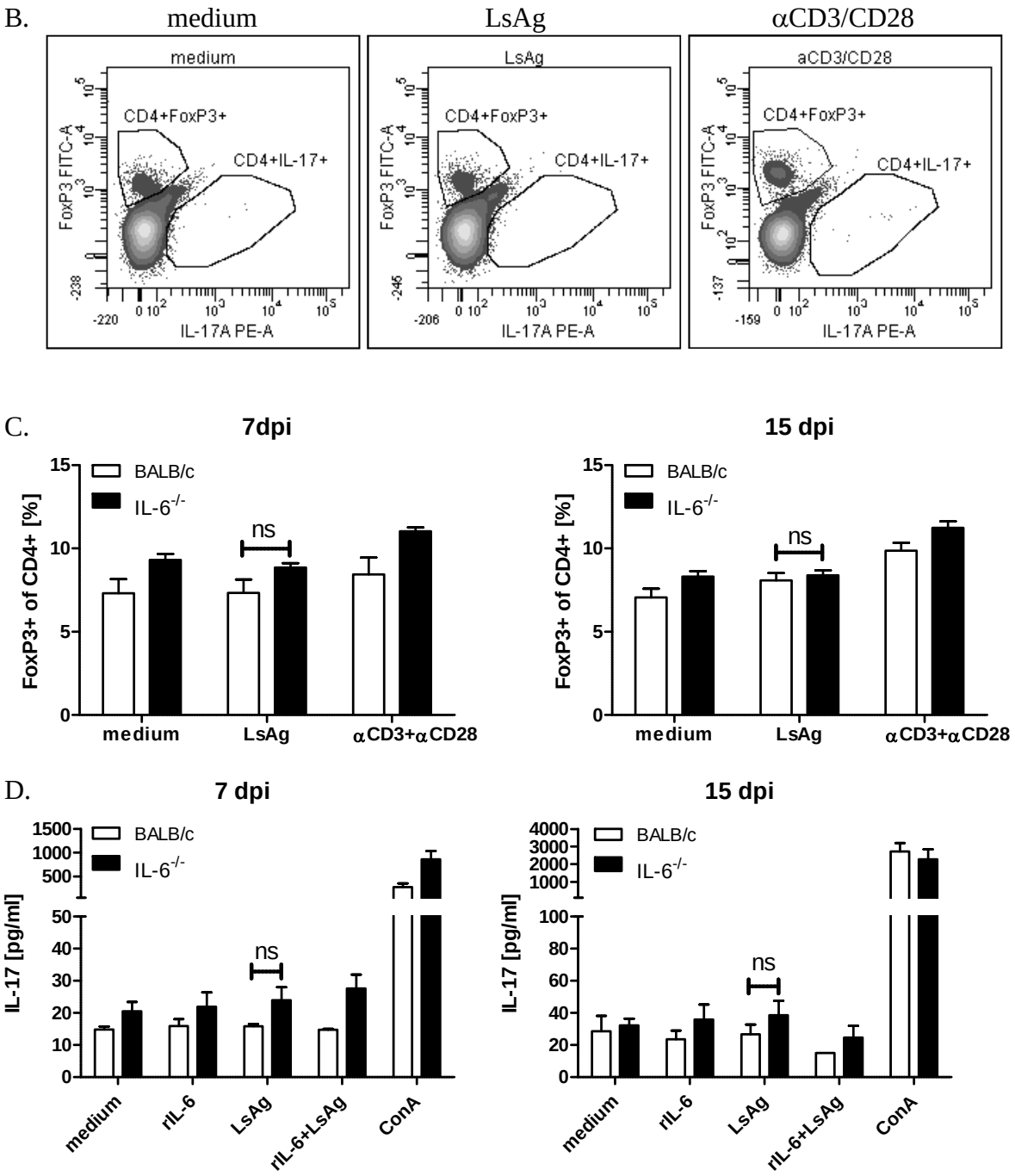


Figure 3.16. IL-6 deficiency does not affect T_{reg} numbers and IL-17 levels during *L. sigmodontis* infection. Splenocytes from *L. sigmodontis* infected IL-$6^{-/-}$ and BALB/c mice were incubated with medium, LsAg and αCD3/CD28 for 4 hours with monensin and intracellularly stained for FoxP3 and IL-17A. Population of $CD4^+$ was determined by FACS (top), followed by gating of $FoxP3^+$ (second row, left) and IL-$17A^+$ (second row, right) cells using the FMO approach (A). Gating of $CD4^+FoxP3^+$ and $CD4^+$IL-$17A^+$ cells after stimulation with medium (left), LsAg (middle) and αCD3/CD28 (right) (B). Frequency of $CD4^+FoxP3^+$ cells at 7 (left) and 15 (right) dpi (C). IL-17 secretion from splenocyte culture after stimulation for 72 hours (D). (Representative of a minimum of 2 experiments with at least 10 mice per group. Data is shown as means ± SEM. ns: not significant).

The immune responses against *L. sigmodontis* at the site of infection (pleural cavity) in IL-6$^{-/-}$ mice have been described above. In the next sections, investigations are presented that focused on events before the larvae reach the pleural cavity and potentially caused an increased worm burden in IL-6$^{-/-}$ mice at early time points of infection.

3.17. *L. sigmodontis* infected IL-6$^{-/-}$ mice have an increased worm burden despite blockade of mast cell degranulation

Several mechanisms might affect worm migration to the pleural cavity. Previously, it was shown that mast cell degranulation and histamine release increased vascular permeability and allowed better worm migration (Specht et al. 2011). Blocking mast cell degranulation with the mast cell stabilizer cromolyn still resulted in a significantly increased worm burden in IL-6$^{-/-}$ mice compared to the BALB/c controls (figure 3.17 B). However, three out of four experiments showed a trend (once reaching statistical significance with $p<0.05$) to a reduced worm burden in the cromolyn-treated IL-6$^{-/-}$ mice in comparison to the NaCl treated IL-6$^{-/-}$ controls (figure 3.17). This suggests that differences in mast cell degranulation in IL-6$^{-/-}$ mice are not the only mechanism that leads to the increased worm burden in IL-6$^{-/-}$ mice.

