Immune-modulation of filarial-derived antigens on *Mycobacterium ulcerans*-activated cell populations

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Table of contents

List of abbreviations

α	anti-
AAM	Alternatively activated macrophages
Ab	Antibody
ACT	Ammonium Chloride-tris
Ag	Antigen
ANOVA	Analysis of variance
APCs	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
BCR	B-cell receptor
BMDCs	Bone-marrow derived dendritic cells
BSA	Bovine serum albumin
BuAg	*Mycobacterium ulcerans* stimulation extract
CD	Cluster of differentiation
CpG	Cytosine-phosphatidyl-guanine
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell scanning/sorting
Fc	Fragment crystallizable region
FCS	Fetal calf serum
g	Gravitational force

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HKLM	Heat-killed *Listeria monocytogenes*
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove´s Modified Dulbecco Media
IRF	Interferon regulatory factor
LPS	Lipopolysacharide
LsAg	*Litomosoides sigmodontis* stimulation extract
MACS	Magnetic Activated Cell Sorting
MAL	MyD88 adaptor-like
MAPK	Mitogen activated protein kinases
Mf	Microfilaria
MHC	Major histocompatibility complex
mLN	Mediastinal lymph node
MyD88	Myeloid differentiation primary response gene 88
NEAA	Non-essential amino acid
NFκB	Nuclear factor-κB
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
ns	Non-significant
P_3Cys	Palmitoyl-cysteine ((RS)-2,3-di(palmitoyloxy)-propyl
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PL	Peritoneal lavage

PRR	Pattern recognition receptor
PW	Pleura wash
rpm	Revolutions per minute
RPMI	Cell culture medium developed by Roswell Park Memorial Institute
SEM	Standard error of mean
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	Specific pathogen free
ssRNA	Single-stranded RNA
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
TCR	T-cell receptor
TEMED	Tetramethylethylendiamin
TGF	Transforming growth factor
TH cells	Helper T-cells
TIR	Toll-IL-1 receptor
TLR	Toll-like receptors
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cells
TRIF	TIR domain-containing adaptor-inducing IFNβ
Tris	Tris(hydroxymethyl)aminomethane
v/v	Volume per volume
w/v	Weight per volume

1. Introduction

1.1 The immune system

The immune system is the body's defence against pathogens and the damage they cause and consists of different tissues, effector cells, and molecules. Its task is to recognize a pathogen or infection whilst distinguishing between "foreign" and "self" in the hosts body, which then contain the infection and, if possible, eliminates the source. Furthermore, it has the capacity to regulate itself and to develop an immunological memory which provides a life-long protection. Immune system can be classified into either innate or adaptive immune responses (Alberts et al., 2008; Murphy and Weaver, 2017).

1.1.1 The innate immune system

Innate immunity is evolutionary the older host defence system and already present at birth. It is non-specific but fast, and thus the first reaction of the body to an unknown pathogen. The system consists of anatomical and chemical as well as of cellular and inflammatory barriers. Through inflammation, complement activation, phagocytosis, and destruction of pathogens, the innate immune system can act immediately to protect the body from infection. Furthermore, it has the capacity to amplify its response as well as to create an important bridge to the adaptive immune system (Alberts et al., 2008; Murphy and Weaver, 2017). The cells of the innate immune system play a key role. Sensor-cells such as macrophages, neutrophils, eosinophils and dendritic cells can detect microbes or the cellular damage produced by them through pattern recognition receptors (PRRs). These receptors can detect pathogen-associated molecular patterns (PAMPs) that can be found in many microorganisms but not in the host's cells, thus distinguishing between "foreign-" and "self-" antigens (Janeway, 1989). They have a crucial function for immunity, including opsonization and phagocytosis, the induction of apoptosis as well as the activation of complement and pro-inflammatory signalling pathways (Janeway, 1989; Medzhitov and Janeway, 1997).

PRRs can be found as cytoplasmic proteins, such as NOD-like receptors (NLRs) as well as transmembrane protein, such as Toll-like receptors (TLRs) (Medzhitov, 2001).

1.1.1.1 TLR-signalling

First postulated by Charles Janeway, Jr. (Janeway, 1989) and then confirmed by Jules Hoffman in *Drosophila melanogaster* (Lemaitre et al., 1996), TLRs and their signalling pathways have been at the forefront of understanding primary immune responses and therefore play a central role in the innate immune system. They are perhaps the most important subgroup of PRRs (Pasare and Medzhitov, 2005).

Currently, ten members of the TLR-family are known in humans and 12 in mice (Murphy and Weaver, 2017). TLRs are expressed either on the cell surface or intracellular and each can recognize a distinct set of PAMPs, leading to a complex activation of the cell as depicted in Fig. 1.

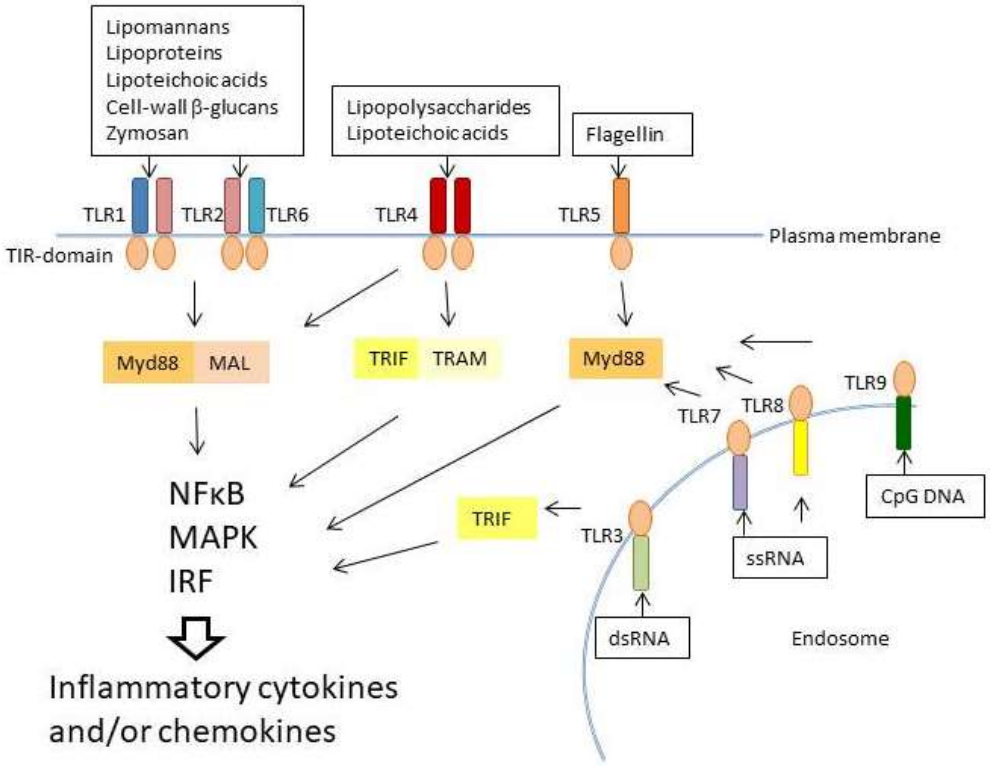

Fig. 1: TLR signalling of recognized PAMPs.