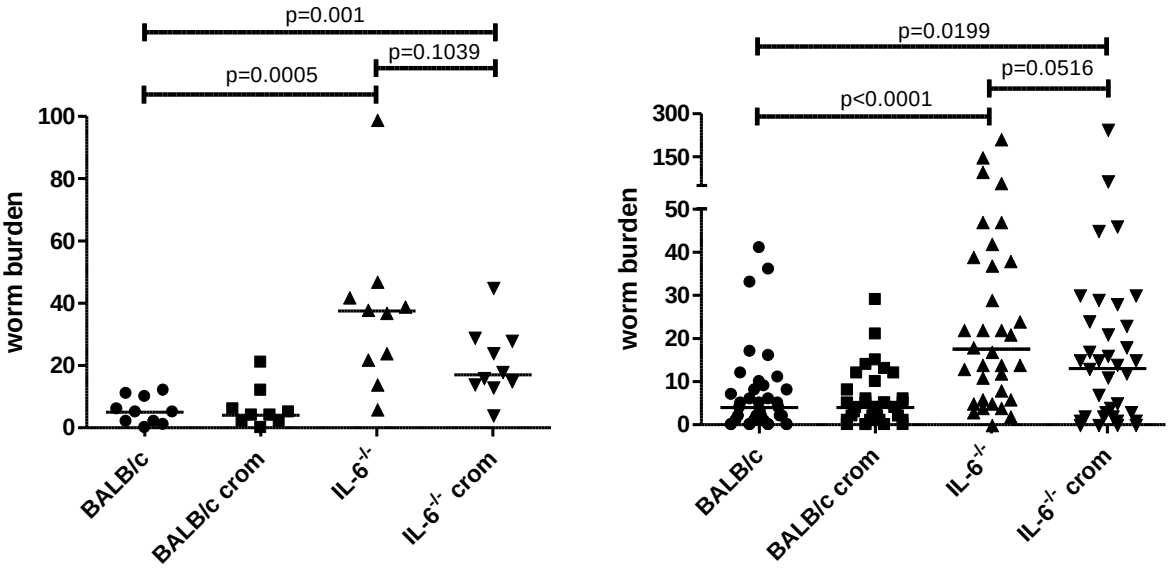
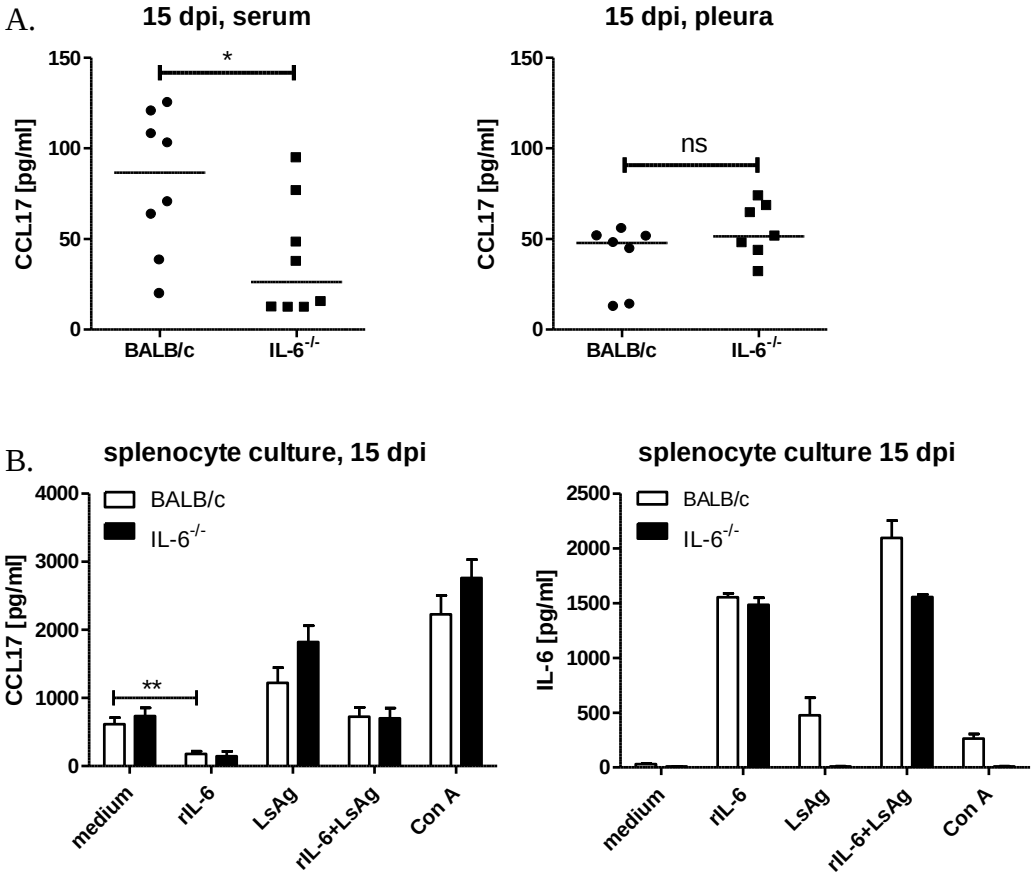


Figure 3.17. *L. sigmodontis* infected IL-6$^{-/-}$ mice have an increased worm burden despite blockade of mast cell degranulation. BALB/c and IL-6$^{-/-}$ mice were injected with cromolyn (crom) or NaCl as control daily from 6 days prior to infection until sacrificing. Worm burden at day 14 post *L. sigmodontis* infection is shown. Representative result out of four experiments (A). Pooled results of 4 similar experiments (B). (Data is shown as median).

3.18. Increased worm burden in IL-6$^{-/-}$ mice is not due to an increased vascular permeability caused by histamine release or reduction of CCL17 levels

Specht and colleagues (Specht et al. 2011) previously showed that CCL17 deficiency led to an increased vascular permeability and increased worm burden in CCL17$^{-/-}$ mice compared to BALB/c mice. In this study, CCL17 production in the pleural lavage was similar in BALB/c compared to IL-6$^{-/-}$ mice, although reduced levels of CCL17 were found in the serum of 15 days infected IL-6$^{-/-}$ mice (figure 3.18.1 A). Stimulation of splenocytes with LsAg, ConA or LsAg + rIL-6 did not reveal any differences in CCL17 production between IL-6 deficient and competent mice (figure 3.18.1 B). Interestingly, splenocyte cultures from both mouse strains produced significantly less CCL17 *in vitro* after addition of rIL-6 into the supernatant (figure 3.18.1.B). Furthermore, IL-6 deficiency led to an increased production of CCL17 after DC stimulation. This increase was dependent on the concentration of IL-6 present in the culture (figure 3.18.1.C), suggesting that *in vitro* CCL17 production is reduced in an IL-6 dependent manner.



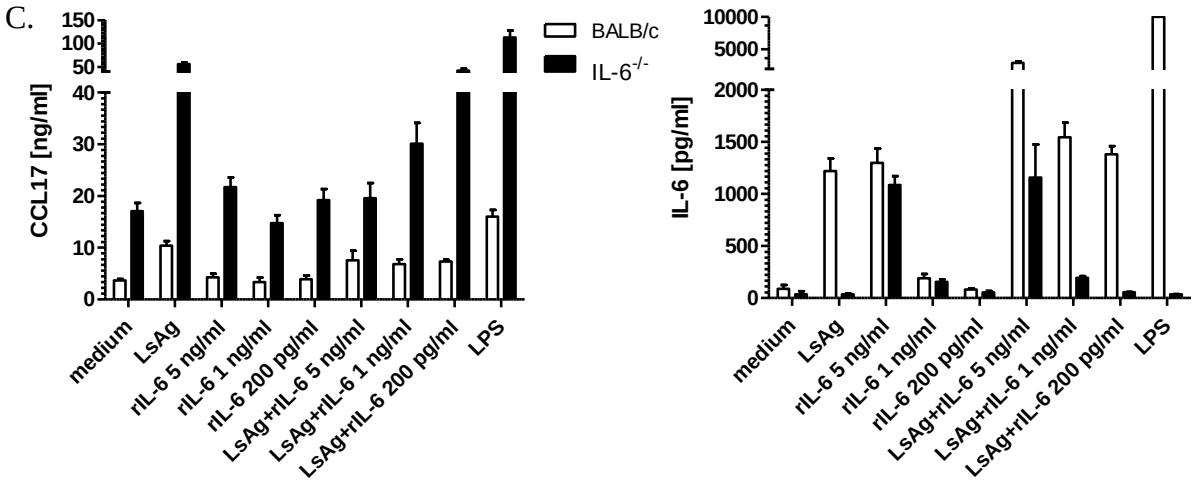


Figure 3.18.1. ***In vitro*** **CCL17 production is reduced in an IL-6 dependent manner**. Levels of CCL17 in the serum (left) and pleural lavage (right) 15 days post *L. sigmodontis* infection (A). Splenocytes from 15 days infected mice were stimulated with rIL-6, LsAg, rIL-6 + LsAg and ConA for 72 hours and CCL17 (left) and IL-6 (right) production is given (B). DCs were derived from bone marrow of naive mice and cultured for 18 hours with different stimuli and CCL17 (left) and IL-6 (right) production is shown (C). (Representative of 2 experiments with a minimum of 6 (A and C) or 3 (B) mice per group. Each stimulation was conducted in triplicate. Data is shown as median [*in vivo*] or means ± SEM [*in vitro*]. * $p < 0.05$, ** $p < 0.01$, ns: not significant).