Each TLR is able to recognize a distinct set of PAMPs. The TIR (Toll-IL-1 receptor) domain of the different TLR receptors interacts with adaptor molecules MyD88 (myeloid differentiation primary response gene 88), MAL (MyD88 adaptor-like), TRIF (TIR domain-containing adaptor-inducing IFNβ) and/or TRAM (TRIF-related adaptor molecule). The consecutive signalling by most TLRs activates either the transcription factors NFκB (nuclear factor-κB) and IRF (interferon regulatory factor) and/or MAPKs (mitogen activated protein kinases) inducing the production of pro-inflammatory proteins. Adapted from Medzhitov (2001) and Murphy and Weaver (2017).

TLRs expressed on the cell surface are important for recognizing bacterial components. Well known examples are the TLR-2 heterodimers that recognize the peptidoglycan of Gram-positive bacterial cell walls, bacterial lipoproteins, and the lipoarabinomannan of Mycobacteria, and TLR4 that recognizes the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria. Intracellularly expressed TLRs, recognize nucleic acids after the microbe has been broken down (Medzhitov, 2001; Murphy and Weaver, 2017). The ligand can bind either directly or indirectly and lead to the dimerization of the TIR (Toll-IL-1 receptor) domain. This domain then interacts with similar domains in cytoplasmic adaptor molecules (MyD88, MAL, TRIF, TRAM) that initiate intracellular signalling. Two pathways can be distinguished, the MyD88-dependent and the TRIF-dependent pathways. MyD88 (myeloid differentiation primary response gene 88) is probably the most important adaptor molecule, since all TLRs except TLR3 interact with MyD88. MAL (MyD88 adaptor-like) mediates the activation of the MyD88-dependant pathway downstream of TLR2 and TLR4. TRIF (TIR domain-containing adaptor-inducing IFNβ) is activated downstream of TLR3 and, mediated by TRAM (TRIF-related adaptor molecule), also downstream of TLR4 (Kawai and Akira, 2007). Through multiple downstream intracellular signalling genes, regulatory proteins such as NFκB are then activated, leading to the production of inflammatory and antiviral cytokines as well as chemotactic factors and antimicrobial peptides. These, in turn, not only help fight the infection directly, but also stimulate innate effector responses and help initiate an adaptive immune response (Alberts et al., 2008; Murphy and Weaver, 2017).

1.1.2 The adaptive immune system

The adaptive immune system, also known as acquired immune system, is a highly specific and efficient system of defence, even though slower than the innate immune system, and is able to provide long-lasting protection. Lymphocytes play the most important role and have evolved to recognize a great variety of antigens, an antigen being any molecule or part of a molecule capable of eliciting an adaptive immune response (Alberts et al., 2008; Murphy and Weaver, 2017).

There are two different classes of lymphocytes that represent the adaptive immune system: The T lymphocytes (T-cells) and the B lymphocytes (B-cells). They both originate out of the same lymphatic progenitor cell and mature in the primary lymphoid

organs (T-cells in the thymus and B-cells in the bone marrow). Of the fully differentiated lymphocytes, only 2 % reside in the blood while the rest can be found in secondary lymphoid organs that comprise the lymph nodes, the spleen and the mucosa-associated lymphoid tissues (Lüllmann-Rauch, 2009).

B-cells build the humoral immune response. They express immunoglobulins (Ig) on their surfaces that serve as B-cell receptors (BCR). Upon interaction with an antigen, B-cells will proliferate and differentiate into plasma cells. These plasma cells produce antibodies with the same antigen specificity as the original BCR. There are five different classes of antibodies (IgM, IgD, IgG, IgA and IgE) that are distinguished by different structures and properties.

Antibodies bind antigens in a highly specific manner, leading to neutralization, opsonization, and complement activation, thus destroying intracellular microorganisms and their products and preventing the spread of intracellular infection.

T-cells are responsible for cell-mediated immune responses of adaptive immunity. Through their T-cell receptor (TCR), T-cells are able to recognize and bind fragments of antigens that are presented to them on molecules of the major histocompatibility complex (MHC molecules) by other cells (Germain, 1994). T-cells can be further distinguished by molecules of the CD-system that are expressed on the cell surface, most importantly CD4 and CD8. These play a role as co-receptors to the T-cell receptor and interact with two different classes of MHC molecules. $CD8^+$ T-cells, also called cytotoxic T-cells, are able to recognize MHC class I molecules that are expressed on most cells of the body. These display fragments of proteins synthesized in the cytosol, thus allowing recognition and destruction of cells infected with viruses or other intracellular pathogens. $CD4^+$ T-cells activate other cells. They recognize antigen presented in MHC class II molecules which are expressed on antigen presenting cells (APCs) such as macrophages, dendritic cells and B-cells. Upon interaction, $CD4^+$ T-cells differentiate into CD4 effector subsets such as TH1, TH2, TH17 and Treg, also called helper T-cells (TH cells), which activate their target cells through direct contact and the release of cytokines, and regulatory T-cells (Treg) which are able to inhibit the extend of immune activation (Alberts et al., 2008; Murphy and Weaver, 2017).

1.1.2.1 The role of DCs for T-lymphocyte activation

To proliferate and differentiate into effector cells, naïve T-cells need to be activated. After the discovery of dendritic cells (DCs), (Steinman and Cohn, 1973), DCs have been shown to play a central role in T-cell activation (Banchereau and Steinman, 1998). DCs, as important representatives of APCs are able to capture and process antigens, and then migrate to lymphoid tissue. Here they present processed antigens complexed with MHC proteins to other cells, express co-stimulatory proteins as well as cell-cell adhesion molecules and secrete cytokines to initiate immune responses (Fig. 2).

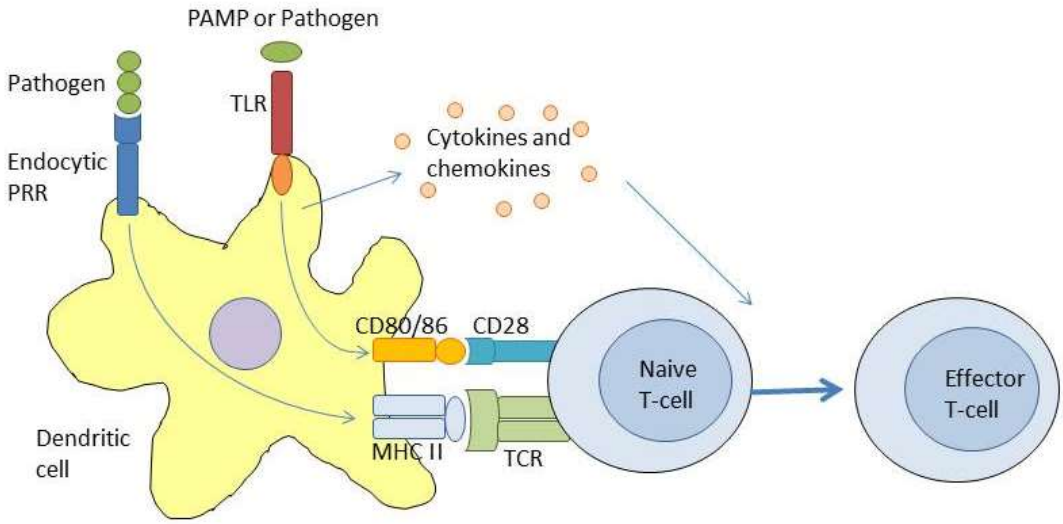

Fig. 2: Induction of adaptive immunity by DCs.