Given that histamine mediates vasodilatation that may facilitate the migration of *L. sigmodontis* larvae to the pleural cavity (Specht et al. 2011), the role of histamine was analyzed by blocking histamine receptors *in vivo*. Usage of histamine receptor antagonists 1 and 2 did not reduce the worm burden in IL-6$^{-/-}$ mice compared to the control groups (figure 3.18.2).

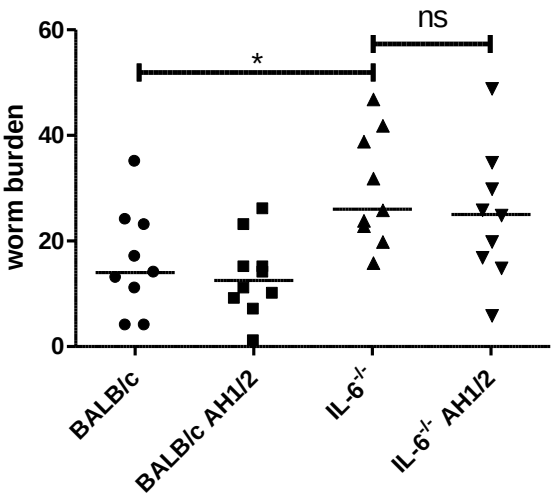


Figure 3.18.2. Blocking of histamine receptors does not decrease *L. sigmodontis* worm burden in IL-6$^{-/-}$ mice. BALB/c and IL-6$^{-/-}$ mice were injected with histamine receptor antagonists pyrilamine and cimetidine in PBS (AH1/2) or PBS alone two hours prior to and 1 hour post infection, followed by daily injections until 5 dpi. Worm burden was determined at 14 dpi. (Representative of 2 experiments with a minimum of 9 animals per group. Data is shown as median. * $p<0.05$ ns: not significant).

In order to investigate a role of vascular permeability in increasing the worm burden in IL-6$^{-/-}$ mice at an early time post infection, the role of IL-6 in vascular permeability of the skin was analyzed. BALB/c and IL-6$^{-/-}$ mice were injected with LsAg i.c. followed by i.v. Evans Blue injection and dye leakage from blood vessels into the tissue was quantified. There was no difference of Evans Blue leakage in IL-6$^{-/-}$ compared to BALB/c mice. However, significantly increased levels of Evans Blue were observed after LsAg and anti-FcεR1 (MAR-1) treatment compared to PBS injection in both types of mice (figure 3.18.3).

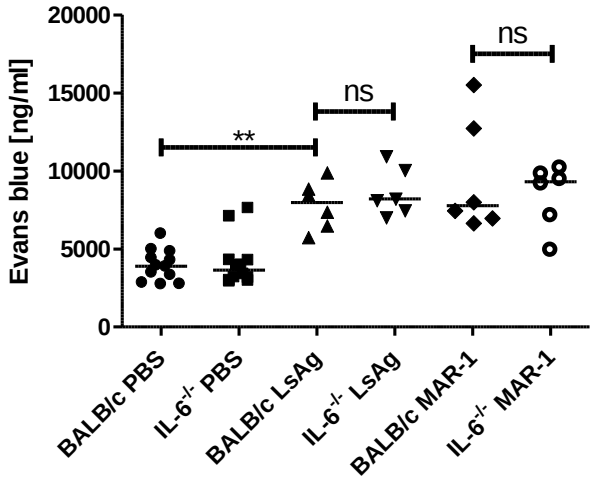


Figure 3.18.3. Vascular permeability is similar between BALB/c and IL-6$^{-/-}$ mice. BALB/c and IL-6$^{-/-}$ mice were injected i.c. into the ears with LsAg or MAR-1 in PBS or PBS alone, followed by Evans Blue injection i.v. 3 minutes later. After 10 minutes, the complete ears were cut off and Evans Blue leakage was quantified at 620 nm using a plate reader. (Representative of 3 experiments with different settings and a minimum of 6 animals per group. Data is shown as median. ** $p<0.01$ ns: not significant).

3.19. Subcutaneous L3 inoculation leads to a similar worm recovery at 15 dpi

It has been shown above that increased vascular permeability is not the cause for the increased worm burden in IL-6$^{-/-}$ mice. Due to the fact that the increased worm burden in IL-6$^{-/-}$ mice occurred at a very early time point post infection, it was hypothesized that the increased worm burden in IL-6$^{-/-}$ mice may be due to an increased worm survival or migration in IL-6$^{-/-}$ mice when they pass the first barrier within the mouse, the skin. To investigate if the skin plays a major role in the increased worm burden in IL-6$^{-/-}$ mice, mice were inoculated with 40 L3 subcutaneously. While after natural infection IL-6$^{-/-}$ mice had a higher worm burden at 15 dpi, s.c. injection did not reveal a difference in worm burden between IL-6$^{-/-}$ and BALB/c mice (figure 3.19). This suggests that immune responses that occur in the epidermis and dermis may play a major role for protection and lead to increased worm survival or migration in IL-6$^{-/-}$ mice.

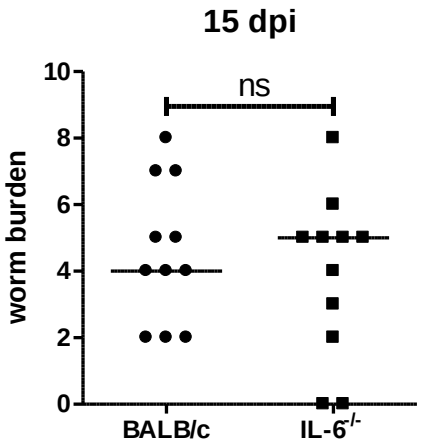


Figure 3.19. Circumventing the skin barrier by s.c. injection results in a similar worm load in BALB/c and IL-6$^{-/-}$ at 15 dpi. Mice were infected with *L. sigmodontis* by injecting 40 L3 s.c. Infection was terminated at 15 dpi and worm burden in the pleural cavity is displayed. (Pooled results from two independent experiments. Data is shown as median. ns: not significant).

4. Discussion

This study showed an increased worm burden in IL-6$^{-/-}$ mice during the early phase of infection with *L. sigmodontis* in comparison to BALB/c mice, but not during the chronic phase of infection. Potential mechanisms leading to an increased worm burden early in infection and similar worms in later times post infection are: (1) dampening of the host immune response in IL-6$^{-/-}$ mice during acute infection, (2) increased host immune response in IL-6$^{-/-}$ mice during later time points post infection, (3) increased survival/reduced destruction of L3 in IL-6$^{-/-}$ mice during the early phase of infection, (4) reduced survival/increased destruction of L4 or adult worms in IL-6$^{-/-}$ during the late phase of infection, or (5) increased migration of L3 into the pleural cavity.