Recognition of pathogens or pathogen-associated molecular patterns (PAMP) by Toll-like receptors (TLR) leads to the upregulation of expression of major histocompatibility complex class II (MHC II) molecules as well as co-stimulatory molecules (CD80 and CD86) on dendritic cells. Through these molecules expressed on the cell surface, dendritic cells are able to activate naïve T-cells to differentiate into effector T-cells specific for the pathogen. Differentiation of the naïve T-cells is furthermore influenced by the secretion of cytokines and chemokines by the dendritic cell. (Adapted from Medzhitov (2001).

Through these mechanisms, DCs are able to either activate or supress T-cells. They also help induce self-reactive T-cells to become tolerant. To be able to activate naïve T-cells, DCs need to be activated themselves. This activation occurs through pattern recognition receptors but also by tissue injury and by activated effector helper T-cells in turn. Thus, DCs help to promote immune reactions and create an important link between

innate and adaptive immunity. (Alberts et al., 2008; Banchereau and Steinman, 1998; Murphy and Weaver, 2017)

1.1.3 Cytokines

Cytokines are small proteins that are released in response to an activating stimulus and induce responses through binding of specific receptors. Depending on their structure, cytokines can be divided into four families: IL-1, haematopoietin, interferons, and TNF family. They can be released as a reaction from the innate or the adaptive immune system. As a part of the innate immune system for example, dendritic cells and macrophages produce important cytokines, such as TNF-α, IL-6, IL-1β and IL-12, in response to bacterial products. These cytokines induce and maintain inflammation, coordinate cellular responses such as recruitment, and lead to systemic reaction such as the release of acute-phase proteins (Alberts et al., 2008; Murphy and Weaver, 2017).
As a part of the adaptive immune system, the most important representatives are probably the helper T-cells TH1 and TH2 that are distinguished by the cytokines they produce (Mosmann, 1989; O'Garra, 1998). TH1 cells mainly secrete IFN-γ and TNF-α. These cytokines are responsible for defending against intracellular microbes that persist in macrophage vesicles, such as viruses, protozoans and intracellular bacteria like mycobacteria. Additionally, they also stimulate B cells and activate complement, thus helping to eliminate some extracellular microbes as well. TH2 cells secrete a variety of cytokines including the interleukins IL-4, IL-5, IL-13 and IL-10. They are responsible for defence against extracellular pathogens, including helminth parasites through activation of other cells such as mast cells, basophils, and eosinophils promoting mucosal barrier immunity. Furthermore, cytokines from both TH1 and TH2 cells are able to supress the development of the other cell type, thus reinforcing the predominance of either a TH1 or TH2 response (Alberts et al., 2008; Murphy and Weaver, 2017).

1.2 Human filariasis

Filariasis refers to a group of different diseases provoked by different filarial nematodes. Nematodes (roundworms) form one of the three major groups of helminth infection together with Cestodes (tapeworms) and Trematodes (flukes). Filarial infections remain a major public health problem in endemic countries. In humans, four major groups of

filarial infections can be distinguished: Lymphatic filariasis (*Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori)*, Onchocerciasis (*Onchocerca volvulus)*, Loiasis (*Loa loa)* and Manonellosis (*Mansonella perstans*, *Mansonella ozzardi* and *Mansonella streptocerca)*. Together they are estimated to infect approximately 250-300 million people in Africa, Southeast Asia and southern and central America (Simonsen et al., 2011; Taylor et al., 2010; World Health Organization, 2019a, 2019b).

These diseases vary in their clinical presentation and in some cases can present with severe pathology such as elephantiasis, hydrocele or blindness. However, in the majority of individuals, infections with filarial nematodes result in chronic infections with comparatively little morbidity, and the parasites can persist for many years, due to their capacity to modulate the host's immune system (Hoerauf et al., 2005). Filarial infection requires a biting vector (e.g. mosquitoes) for transmission. During a blood meal, the vector L3 larvae enter the mammalian host. These develop into adult worms, producing microfilariae, the transmission life-stage that can be engulfed by a vector during a second blood meal. The most common diagnostic tool for all filarial infection is the detection of microfilariae in blood or skin snips. However, this method requires trained personnel and thus sometimes results in poor sensitivity and thus, immune-diagnostics and molecular diagnostics have been established for further quantification (Ta-Tang et al., 2018; Taylor et al., 2010). Different anti-helminthic drugs such as diethyl-carbamazine, ivermectin, and albendazole are used to treat filarial infections and employed as mass drug administration in the global programme to eliminate lymphatic filariasis (World Health Organization, 2017). However, also depletion of the *Wolbachia* endosymbionts with antibiotic treatment (e.g. doxycycline, quinazoline or corallopyronin A) has shown promising results with higher anti-parasitic efficacy than classic anti-helminthic drug treatment (Hoerauf, 2008; Hübner et al., 2020; Schiefer et al., 2020).

1.2.1 Immune responses in filariasis

Helminths are known to polarize the immune system to survive in their host. Several studies have shown that filarial infections lead to hypo-responsiveness towards filarial-derived antigens as well as bystander antigens (Colley et al., 1986; King et al., 1992; Piessens et al., 1980) and that drug clearance of the infection leads to the recovery of antigen-specific responses (Kamradt, 2005; Sartono et al., 1995).

Helminths in general are known to elicit a TH2 immune response with elevated levels of typically associated cytokines (IL-5, IL-13, IL-4), as well as eosinophilia, mastocytosis and production of IgE. Also TH1 responses have been shown to play a role, and a balanced combination is considered a requirement for resolution of infection (Maizels and Yazdanbakhsh, 2003). Once infected, however, it is in the interest of both host and parasite that these immune responses are dampened. A balance is needed to limit damage to the host, often resulting in the acceptance of the infection. Indeed, asymptomatic states of filarial infections allow the parasite to remain in their host for extended periods of time in which transmission is maintained, show an extensive amount of immune-regulation, while a breakdown of the regulatory system leads to morbidity (Maizels and Yazdanbakhsh, 2003; Rajasekaran et al., 2017). Immune responses to filarial infections have been shown to be stage specific and dependent on the presence of microfilaria (Babu et al., 2006; S. Mahanty et al., 1996). Cells of patients in chronic stages of Onchocerciasis or lymphatic filariasis with little pathology and high microfilarial (Mf) loads show increased production of regulatory cytokines IL-10 and TGF-β (Doetze et al., 2000; Mahanty et al., 1996), while cells of patients with severe pathology and low Mf loads show increased production of the pro-inflammatory cytokine IFN-γ as well as the typically associated Th2 cytokines IL-5 and IL-4 (Doetze et al., 1997; Sartono et al., 1997). Comparing immune profiles of patients with patent (Mf+) and latent (Mf-) *Wuchereria bancrofti* infection, Arndts et al. (2012) were also able to show that immune-suppression was stronger in Mf+ compared to Mf- individuals, whilst Katawa et al. (2015) were able to demonstrate congruent results comparing profiles of Onchocerciasis patients with little and severe pathology. Regulatory T-cells (Tregs) have been shown to be mainly responsible for maintaining tolerance and preventing severe pathology (Maizels and McSorley, 2016). Filarial-infected individuals generally show increased amounts of Tregs that are the main source of the anti-inflammatory mediators IL-10 and TGF-β but these levels are diminished in patients with severe pathology (Doetze et al., 2000; Katawa et al., 2015; Ritter et al., 2019). Filaria appear to drive Treg responses directly and indirectly and depletion of the cells in mouse model systems results in clearance of the infection (Maizels and McSorley, 2016; Taylor et al., 2005). Also IL-10 producing regulatory B-cells have been shown to be up-regulated in filarial infection, leading to dampened systemic innate and adaptive immune responses (Ritter

et al., 2019, 2018). B-cells promote and support TH2 type immune responses and are the source of IgE and IgG4. Under the influence of IL-10, a switch occurs from IgE to the non-inflammatory IgG4 that has been shown to be a marker for chronic filarial infections with little pathology (Adjobimey and Hoerauf, 2010; Maizels and McSorley, 2016). Prodjinotho and colleagues (2017) were also able to show that IgG4-mediated inhibition of granulocyte activation influence pathological manifestations in lymphatic filariasis. Innate immune cells such as DCs and macrophages have been shown to promote a modulated TH2 response, but also to induce Tregs in filarial infections (Chaussabel et al., 2003; Maizels and Yazdanbakhsh, 2003; Osborne and Devaney, 1999). Especially alternatively activated macrophages (AAM) play a critical role in immune modulation by filarial infection (Taylor et al., 2006; Turner et al., 2018). On DC different filaria and filaria-derived products have been shown to trigger TLR and to polarize immune responses by altering TLR expression and TLR-mediated responses (Mukherjee et al., 2019; Semnani et al., 2008a; Venugopal et al., 2009).

For the host, the polarization of the immune system results in intended and unintended consequences that can be advantageous such as the suppression of collateral damage during filarial infection and the reduction of allergic, autoimmune, and inflammatory reactions, but also disadvantageous consequences such as reduction of vaccine responses and increase of susceptibility to coinfection as discussed in section 1.4 (Maizels and McSorley, 2016; Rajasekaran et al., 2017).

1.2.2 *Litomosoides sigmodontis* as a murine model of filariasis

Litomosoides sigmodontis is a filarial nematode used as an established model of filarial infections in rodents, including laboratory mice. Its natural host is the cotton rat *Sigmodon hispidus*, but it has been shown to undergo a full life-cycle and produce patent infections in immune-competent BALB/c mice (Petit et al., 1992). This allows one to investigate stage-specific responses and easily recover individual life-cycle stages during the complete course of infection (Hoffmann et al., 2000; Hübner et al., 2009). C57BL/6 mice on the other hand are resistant to complete parasite development, eliminating worms after the final moulting stage, allowing one to study immune pathways required for worm elimination (Babayan et al., 2003; Hoffmann et al., 2000). BALB/c mice are known as TH2-dominant and C57BL/6 mice as TH1-dominant mouse strains

(Watanabe et al., 2004). As depicted in Fig. **3**, the laboratory life cycle is similar to that of other filarial infections.

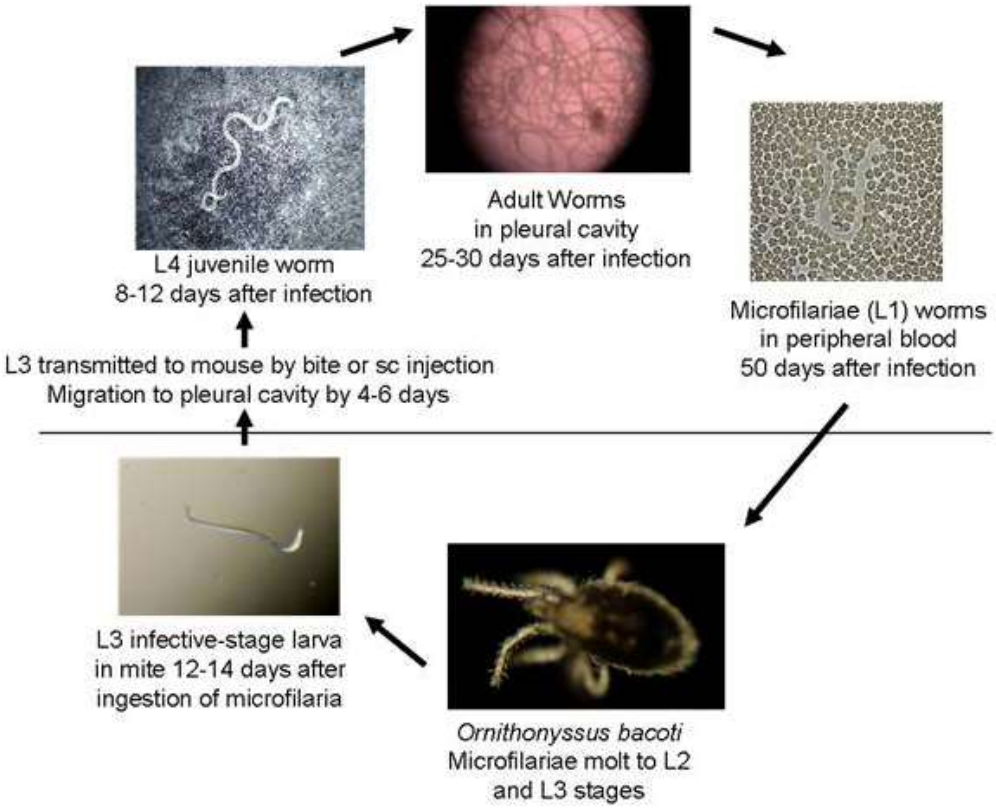

Fig. 3: Laboratory life cycle of *Litomosoides sigmodontis* in BALB/c mice

In brief, infection occurs through the bite of infected mites *(Ornithonyssus bacoti*) or by direct injection, transferring the infective-stage L3 larvae into the mammalian hosts. The larvae migrate through the lymphatic system to the pleural cavity by 4-6 days after infection. There, 8-12 days after infection, larvae molt to L4 stage larvae and become adults 25-30 days after infection. Fertile worms produce microfilariae (patency stage), the L1 larvae, which circulate in the blood from 50 days after infection. These can be taken up again by mites during another blood meal and develop though the subsequent L2-3 stages 12-14 days after ingestion. Adapted from Hübner et al. (2009).