4.1. Immune responses against *L. sigmodontis*

4.1.1. IL-6 signaling during *L. sigmodontis* infection

This study shows for the first time that during acute *L. sigmodontis* filarial infection signaling of IL-6 involves trans-signaling. As was previously shown in a review by Scheller and colleagues, IL-6 trans-signaling is mediated by sIL-6R, which is essential for pro-inflammatory immune responses, and differs from mIL-6R-signaling that is important during anti-inflammatory responses (Scheller et al. 2011). Although this has been shown for several pathogens (Rose-John 2012; Scheller et al. 2011), the occurrence of IL-6 trans-signaling during nematode infection was unknown. sIL-6R was produced in low amounts in the pleural lavage of naive mice but increased during acute infection with *L. sigmodontis*. This is may be due to increased neutrophil apoptosis in the pleural cavity following infection, which correlates with slightly reduced neutrophil numbers in the pleural cavity during acute infection compared to naive animals. As was previously described (Scheller et al. 2011), sIL-6R is generated by two mechanisms; first by proteolytic cleavage of mIL-6R mainly after apoptosis of neutrophils and second by secretion from neutrophils and monocytes of an alternatively sliced mIL-6R product. *In vitro* stimulation of neutrophils from naive mice with LsAg increased the level of sIL-6R compared to unstimulated neutrophils, although this difference did not reach statistical significance. This suggests that sIL-6R may be involved in immune responses during acute *L. sigmodontis* infection. Several proteins that are crucial in the IL-6 trans-signaling pathway such as gp130 and STAT3 should be addressed in the future to obtain a complete

overview of IL-6 trans-signaling during filarial infection. Differences in sIL-6R between BALB/c and IL-6$^{-/-}$ mice both *in vivo* and *in vitro* were not observed due to the fact that IL-6$^{-/-}$ was generated by disrupting the IL-6 gene, but not the IL-6R gene (Kopf et al. 1994).

4.1.2. Neutrophils

The role of neutrophils in worm survival both in the early and late phase of infection has been previously described (Makepeace et al. 2012; Martin et al. 2000a; Saeftel et al. 2001, 2003). In the acute stage of infection, neutrophils are reported to be the main cell type that is crucial for worm destruction (Martin et al. 2000a). In contrast, Saeftel and colleagues (Saeftel et al. 2001, 2003) showed that neutrophils are crucial for the encapsulation process at the late stage of infection in an IFNγ-dependent manner (50 and 80 dpi). Since, IL-6 is required for neutrophil mobilization from the bone marrow to the periphery and to the site of infection during acute inflammation (Murphy 2012; Scheller et al. 2011), it is important to determine the role of IL-6 on neutrophil migration during filarial infection and their ability in worm destruction both at early and late phases of infection.

In the current study IL-6 deficiency had no impact on neutrophil migration to the periphery and pleural cavity at 7 and 15 dpi as well as at the late stage of *L. sigmodontis* infection. Neutrophil migration into the pleural cavity was low after the first week of infection, both in BALB/c and IL-6$^{-/-}$ mice. This was confirmed by a time course experiment in *L. sigmodontis*-infected BALB/c mice showing increased neutrophil numbers in the pleural cavity after approximately 28 dpi. Additionally, neutrophil activation was not affected by IL-6 deficiency as the production of the neutrophil associated cytokines TNFα and MIP-2 was similar between BALB/c and IL-6$^{-/-}$ mice.

IFNγ, which induces neutrophil activation and phagocytosis (Saeftel et al. 2001, 2003) was not detectable during infection *in vivo,* both in serum and pleural lavage, although IFNγ secretion *in vitro* was increased at early but not later time points in IL-6$^{-/-}$ mice. Given that IFNγ is implemented in neutrophil-mediated worm destruction during chronic infection but not early time points post infection (Al-Qaoud et al. 2000; Saeftel et al. 2001, 2003) it is unlikely that IFNγ led to the increased worm elimination in IL-6$^{-/-}$ mice after 15 dpi. Therefore depleting IFNγ before 15 dpi until 30 dpi should be conducted in order to address the role of IFNγ in neutrophil-mediated worm destruction in IL-6$^{-/-}$ mice at 30 dpi.

4.1.3. Macrophages

Another phagocytic cell population that might be involved in worm killing at early infection time points is macrophage. As previously described, macrophages are capable to kill filarial larvae but not adult worms by releasing toxic radicals (Ou et al. 1995). As L3 of *B. pahangi* induce macrophage activation *in vitro* (Jeffers et al. 1984), it can be assumed that an increased worm burden during acute infection and reduced survival of adult worms at the chronic stage in IL-6$^{-/-}$ mice might be mediated by macrophage modulation. However the total number of macrophages at the site of infection did not differ between IL-6$^{-/-}$ and BALB/c mice. Similarly, the total number of AAMs as determined at 30 dpi was not different between BALB/c and IL-6$^{-/-}$ mice. Additionally, activation of macrophages was not affected by the lack of IL-6 suggesting that IL-6 is not important for macrophage function during filarial infection. This may be further confirmed by measuring the production of toxic radicals such as ROS and NO derivates by macrophages in response to *L. sigmodontis* larvae or antigen in BALB/c and IL-6$^{-/-}$ mice.

4.1.4. B cells

B cells, as reported with µMT mice, are not involved in worm elimination (Martin et al. 2001), although a study by Al-Qaoud et al. showed that worm burden in B1 deficient BALB/c mice (Xid mice) was significantly increased compared to BALB/c controls at 28 dpi, probably due to a lack of IgM -producing B1 cells (Al-Qaoud et al. 1998). In IL-6$^{-/-}$ mice, I observed a reduced relative but not absolute number of B cells in the blood and pleural cavity during early infection. Additionally, the percentage of B2 cells was decreased in IL-6$^{-/-}$ mice. Reduced frequencies of B1 cells in IL-6$^{-/-}$ mice may mediate the increased worm burden due to a reduced production of IgM. Reduction of B2 cells in IL-6$^{-/-}$ mice may also facilitate worm survival, as a study by Mwinzi and colleagues revealed that B2 cells are important for resistance against *S. mansoni* by regulating IgE production (Mwinzi et al. 2009). However, whereas IgM is produced early during infection induction of IgE does not appear before 4 weeks post infection (Torrero et al. 2010) suggesting that B2 cells may have an effect on late infection time points, but do not cause the difference of *L. sigmodontis* worm burden observed at 15 dpi.

Another variable which is important for immunity against helminth infection is IgG production (Specht and Hoerauf 2012). *L. sigmodontis*-specific IgG1 and IgG2a were

detected before L3 challenge (0 dpi) and increased until 10 dpi in vaccinated mice compared to primary infected mice (Babayan et al. 2006; Le Goff et al. 2000b). At later time points of infection (60 dpi), production of *L. sigmodontis*-specific IgG1 and IgG2a peaked and no difference of their levels were measured between vaccinated and primary infected mice (Le Goff et al. 2000b).