The vector for transmission is the mite *Ornithonyssus bacoti* that transfers L3 larvae into their host during a blood meal. The L3 larvae penetrate the lymphatic system and migrate to the pleural cavity of their host. Here they develop into L4 larvae and eventually adults. In all BALB/c mice worms reach sexual maturity and develop the chronic stage of the disease around 70 days post infection, but only about 50 % of the mice exhibit blood microfilaria (Petit et al., 1992; Rodrigo et al., 2016). In C57BL/6 mice no patency is achieved and eradication of the worms is completed 40 days post infection

(Graham et al., 2005; Hoffmann et al., 2000). A cyclical passage between cotton rats (*Sigmodon hispidus*) or jirds (*Meriones unguiculatus*) and mites allows to maintain the life-cycle and which the microfilariae develop through the different L1-3 larvae stages (Graham et al., 2005; Hübner et al., 2009). *Litomosoides sigmodontis* infected mice show similar immune response to the one observed in humans infected with filaria, with patent and non-patent states and distinct immune-profiles associated with them (Arndts et al., 2012; Rodrigo et al., 2016). BALB/c mice also develop a chronic immunotolerant state of infection, associated with regulatory T-cells and production of IL-10, and show the typical skewed TH2 immune response (Babayan et al., 2003; Hoerauf et al., 2005; Hoffmann et al., 2000). Nevertheless, certain signalling pathways and receptors have been shown to enhance worm burden control or Mf counts (Frohberger et al., 2020; Ritter et al., 2017; Rodrigo et al., 2016).

1.3 Buruli ulcer disease

Buruli ulcer is a disabling cutaneous disease caused by *Mycobacterium ulcerans (M. ulcerans)*. It is the third most common mycobacterial infection after tuberculosis and leprosy (Weir, 2002). Buruli ulcer was first described in 1948 in Australian patients (MacCallum et al., 1948). Currently at least 33 countries in Africa, South America and the Western Pacific regions have reported cases of Buruli ulcer, most cases being reported in Ghana, Australia and Nigeria (World Health Organization, 2019c). Buruli ulcer occurs in patients of all ages, but it has a particularly high prevalence in children as the most vulnerable population. About 48 % of reported cases in Africa occur in children under 15 years old, increasing the public health importance in tackling this disease (Yotsu et al., 2018).

Buruli ulcer often presents itself with painless nodules, sometimes with plaques or oedemas, localized mainly on the extremities. Within weeks, the lesions will necrotize and progress into ulcerations which can show typical wide-reaching undermined borders. Ulcerations can be very deep, even affecting bone and causing grave deformities if left untreated (Guarnera, 2018; World Health Organization, 2019c). The lesions usually progress with no pain or fever, due to the immunosuppressive properties of the mycolactone. However, secondary bacterial infections often occur, leading to pain and delayed healing, and entailing prolonged hospital stay (Van Leuvenhaege et al.,

2017). The pathogenesis of Buruli ulcer relies on the macrolide mycolactone produced by *M. ulcerans*, a toxin which causes tissue damage and inhibits the immune response (Demangel, 2021; George et al., 1999). Different kinds of mycolactone can be distinguished. Mycolactone A and B, mostly produced by strains in Africa are more potent in causing necrosis than mycolactone C, the predominant mycolactone in Asian and Australian strains that tend to be less virulent (Guarnera, 2018).

Mycobacterium ulcerans is an environmental bacterium but the mode of transmission remains unclear. A concentration of cases around slow-moving or stagnant bodies of water, combined with the clinical presence of the disease on exposed areas of the body, support the current hypothesis that the disease is transmitted from these environments to humans. However, current studies have remained inconclusive, and there may be several routes of transmission (Merritt et al., 2010; Yotsu et al., 2018). The clinical diagnosis, especially of early cases, can sometimes be difficult and can be confirmed by smear for direct detection of acid-fast bacilli or culture, by histopathological examination, and by PCR. However, a positive culture requires incubation for at least six to eight weeks under appropriate conditions. In addition, access to facilities appropriate to conduct these tests as well as the test itself, can be challenging in many of the endemic areas (Buntine et al., 2001; Werf et al., 2005; Yotsu et al., 2018). The current World Health Organization (WHO) recommendations for treatment comprise (1) a combination of specific antibiotics (rifampicin and streptomycin) for 8 weeks as first-line treatment for all forms of active disease, complemented with (2) wound care, prevention of disability, and surgery to remove necrotic tissue, to cover large skin defects and to correct deformities (World Health Organization, 2012). But even though introduction of this treatment has substantially improved treatment, therapy is still often started too late, and the time required for treatment, the frequent need of hospitalization, and the financial burden remains a problem (Chukwu et al., 2017; Yotsu et al., 2018). Some work on vaccination has been done so far e.g. vaccination with Bacillus Calmette-Guérin (BCG) but the results are controversial (Zimmermann et al., 2018). At present, protection against possible environment exposure or control thereof are the only preventive measure (Yotsu et al., 2018).

1.3.1 Immune response to Mycobacterium ulcerans

Similar to infections with tuberculosis, not all *M. ulcerans*-infected individuals appear to develop Buruli ulcer. There is evidence of exposure without development of the clinical disease (Diaz et al., 2006; Yeboah-Manu et al., 2012) as well as variable outcomes from severe progress to spontaneous healing (Gordon et al., 2011). Case reports of severe infections in patients co-infected with HIV suggest the importance of a cellular immune response (Werf et al., 2005). Comparing immune profiles of infected and healthy individuals having been in contact with *M. ulcerans*, Gooding et al. (2002) proposed that a TH1 response to *M. ulcerans* may prevent the development of Buruli ulcer in people exposed, while a TH2 response does not.

Activation of the innate immune system by an infection with *M. ulcerans* at the site of infection leads to an initial TH1-based response and appears to be critical in determining the outcome of the infection (Phillips et al., 2006b; Röltgen and Pluschke, 2020). Especially an appropriate IFNγ- response is crucial for protection against most mycobacterial infections (Demangel et al., 2009).

This response, however, appears to be down regulated by *M. ulcerans* in early stages allowing it to spread. Mycolactone appears to play a central role in hindering immune responses of macrophages and other phagocytic cells through different pathways (Fevereiro et al., 2019; Sarfo et al., 2016), altering functions such as mobility of the cells and production of cytokines such as IFN-γ (Guarnera, 2018). Cellular immunity is suppressed both locally through cytotoxicity, and thus killing of DCs and inflammatory infiltrates, as well as at a systemic level, by reducing the ability of DCs and T-cells to respond to stimulation (Boulkroun et al., 2010; Demangel et al., 2009).