As IL-6 is known to be crucial in the end stage of plasma cell differentiation (Kishimoto 2010; Scheller et al. 2011) and IgG production by promoting IL-21 production (Neveu et al. 2009), it is important to address whether IL-6 deficiency may affect IgG production during *L. sigmodontis* infection. Production of both *L. sigmodontis*-specific IgG1 and IgG2a was not different between BALB/c and IL-$6^{-/-}$ mice at 30 and 60 dpi, when no difference in worm burden and B cells was observed. This is probably due to the fact that the function of IL-6 in plasma cell differentiation and IgG production can be substituted by IL-21 as previously described (Eto et al. 2011). Therefore, it would be interesting to further investigate the role of IL-21 during *L. sigmodontis* infection of IL-$6^{-/-}$ mice.

4.1.5. CD4 T cells

CD4 T cells play a significant role in protective immunity against filarial parasites (Hoerauf et al. 2005; Specht and Hoerauf 2012). Al-Qaoud and colleagues showed a role for CD4 T cells in protection against filarial infection, as depletion of CD4 T cells led to increased worm burdens at 28 dpi (Al-Qaoud et al. 1997). The presented study did not observe a different number of CD4 T cells between BALB/c and IL-$6^{-/-}$ mice, neither in the blood nor in the pleural cavity. Since activation, cytokine production and proliferation of CD4 T cells in IL-6 deficiency were not determined in this study, the role of IL-6 in activation and proliferation as well as its exact role in CD4 T cell-mediated protection against filarial worms may be elucidated in further studies.

4.1.5.1. T_{reg} cells

Although the role of IL-6 in inhibiting TGFß-induced T_{reg} differentiation has been reported several years ago (Bettelli et al. 2006), its role in T_{reg} initiation during filarial infection is unknown. This study demonstrated that in the absence of IL-6, the number of splenic T_{reg} after re-stimulation with LsAg was not different compared to BALB/c controls. This suggests that IL-6 signaling during *L. sigmodontis* infection has little or no impact on the

number of T_{reg} in BALB/c mice, which is probably due to the low amount of IL-6 produced during the infection in BALB/c mice, or that IL-6 deficiency did not result in increased T_{reg} numbers during filarial infection due to another T_{reg} inhibitory mechanism.

Another reason for the similar number of T_{reg} in both types of mice might be due to the level of TGFß that was secreted during infection and restimulation. As has been previously described, in certain environmental conditions including filarial infection and presence of TGFß, naive T cells differentiate into T_{reg} (Belkaid 2007; Hoerauf et al. 2005). In the presence of IL-6, however, this differentiation shifts towards the Th17 lineage (Kimura and Kishimoto 2010).

4.1.5.2. IL-17-producing Th17 cells

The role of Th17 differentiation during filariasis is not yet completely known, although a study by Babu and co-workers showed that IL-17 is one of the cytokines that is responsible for lymphedema in human *B. malayi* infection (Babu et al. 2009). Despite the role of IL-6 in IL-17 production, IL-6 deficiency did not affect IL-17 responses in this study. Accordingly, a study by Kimura and colleagues revealed that differentiation of IL-17 producing Th17 cells can occur in the absence of IL-6 (Kimura et al. 2007). They showed that lack of IL-6 signaling either by using IL-$6^{-/-}$ or administration of anti-gp130 or anti-IL-6R did not prevent the production of IL-17 by naive T cells stimulated with conditioned medium from LPS-stimulated bone marrow-derived DCs. They suggested another factor than IL-6, TGFß and IL-23 to regulate Th17 differentiation; however, they could not address this factor in their paper (Kimura et al. 2007). A study by Yang and colleagues revealed that IL-21 may substitute IL-6 to induce Th17 differentiation (Yang et al. 2008). Therefore, IL-21 may compensate for the lack of IL-6 during *L. sigmodontis* infection and induce Th17 cells and IL-17 production.

4.1.5.3. Th2 responses

There are two phases of IL-5-mediated worm reduction which are previously described during murine filarial infection; the first phase occurs in the first two days after infection and the second phase occurs after three weeks post infection when specific IgG starts to be produced (Babayan et al. 2005; Martin et al. 2000b, 2000a). Worm destruction in the first phase tends to be fast, whereas in the second phase, the destruction is slow because the

worm size increased (Martin et al. 2000a). Optimal destruction and killing of the worms requires adequate levels of IL-5, eosinophils and antibodies (Le Goff et al. 2000b, 2000a; Martin et al. 2000b, 2000a; Specht and Hoerauf 2012).

Effective destruction and killing of the worms during the first phase, for instance, occurred in mice vaccinated with L3. Vaccinated mice have a lower worm rate compared to primary infected mice as early as 2 dpi due to increased worm destruction and killing, and the rate was stable for several weeks (Babayan et al. 2006; Martin et al. 2000a). Several studies have shown the role of IL-5, eosinophils and IgE in this worm destruction (Le Goff et al. 2000b; Martin et al. 2000b, 2000a; Specht and Hoerauf 2012). Production of IL-5 was increased after splenocyte stimulation with adult worm extract prior to worm inoculation (0 dpi) in vaccinated mice compared to primary infected mice (Le Goff et al. 2000b). Additionally, IL-5 production by splenocytes was also increased after either adult, L3 and microfilaria extracts challenge in vaccinated mice at 2 dpi (Babayan et al. 2006). Furthermore, IL-5 levels were found in higher concentrations in the pleural lavage of vaccinated mice than in the pleural cavity of primary infected mice after worm challenge at 10 dpi, although levels of IL-5 in the serum is independent of vaccination (Babayan et al. 2006; Martin et al. 2000a).

As the numbers of eosinophils in the pleural cavity, the levels of IL-5 *in vitro* after splenocyte culture and the IgE concentrations in the pleural lavage and serum were significantly increased in IL-$6^{-/-}$ mice at early time points during infection in this study, it is unlikely that this result is due to reduced larval destruction in the pleural cavity by eosinophils, IL-5 and IgE. In fact, increased IL-5, eosinophils and IgE in this study were probably just an effect of, not a cause for the increased worm burden in IL-$6^{-/-}$ mice. This was confirmed by the depletion of IL-5 before and during infection. Depletion of IL-5 resulted in reduced eosinophil numbers in the pleural cavity but did not reduce the worm burden in IL-$6^{-/-}$ mice at 15 dpi. This suggests that the increased worm burden in IL-$6^{-/-}$ mice during early infection is not mediated by eosinophils, IL-5 and IgE.

Elevated IL-5 levels *in vitro* early during infection were probably due to an increased polarization towards Th2 in IL-$6^{-/-}$ caused by the higher worm burden. Babu and Nutman reported in an *in vitro B. malayi* study that activation of naive T cell occurred already within 1 dpi, mainly towards a Th1 response (Babu and Nutman 2003). After 7 dpi, host immune polarization shifts towards Th2 dominance due to incoming L3 as was shown in a study by Porthouse and colleagues using *B. pahangi* (Porthouse et al. 2006). Synergistic

responses of Th1/Th2, however, are needed during infection in order to inhibit L3 establishment and adult *L. sigmodontis* survival (Saeftel et al. 2003; Specht and Hoerauf 2012). This may explain the increased levels of both IL-5 and IFNγ *in vitro* in IL-6$^{-/-}$ mice at early infection, but not during later time points.