Histological, microbiological and immunological findings appear to show a distinction of T-helper response as well as bacilli count between different stages of the disease. In early stages, large numbers of extracellular mycobacteria are seen, while in chronic and healing stages with granuloma formation, only few or no bacilli are present (Guarner et al., 2003; Hayman and McQueen, 1985). In these early ulcerative stages with a high amount of bacilli, macrophages and lymphocytes usually express high amounts of IL-10 and little IFN-γ, while lesions with granuloma show high expression levels of IFN-γ (Kiszewski et al., 2006). Measuring cytokine mRNA expression in human skin, Phillips et al. (2006) was also able to show elevated IFN-γ as well as IL-1β, IL-12p35, IL-12p40, IL-

15 and TNF-α in tissue samples containing granulomas. This response is similar to other Mycobacteria, despite the presence of mycolactone (Phillips et al., 2006b). Prévot et al. (2004) distinguished between nodular und ulcerative earlier stages of the disease. Measuring systemic and intralesional cytokine production, patients with ulcers compared to nodules show lower IFN-γ levels, while IL-10 appears to be elevated in patients with ulcerative form. There have been some controversial findings in the literature as to the IFN-γ production in nodular and ulcerative stages (Phillips et al., 2006b), but this may be explained through the fact that granuloma formation was not taken into account, or due to differences in bacterial strains (Schipper et al., 2007). Highly virulent and aggressive forms of the disease with large ulcerations are associated with impairment of effective immune responses through mycolactone, even though also mycolactone-independent mechanisms to evade the immune system appear to play a role (Fevereiro et al., 2019; Oliveira et al., 2005).

Nevertheless, this evidence suggests that in some of the earlier stages a primary TH1 response is down regulated through TH2 preponderance, IL-10 overproduction or by TH1 downregulation per se, allowing the progression of the disease (Werf et al., 2005).

Filarial infections are known to lead to a predominant TH2-based immune response, leading away from the protective TH1-based immune response, and to actively downregulate TH1 and TH2 pro-inflammatory responses in chronic infections (section 1.2.1). Such findings suggest that co-infections with filaria may have an impact on the outcome of Buruli ulcer.

1.4 Coinfections between mycobacteria and filaria

Mycobacterial and filarial infections have been shown to be highly co-endemic. In the case of *M. ulcerans* and filarial infections, both are endemic in Sub-Saharan Africa as well as South America and mostly affect poor, rural populations and often children (sections 1.2 and 1.3). As described in sections 1.2.1 and 1.3.1, their individual impact on the immune system may differentially regulate each other.

Several studies have been conducted on the impact of helminth infection on tuberculosis. Helminth infections, including filarial infection appear to influence diagnostic tests for latent infections in many but not all studies (Babu and Nutman, 2016). In many studies, co-infections were shown to be a risk factor for active

tuberculosis and alter clinical presentation of the disease (Elias et al., 2006; Mhimbira et al., 2017; Resende Co et al., 2007; Tristão-Sá et al., 2002). However, a large longitudinal study in India showed no significant effect on progression or severity of tuberculosis (Chatterjee et al., 2014), and epidemiological studies are often inconclusive (Taghipour et al., 2019). On the other hand, clear evidence of an impairment of immunogenicity of BCG vaccines could be demonstrated (Elias et al., 2008). Also *in-vitro* studies with different co-infecting helminth species suggest a modulation of a variety of tuberculosis-specific responses but it remains unclear whether this leads to the development of active tuberculosis (Babu and Nutman, 2016). For leprosy, Diniz et al. (2010) were able to demonstrate a progression to more severe, multibacillary forms of the disease in the presence of intestinal helminths. Few studies have been conducted so far on co-infections with helminths and *M. ulcerans*. Stienstra et al. hypothesized that schistosomiasis might have an impact on Buruli ulcer (2001) but were unable to find an enhanced susceptibility to *M. ulcerans* infections by a co-infection with schistosomes (2004). Phillips et al. (2014) conducted a study on co-infection with *M. ulcerans* and *M. perstans* in Ghana and were able to show that 23 % of Buruli ulcer patients were co-infected with *M. perstans* while only 13 % of controls had *M. perstans* infections. Even though these findings were not significant they concluded that filarial co-infections should be considered in the diagnosis and treatment of Buruli ulcer.

Controversial findings in all of these different mycobacterial and helminth co-infections might be due to the type of helminth and mycobacterium, geographic region, method employed and power of the study. Even though the data so far is not conclusive, there appears to be a link between mycobacterial and filarial infections and further studies are needed.

1.5 Aim and objectives

Very little is known about the effect of a co-infection with *M. ulcerans* and filarial infections. As mentioned above, infections with both pathogens can occur in co-endemic areas and filaria have been shown to have an impact on other mycobacterial co-infections. A successful clearance of and immunity against Buruli ulcer disease appears to be dependent on a strong TH1-based immune response, and especially the release of the pro-inflammatory cytokine IFN-γ. Filarial infections, on the other hand, are known to

lead to a TH2-based immune response and to dampen pro-inflammatory responses. Different studies have suggested an effect of filarial infection on Buruli ulcers disease, however, an impact on Buruli ulcer disease has not been shown so far.

Therefore, the main aim of this thesis was to study the immune-modulatory capacity of filarial infection on the immune responses elicited by *M. ulcerans in-vitro*. To obtain a better understanding of the complex mechanism of the immune response in an *M. ulcerans*-filarial-co-infection scenario, the murine infection model of *L. sigmodontis* was used. *In-vitro* assays with murine immune cells were performed, using stimulation extracts derived of *M. ulcerans* and *L. sigmodontis* or live *L. sigmodontis*.

In the first part of this thesis cytokine profiles of murine immune cells upon exposure to *M. ulcerans* were established and the role of Toll-like receptors for activation of these immune cells by *M. ulcerans* was investigated. In the second part the immune-modulatory capacity of filarial antigen on the established *M. ulcerans*-induced immune responses was analysed. Finally, in the third part the modulation of *M. ulcerans*-induced immune responses by infection with *L. sigmodontis ex-vivo* was investigated.

2. Material and methods

This section presents first the variety of materials and then the methods that were used throughout this study.

2.1 Materials

Different materials were employed during this thesis. This section lists all equipment, software, and consumables, as well as the chemicals, reagents, kits and antibodies used in the study, as well as the protocols employed for preparing buffers, media and solutions.

2.1.1 Equipment

BD FACSCanto™ II flow cytometer	BD Biosciences, Heidelberg, Germany
C-DiGit® Blot Scanner	Li-COR Biosciences GmbH, Bad Homburg, Germany
Centrifuge (Eppendorf 5415 R)	Eppendorf AG, Hamburg, Germany
Centrifuge (Eppendorf 5810 R)	Eppendorf AG, Hamburg, Germany
Centrifuge (Multifuge 4 KR)	Fischer Scientific GmbH, Schwerte, Germany
ELISA microplate reader (Spectra Max 340PC384)	Molecular Devices, Sunnyvale, USA
Freezer (-20°C)	Bosch GmbH, Stuttgart, Germany
Freezer (-80°C)	Heraeus Holding GmbH, Hanau, Germany
Fridge	Bosch GmbH, Stuttgart, Germany
Glassware	Schott AG, Mainz, Germany
Ice machine (Scotsman AF 80)	Gastro Handel GmbH, Wien, Austria
Incubator	Binder GmbH, Tuttlingen, Germany
Incubator (Hera cell 240)	Fischer Scientific GmbH, Schwerte, Germany
Microscope (Diavert)	Leica Microsystems GmbH, Wetzlar, Germany

Microscope (Leica DM IL)	Leica Microsystems GmbH, Wetzlar, Germany
Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories GmbH, Munich, Germany
Multichannel pipettes (30-300µl)	Biohit, Helsinki, Finland
Neubauer counting chamber	LO Laboroptik GmbH, Bad Homburg, Germany
OctoMACS™ separator	Miltenyi Biotech, Bergisch Gladbach, Germany
pH meter	Mettler Toledo GmbH, Giessen, Germany
PIPETBOY acu 2	INTEGRA Biosciences GmbH, Biebertal, Germany
Pipettes (10-1000µl)	Eppendorf AG, Hamburg, Germany
QuadroMACS™ separator	Miltenyi Biotech, Bergisch Gladbach, Germany
Rocker	Bio-Rad Laboratories GmbH, Munich, Germany
Scissors	Labomedic GmbH, Bonn, Germany
Sieves	Labomedic GmbH, Bonn, Germany
Thermo magnetic stirrer	IKA GmbH & Co.KG, Staufen, Germany
Thermomixer	Eppendorf AG, Hamburg, Germany
Trans-Blot® Turbo™ Transfer System	Bio-Rad Laboratories GmbH, Munich, Germany
Tweezers	Labomedic GmbH, Bonn, German
Vortex mixer (Minishaker)	IKA GmbH & Co.KG, Staufen, Germany
Weighing machine (d=0,1mg)	Sartorius AG, Göttingen, Germany

2.1.2 Additional software

FACSDiva™	FACS acquisition and analysis software	BD Biosciences, Heidelberg, Germany
FlowJo	Flow cytometry analysis software	TreeStar Inc, Ashland, USA
GraphPad Prism 5	Biostatistics, curve fitting and scientific graphing software	GraphPad Software, San Diego, USA

SoftMax® Pro 7	Microplate reader acquisition and analysis software	Molecular Devices, San José, USA
Image Studio™	Blot Scanner acquisition software	Li-COR Biosciences GmbH, Bad Homburg, Germany

2.1.3 Consumables

Cell scraper	Corning B.V. Life science, Lowell, USA
CELLSTAR Tubes (5, 15 and 50 ml)	Greiner GmbH, Frickenhausen, Germany
Culture plates (12, 24 and 96 well)	Greiner GmbH, Frickenhausen, Germany
ELISA plates (96 well)	Greiner GmbH, Frickenhausen, Germany
Eppendorf tubes (0.5-2ml)	Eppendorf AG, Hamburg, Germany
FACS tubes	BD Biosciences, Heidelberg, Germany
Filter	BD Biosciences, Heidelberg, Germany
Gauze	Labomedic GmbH, Bonn, Germany
Glass pipettes (1-20ml)	Brand GmbH & Co.KG, Wertheim, Germany
Gloves	Ansell Healthcare, Bruessel, Belgium
Hypodermic needles (Sterican)	B. Braun Meslungen AG, Melsungen, Germany
MACS® Columns (MS and LS)	Miltenyi Biotech, Bergisch Gladbach, Germany
Microscopic slides/covers	Engelbrecht, Munich, Germany
Nitrocellulose membrane	Bio-Rad Laboratories GmbH, Munich, Germany
Parafilm M®	America National Can, Chicago, USA
Pasteur pipette	Eppendorf AG, Hamburg, Germany
Petri dishes	Greiner GmbH, Frickenhausen, Germany
Pipet tips (1-1000 µl)	Eppendorf AG, Hamburg, Germany
Refuse bags	Brand GmbH & Co.KG, Wertheim, Germany

Syringes (2-20 ml)	B. Braun Meslungen AG, Melsungen, Germany
Thick Blot Filter Paper	Bio-Rad Laboratories GmbH, Munich, Germany

2.1.4 Chemicals and reagents

3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma-Aldrich GmbH, Munich, Germany
Advanced Protein Assay™	Cytosceleton, Denver, USA
Ammonium chloride	Merck KGaA, Darmstadt, Germany
Ammonium persulfate	Bio-Rad Laboratories GmBH, Munich, Germany
BD FACS™ Clean BD	BD Biosciences, Heidelberg, Germany
BD FACS™ Rinse	BD Biosciences, Heidelberg, Germany
Bovine serum albumin (BSA)	PAA Laboratories GmbH, Pasching, Austria
Bromophenol blue	Sigma-Aldrich GmbH, Munich, Germany
coating buffer for BD ELISA	BD Biosciences, Heidelberg, Germany
cOmplete™ Lysis-M	F. Hoffmann-La Roche AG, Basel, Switzerland
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich GmbH, Munich, Germany
Dithiothreitol (DTT)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Disodium phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt, Germany
Eosin yellow	Merck KGaA, Darmstadt, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Fetal calf serum (FCS)	PAA Laboratories GmbH, Pasching, Austria
Formaldehyde	Sigma-Aldrich GmbH, Munich, Germany
Gentamycin	PAA Laboratories GmbH, Pasching, Austria
Glycerine	Sigma-Aldrich GmbH, Munich, Germany
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	PeproTech GmbH, Hamburg, Germany

Heat-killed *Listeria monocytogenes*	InvivoGen, San Diego, USA
Hydrogen peroxide (H2O2)	Sigma-Aldrich GmbH, Munich, Germany
IMDM medium	PAA Laboratories GmbH, Pasching, Austria
Isoflurane	Piramal Enterprises Ltd., Mumbai, India
Isopropanol	Merck KGaA, Darmstadt, Germany
L-Glutamine	PAA Laboratories GmbH, Pasching, Austria
Lipopolysaccharide (LPS)	Sigma-Aldrich GmbH, Munich, Germany
MACS® buffer	Miltenyi Biotech, Bergisch Gladbach, Germany
Methanol (MeOH)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Monosodium phosphate (NaH_2PO_4)	Merck KGaA, Darmstadt, Germany
Non essentielle amino acid (NEAA)	PAA Laboratories GmbH, Pasching, Austria
PageRuler™ Prestained Protein Ladder	Thermo Fisher Scientific, Waltham, USA
P_3Cys	InvivoGen, San Diego, USA
PBS Powder	Thermo Fisher Scientific, Waltham, USA
Penicillin/Streptomycin	PAA Laboratories GmbH, Pasching, Austria
Phenol	Merck KGaA, Darmstadt, Germany
Phosphate buffered saline (PBS)	Thermo Fisher Scientific, Waltham, USA
Powdered milk	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
RPMI medium	PAA Laboratories GmbH, Pasching, Austria
Sodium bicarbonate	PAA Laboratories GmbH, Pasching, Austria
Sodium carbonate (Na_2CO_3)	Merck KGaA, Darmstadt, Germany
Sodium chloride (NaCl)	Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Sodium pyruvate	PAA Laboratories GmbH, Pasching, Austria