Moreover, increased eosinophil numbers in the pleural cavity of IL-6$^{-/-}$ mice might be due to an increased migration of eosinophils from the blood, but did not correlate with overall increased eosinophilia, as eosinophil numbers in the blood of IL-6$^{-/-}$ mice were similar to BALB/c mice. A more pronounced up-regulation of cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and CD62E on endothelial cells in IL-6$^{-/-}$ mice may be the reason for the increased numbers of eosinophils entering the pleural cavity, as similar mechanisms for eosinophil recruitment into the cornea were described in an *Onchocerca volvulus* study by Kaifi and colleagues (Kaifi et al. 2001). A second cause for increased numbers of eosinophils in IL-6$^{-/-}$ mice is increased survival of eosinophils in the pleural cavity of IL-6$^{-/-}$ mice. As has been shown by Ohnmacht and colleagues, *Nippostrongylus brasiliensis* infection increased the number of eosinophils at the site of infection, although their production in bone marrow of infected mice was not different compared to naïve mice. This suggests that parasite-induced eosinophilia is caused by an improved survival and recruitment of eosinophils rather than by an increased generation of eosinophils (Ohnmacht et al. 2007).

In the second phase of IL-5-mediated worm destruction (after 3 weeks post infection), the essential role of IL-5 has been studied using IL-5 transgenic CBA/Ca mice. The study suggested that IL-5 induced clearance of worms occurred after day 10 and before day 30 post challenge (Martin et al. 2000b). Another study suggested that the protective role of IL-5 in primary infection occurs at the late phase (80 dpi), as they found significantly increased pleural cavity eosinophils in primary infected mice at 28 dpi, but this did not result in a reduced worm burden at this time point, but 8 weeks later (Martin et al. 2000a). Similarly, a study by Saeftel and colleagues using *L. sigmodontis* showed an increased worm burden in IL-5$^{-/-}$ mice at 50 and 80 dpi (Saeftel et al. 2003).

In this study depletion of IL-5 during early infection did not reduce the worm burden in IL-6$^{-/-}$ mice, but tended to further increase the worm burden in IL-6$^{-/-}$ mice. The increased levels of IL-5 observed *in vitro* and the elevated eosinophil numbers at the site of infection in IL-6$^{-/-}$ mice at 15 dpi might lead to increased worm destruction in those mice, not at that point but rather during the later stage of infection when worms molt to adults and the

differences between BALB/c and IL-6$^{-/-}$ mice disappear. However, this hypothesis could not be confirmed as depleting IL-5 and eosinophils starting at 12 dpi when the L3 molt into L4 until 30 dpi did not maintain the increased worm burden in IL-6$^{-/-}$ mice at 30 dpi. This might be due to the fact that the role of IL-5 induced eosinophils in worm destruction occurs at a later phase of infection (Martin et al. 2000a).

4.2. Worm survival during early *L. sigmodontis* infection

Mechanisms on how the larvae migrate from the skin into the subcutaneous tissue during natural infection and their migration from the subcutaneous tissue into blood vessels and then to the pleural cavity have been previously described by several studies (Babayan et al. 2005; Le Goff et al. 1997; Martin et al. 2000b, 2000a). During natural *L. sigmodontis* infection infective L3 pass the skin, the subcutaneous tissue, blood vessels and finally the lymphatic system until reaching the permanent site of infection: the pleural cavity. The L3 of *L. sigmodontis* usually migrate through the skin within one day and then reach the subcutaneous tissue and pleural space within 2 and 4 days, respectively. However, during s.c. inoculation, the majority of L3 migrate into the lymphatic system within 2 days due to a reduced time needed to pass the skin (Babayan et al. 2005; Martin et al. 2000a, 2001). Therefore, events that affect entry or exit of those tissues play an important role in worm establishment during *L. sigmodontis* infection (Babayan et al. 2005; Le Goff et al. 1997, 2000a; Martin et al. 2000a, 2001).

In the skin, increased migration and worm survival mainly involve vascular permeability (Specht et al. 2011) and protective immune responses mediated by phagocytic cells (Le Goff et al. 2000b; Martin et al. 2000a; Specht and Hoerauf 2012). Additionally, the complement system may play a role in worm establishment as was shown by several studies using *B. malayi* (Carter et al. 2007; Rees-Roberts et al. 2010; Thomas et al. 2012). Increased survival of L3 in the skin depends on the ability of the larvae to escape the destruction which is mediated by the early inflammatory host responses mainly involving eosinophils and neutrophils. In the subcutaneous tissue, blood vessels and lymphatic system, dilatation of blood and lymphatic vessels as well as active movement of worms to enter the vessels on their way to the pleural cavity might play a significant role (Babayan et al. 2005; Le Goff et al. 1997, 2000a; Martin et al. 2000a, 2001).

Increased migration of worms from the skin of *L. sigmodontis* infected mice into the blood vessels due to increased permeability has been described by Specht and colleagues (Specht et al. 2011). They demonstrated that in CCL17$^{-/-}$ mice, the worm burden was increased at 10 dpi due to increased vascular permeability caused by increased mast cell degranulation. *Wolbachia* released by L3 stimulate TLR2 which induces mast cell degranulation and histamine release. That in turn increases vascular permeability in the skin of infected mice which facilitates worm migration into subcutaneous tissue and, thus, increases the worm burden in the pleural cavity of CCL17$^{-/-}$ mice (Specht et al. 2011).

Since IL-6 is important for triggering production of CCL17 via trans-signaling, IL-6 deficiency might result in decreased CCL17 production (Scheller et al. 2011) and increased vascular permeability and worm burden at early time points. It was therefore hypothesized that a similar phenotype of IL-6$^{-/-}$ compared with CCL17$^{-/-}$ mice exists that is due to a similar mechanism. However, CCL17 production was not decreased in IL-6$^{-/-}$ mice compared to BALB/c mice neither *in vivo* in the pleural cavity nor *in vitro* after DC stimulation. CCL17 production *in vitro* even increased in DCs derived from IL-6$^{-/-}$ mice and was reduced when rIL-6 was added to the stimulation. This suggests that the increased worm burden in IL-6$^{-/-}$ mice at early time points post infection is independent of CCL17.

Accordingly, in this study vascular permeability and histamine release were not found to be affected in IL-6$^{-/-}$ mice. Evans blue leakage as a surrogate marker of vascular permeability was not altered in IL-6$^{-/-}$ mice. Additionally, worm burden in IL-6$^{-/-}$ mice was not reverted to normal levels after blocking histamine receptors. However, an increased vascular permeability after LsAg injection in both BALB/c and IL-6$^{-/-}$ mice could be confirmed suggesting that filariae and their antigens increase vascular permeability of the skin thus facilitating their migration within the host.

Although vascular permeability and histamine release are not as important in IL-6$^{-/-}$ mice compared to CCL17$^{-/-}$ mice, mast cell degranulation has been affected in this study. This could be demonstrated by the reduction of worm burden at 14 dpi when IL-6$^{-/-}$ mice were repeatedly treated with the mast cell stabilizer cromolyn. When activated, mast cells degranulate and release several mediators such as like histamine and serotonin, lipid inflammatory mediators like prostaglandins and leukotrienes, chemokines and pro-inflammatory cytokines like TNFα and IL-6 (Marshall 2004; Voehringer 2013).

Mast cells are probably important during migration from the subcutaneous tissue into the lymphatic system. Mast cells also promote lymphangiogenesis in several diseases by releasing TNFα and VEGFs (Baluk et al. 2009; Syväranta et al. 2012; Tammela and Alitalo 2010; Utrera-Barillas et al. 2010), although another study in murine and human arthritis showed that TNFα can inhibit lympangiogenesis (Polzer et al. 2008). Additionally, mast cell-derived TNFα modulates B and T cell migration from endothelial venules to the lymph nodes (LN) and causes LN enlargement (Kunder et al. 2011).

Furthermore, in filarial infection, depletion of *Wolbachia*, which reduces the level of VEGF-C in plasma, leads to the reduction of lymph vessel dilatation (Debrah et al. 2006). This is probably due to increased production of pro-inflammatory cytokines such as TNFα and IL-1ß that is mediated by *Wolbachia.* Up-regulation of TNFα and IL-1ß production then leads to increased production of VEGFs (Pfarr et al. 2009). VEGFs have long been known to be crucial in filarial lymphedema by inducing angiogenesis and lympangiogenesis that lead to increased lymph vessel dilatation (Bennuru and Nutman 2009; Pfarr et al. 2009). Dilation of lymph vessels may increase migration of worms from subcutaneous tissues to the lymphatic system (pleural cavity). Taken together, it is hypothesized that the release of *Wolbachia* results in mast cell degranulation and the release of mediators. This could be further examined by stimulating mast cells from *L. sigmodontis* infected BALB/c and IL-$6^{-/-}$ mice with LsAg *in vitro* and measuring the release of different mediators *in vitro*. Once critical mediators are identified, the relevance could be tested *in vivo* during *L. sigmodontis* infection. Mast cell cytokine release (e.g. TNFa, IL-1ß) may lead to lymph vessel dilatation that facilitates worm migration in our model and could result in increased worm burden in the pleural cavity of IL-$6^{-/-}$ mice. However, this study did not find an increased TNFα production by neutrophils and macrophages from IL-$6^{-/-}$ compared to BALB/c mice *in vitro*. Additionally, production of TNFα and IL-1ß could not be detected in either serum or pleural lavage of *L. sigmodontis* infected mice. This suggests that TNFα and IL-1ß are probably not the essential mediators affecting the establishment of filarial infection in this study.

Another possibility of increased worm migration/survival within the skin is due to a reduced number and activation of Langerhans cells (LCs) in IL-$6^{-/-}$ mice (Semnani et al. 2004; Specht and Hoerauf 2012). In a study using *B. malayi*, LCs were shown to take up soluble L3 antigens from live L3 and led to increased LC migration from the epidermis to the LN. This migration involved the production of pro-inflammatory cytokines including

TNFα and IL-1ß. Furthermore, the authors showed that live L3 decreased MHCI and MHCII expression on LC as well as IL-8 production which reduced the ability of LC to induce CD4 T cell proliferation and IFNγ and IL-10 production (Semnani et al. 2004).

As previously described by Porthouse and colleagues, the skin barrier plays an important role during early worm establishment in *B. pahangi* infection (Porthouse et al. 2006). As vascular permeability is not altered in IL-6$^{-/-}$ mice, increased worm burden is potentially determined immediately when the worms enter the host via the skin during the bite of mites. Such an impaired skin barrier function was suggested in this study to cause the increased worm burden in IL-6$^{-/-}$ mice, as inoculation of L3 led to a similar worm number in IL-6$^{-/-}$ compared to BALB/c mice at 15 dpi. Protection via the skin barrier is probably mediated by several innate cells such as neutrophils and eosinophils as well as the complement system thus impairing the passage of worms through the skin and subcutaneous tissue. Lack of IL-6 may therefore impair protective immune responses in the skin and improve the larval migration through the skin and subcutaneous tissues thus leading to increased worm numbers in the pleural cavity. Immune responses in the skin may either delay the worm migration in immunocompetent mice or improve larval elimination thus causing the observed difference in pleural worm burdens at early time points of infection. Porthouse and colleagues reported that neutrophils are the main cell type that is found around the larvae at the site of *B. pahangi* inoculation after 3 and 24 hours (Porthouse et al. 2006). They also observed that expression of IL-6 and TNFα mRNA peaked at those time points (Porthouse et al. 2006). Accordingly, an increased worm burden in IL-6$^{-/-}$ mice in this study may be due to an impaired neutrophil-mediated destruction of invading larvae within the skin during the first days post infection. Although IL-6 deficiency did not affect neutrophil activation *in vitro* as determined after casein-induced peritoneal neutrophil stimulation, it has to be elucidated in the future if neutrophil activation in the skin differs or whether recruitment of neutrophils to the site of infection is delayed or impaired.

4.3. Model of IL-6 during *L. sigmodontis* infection

Invading larvae have to migrate from the site of infection within the skin through the subcutaneous tissues via the lymphatics to the pleural cavity. As was shown in this thesis, IL-6 is essential for early protective immune responses. Recruitment and activation of

neutrophils and eosinophils to the site of infection within the skin are probably essential for the clearance of invading larvae and I hypothesize that this is impaired by the lack of IL-6. Thus, the entrance of larvae to the subcutaneous tissues is facilitated. Following barriers that have to be passed by the L3 are not affected by the lack of IL-6, as vascular permeability and histamine release were not observed to be different. Thus, numbers of larvae in IL-$6^{-/-}$ mice were increased already at the time point when they reached the pleural cavity and bypassing the skin barrier abolished this effect. Mast cell degranulation is further contributing to the increased worm burden at early time points. Differences between IL-$6^{-/-}$ mice and controls in the immune response within the pleural cavity are unlikely to mediate the increased worm burden in the IL-$6^{-/-}$ mice during early infection since worm loads were already increased when the larvae enter the pleural cavity. Accordingly, differences in adult worm burdens vanished after the molt to adult worms and microfilarial production was similar between IL-$6^{-/-}$ and control mice at those later time points.

4.4. Outlook

To further elucidate whether the increased worm burden in IL-$6^{-/-}$ mice is due to an impaired immune response within the skin, local natural infection and. i.c. inoculation of infective L3 will be established. Recruitment of neutrophils, eosinophils, macrophages, mast cells, and LCs to the site of the infection will be determined within the first six hours post local infection in IL-$6^{-/-}$ mice and controls by histology. Additionally, neutrophil recruitment to the infection site will be compared *in vivo* using an IVIS fluorescence imaging system and a neutrophil specific fluorescent imaging reagent.

5. Summary

Lymphatic filariasis is a neglected tropical disease that is caused by parasitic helminths and affects 120 million people worldwide, while 1.3 billion people live in endemic areas. Three species of tissue dwelling nematodes cause this disease: *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Endosymbiotic *Wolbachia* bacteria are implicated in disease pathology as they activate the host's immune system and induce the production of pro-inflammatory cytokines like IL-6.

In this study, I investigated the role of IL-6 using the rodent filarial nematode *Litomosoides sigmodontis* model that closely resembles immune responses caused during human filarial infection.

IL-6-deficient (IL-$6^{-/-}$) and immunocompetent control BALB/c mice were infected via with *L. sigmodontis* the natural vector and parasitological and immunological analysis were performed at different time points post infection. Infectious third stage larvae (L3) are transferred by infected mites during the blood meal, pass the skin barrier and subcutaneous tissue and migrate via the lymphatics to the pleural cavity where they develop into adult worms and reside. The worm burden in the pleural cavity of IL-$6^{-/-}$ mice was found to be increased at 7 and 15 days post infection (dpi), but not after the molt to adult worms (30 dpi) and during chronic infection. This increased worm recovery during early infection in IL-$6^{-/-}$ mice correlated with a reduced relative but not absolute number of total B and B2 cells in the pleural cavity, whereas the number of regulatory T cells and Th17 cells was not altered by IL-6 deficiency during *L. sigmodontis* infection. Eosinophils that are known to be involved in the clearance of filarial infection were increased in the pleural cavity of infected IL-$6^{-/-}$ mice during those early time points. This increase in eosinophil numbers did not mediate the enhanced clearance of *L. sigmodontis* larvae after 14 dpi in IL-$6^{-/-}$ mice, as depletion of IL-5 and therefore reduction of eosinophil numbers, did not lead to the maintenance of an increased worm burden after the molt to adult worms. Increased vascular permeability as induced by augmented mast cell degranulation (e.g. release of histamine) that might allow a better worm migration to the pleural cavity in IL-$6^{-/-}$ mice, was not the mechanism that resulted in an inceased worm burden in IL-$6^{-/-}$ mice at an early time point of infection. Thus, blocking of histamine receptors did not reduce the worm burden in IL-$6^{-/-}$ mice and measurement of vascular permeability in response to parasite antigens revealed no difference between IL-$6^{-/-}$ and BALB/c mice. However, stabilizing mast cells using cromolyn sodium salt reduced the worm burden partially in IL-$6^{-/-}$

indicating that mast cells of IL-6$^{-/-}$ mice may facilitate worm migration, although not via the release of histamine or by increasing the vascular permeability.

Those results indicate that protective immune responses that are impaired by the IL-6-deficiency are likely to occur before the entrance of the infectious larvae into the vascular system and the pleural cavity. Accordingly, bypassing the skin barrier by inoculating infectious L3 subcutaneously resulted in a worm recovery at 15 dpi that was comparable between the BALB/c and IL-6$^{-/-}$ mice. This suggests that during natural infection, protective immune responses in the skin against infectious L3, e.g. neutrophil and eosinophil responses are potentially impaired by the lack of IL-6 and facilitate the migration of the larvae to the pleural cavity. This hypothesis is now being tested by local infections and analysis of the cell recruitment to the site of infection.

Zusammenfassung

Elephantiasis gehört zu den „vernachlässigten Tropenkrankheiten - neglected tropical diseases“, die durch parasitäre Würmer verursacht wird. Derzeit sind weltweit 120 Millionen Menschen mit deren Erregern infiziert und weitere 1,3 Milliarden Menschen leben in Endemiegebieten. Elephantiasis wird hautpsächlich von drei Arten von Nematoden verursacht: *Wuchereria brancrofti*, *Brugia malayi* und *Brugia timori*. Endosymbiotische *Wolbachia* Bakterien sind dabei an der Ausbildung des Krankheitsbildes beteiligt, da sie das Immunsystem des Wirts aktivieren und die Produktion von pro-inflammatorischen Zytokinen wie IL-6 induzieren.

In dieser Arbeit wurde die Rolle von IL-6 auf eine Infektion mit der Nagetierfilarie *Litomosoides sigmodontis* untersucht. *L. sigmodontis* ist hierfür ein exzellentes Modell, da suszeptible BALB/c Mäuse eine chronische Infektion entwickeln, deren Immunreaktion mit derer von humanen Filarieninfektionen vergleichbar ist.

IL-6-defizienten (IL-$6^{-/-}$) und immunkompetente BALB/c Mäuse wurden über den natürlichen Vektor, der tropischen Rattenmilbe, mit *L. sigmodontis* infiziert und parasitologische sowie immunologische Analysen zu verschiedenen Zeitpunkten nach der Infektion durchgeführt. Die infektiösen L3 Larven werden dabei durch infizierte Milben mit der Blutmahlzeit auf den Endwirt übertragen. Hier müssen sie die Hautbarriere und das subkutanen Gewebe passieren, um über die Lymphgefäße in die Pleurahöhle zu gelangen, in der sie sich zu Adultwürmern entwickeln. Wie in dieser Arbeit gezeigt werden konnte, war die Wurmlast in IL-$6^{-/-}$ Mäusen 7 und 15 Tage nach der Infektion erhöht, wobei dieser Unterschied nach der Häutung zu adulten Würmern (30 Tage nach Infektion) und im Verlauf einer chronischen Infektion nicht mehr gegeben war. Diese erhöhte Wurmlast in IL-$6^{-/-}$ Mäuse korrelierte während der frühen Infektionsphase mit einer verringerten relativen Anzahl an B-Zellen am Ort der Infektion, der Pleurahöhle. Die Anzahl regulatorischer T- und Th17-Zellen war hingegen nicht in der Abwesenheit von IL-6 während einer *L. sigmodontis* Infektion verändert. Eosinophile, die bekanntermaßen bei der Beseitigung von Filarieninfektionen beteiligt sind, waren in der Pleurahöhle von infizierten IL-$6^{-/-}$ Mäuse zu frühen Zeitpunkten der Infektion erhöht. Dieser Anstieg der Eosinophilen führte jedoch nicht zu einer erhöhten Eliminierung von *L. sigmodontis* zu späteren Zeitpunkten der Infektion in IL-$6^{-/-}$ Mäusen, da die Depletion von IL-5 und die damit einhergehende Reduktion der Eosinophilen nicht zu einer länger anhaltenden erhöhten Wurmlast in IL-$6^{-/-}$ Mäusen führte. Da die IL-$6^{-/-}$ Mäuse bereits nach dem

Erreichen der Pleurahöhle eine erhöhte Wurmlast zeigten, könnte eine verbesserte vaskuläre Permeabilität durch verstärkte Mastzelldegranulation (z.B. durch Freisetzung von Histamin), die Migration der Würmer zur Pleurahöhle in den IL-$6^{-/-}$ Mäusen erleichtert haben. Diese Hypothese wurde jedoch widerlegt, da sowohl die Blockierung von Histamin-Rezeptoren die Wurmlast in IL-$6^{-/-}$ Mäusen nicht reduzierte als auch die vaskuläre Permeabilität als Reaktion auf Parasitenantigene keinen Unterschiede zwischen IL-$6^{-/-}$ und BALB/c Mäusen aufwies. Mastzellen erleichterten jedoch zu einem gewissen Grade die Migration der Larven in die Pleurahöhle, da eine Mastzell-Stabilisierung mit Cromoglicinsäure (Cromolyn) die Wurmlast partiell in IL-$6^{-/-}$ Mäusen reduzierte.

Diese Ergebnisse lassen vermuten, dass die IL-6-Defizienz protektive Immunantworten beeinträchtigt, die vor dem Eintreten der infektiösen Larvenstadien in das Lymphgefäßsystem und in die Pleurahöhle auftreten. Dementsprechend resultierte das Umgehen der Hautbarriere durch subkutane Injektion von infektiösen L3 Larven in einer vergleichbaren Wurmlast zwischen BALB/c und IL-$6^{-/-}$ Mäusen zum frühen Zeitpunkt der Infektion. Dies deutet darauf hin, dass während einer natürlichen Infektion protektive Immunantworten in der Haut gegen die infektiösen L3 Larven, z. B. durch Neutrophile und Eosinophile, durch das Fehlen von IL-6 beeinträchtigt werden und zu einer erhöhten Wurmlast führen können. Diese Hypothese wird nun durch lokale Infektionen und Analyse der Zellrekrutierung zum Infektionsort untersucht.

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