Sulfuric acid (H2SO4)	Merck KGaA, Darmstadt, Germany
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories GmbH, Munich, Germany
Tris base	Merck KGaA, Darmstadt, Germany
Tris-HCL	Merck KGaA, Darmstadt, Germany
Gibco™ Trypan Blue Solution, 0.4 %	Sigma-Aldrich GmbH, Munich, Germany
Tween®20	Sigma-Aldrich GmbH, Munich, Germany
WesternSure® Pen	Li-COR Biosciences GmbH, Bad Homburg, Germany
WesternSure® PREMIUM Chemiluminescent Substrate	Li-COR Biosciences GmbH, Bad Homburg, Germany

2.1.5 Kits and antibodies

Mouse ELISA kits

Duo Set® ELISA kits (IL-6 and TNF-α)	R&D Systems, Minneapolis, USA
OptEIA™ ELISA sets (IL-5, IL-10, IFN-γ)	BD Biosciences, Heidelberg, Germany

Mouse MACS antibodies and kits

CD4 (L3T4) MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
Monocyte Isolation Kit (BM)	Miltenyi Biotech, Bergisch Gladbach, Germany

Mouse FACS antibodies

CD4-APC	Thermo Fisher Scientific, Waltham, USA
CD11b-APC	BioLegend, San Diego, USA
F4180-PerCpCy5.5	BioLegend, San Diego, USA
Ly6C-PECy7	BioLegend, San Diego, USA

CD3e-PE	BioLegend, San Diego, USA
Ly6G-PE	BioLegend, San Diego, USA
CD45A+B-PE	Thermo Fisher Scientific, Waltham, USA
CD49b-PE	BioLegend, San Diego, USA
CD335-FITC	BioLegend, San Diego, USA

Mouse western blot antibodies and kits

MAPK Family Antibody Sample Kit	Cell Signaling Technology, Danvers, USA
Phospho-MAPK Family Antibody Sampler Kit	Cell Signaling Technology, Danvers, USA
Histone H3 (D1H2) XP® Rabbit mAB	Cell Signaling Technology, Danvers, USA

Others

Anti-Mouse CD16/CD32 (Fc block)	Thermo Fisher Scientific, Waltham, USA
Anti-CD3	Thermo Fisher Scientific, Waltham, USA
Anti-CD28	Thermo Fisher Scientific, Waltham, USA

2.1.6 Buffers, media and solutions

All buffers, media and solutions that were prepared using chemicals and reagents as listed in section 2.1.4. All preparation of cell culture media and buffers was done under sterile conditions.

2.1.6.1 Cell culture media and buffers

Cell culture medium	1x	RPMI
	10 % (v/v)	FCS
	1 % (v/v)	Penicillin/Streptomycin
	1 % (v/v)	L-Glutamine
	0,1 % (v/v)	Gentamycin

BMDC medium	1x	IMDM
	10 % (v/v)	FCS
	1 % (v/v)	Penicillin/Streptomycin
	1 % (v/v)	L-Glutamine
	1 % (v/v)	NEAA
	1 % (v/v)	Sodium bicarbonate
	1 % (v/v)	Sodium pyruvate
Cell culture medium for western blot	1x	RPMI
	1 % (v/v)	Penicillin/Streptomycin
	1 % (v/v)	L-Glutamine
	0,1 % (v/v)	Gentamycin
ACT buffer	2.06 g	Trizma hydrochloride
	8.99 g	Ammonium chloride
	Fill up to 1 l with distilled water (pH7.2)	

2.1.6.2 Buffers and solutions for ELISA

Washing buffer	1 M	PBS (pH 7.2)
	0.05 % (v/v)	Tween 20
Coating buffer (BD) for IFN-γ	0.1 M	Na_2CO_3 (pH 9.5)
For all other BD ELISA	0.1 M	Na_2HPO_4 (pH 9.0)
Blocking buffer	1x	PBS
	1 % (w/v)	BSA

Developing buffer	0.1 M	NAH_2PO_4 (pH 5.5)
TMB substrate	60 mg	TMB
	10 ml	DMSO
Developing solution	5 ml	Developing buffer
	100 µl	TMB substrate
	1 µl	H_20_2
Stopping solution	2 M	H_2S0_2

2.1.6.3 Buffers and solutions for SDS-polyacrylamide-gel electrophoresis

Upper Tris (pH 6.8)	0.5 M	Tris-HCL
	0.4 % (w/v)	SDS
Lower Tris (pH 8.8)	1.5 M	Tris-HCL
	0.4 % (w/v)	SDS
SDS-separating gel 12.5%	2.19 ml	Distilled water
	1.56 ml	40% acrylamide
	1.25 ml	Upper Tris
	50 µl	10% ammonium persulfate
	5 µl	TEMED

SDS-stacking gel	3.13 ml	Distilled water
	0.62 ml	40% acrylamide
	1.25 ml	Lower Tris
	50 µl	10% ammonium persulfate
	5 µl	TEMED
Sample buffer (5x)	2.5 ml	Upper Tris
	1 g	SDS
	100 mg	DTT
	5 ml	Glycerine
	8 µg	Bromophenol blue
	Fill up to 10 ml with distilled water	
Running buffer	2.9 g	Tris base
	14,4 g	Glycerine
	1 g	SDS
	Fill up to 1 l with distilled water (pH8.3)	

2.1.6.4 Buffers and solutions for western blot

Transfer buffer	25 mM	Tris base
	192 mM	Glycerine
	200 mM	MeOH
	Fill up to 1 l with distilled water (pH 8.1-8.5)	

Tris-buffered saline (TBS) (x10)	88 g	NaCl
	24 g	Tris base
	Fill up to 1 l with distilled water (pH 7.6)	
Tris-buffered saline with Tween (TBST)	1x	TBS
	1 % (v/v)	Tween 20
Blocking buffer	7.5 g	Powdered milk
	150 ml	TBST

2.1.6.5 Other buffers and solutions

FACS buffer	1x	PBS
	2 % (v/v)	FCS
Hinkelmann solution	0.5 % (v/v)	Eosin yellow
	0.5 % (v/v)	Phenol
	0,185% (v/v)	Formaldehyde

2.2 Methods

Different parasitological, immunological and cell-biological methods were employed in this thesis and are described in the following sections.

2.2.1 Mice

Strains of C57BL/6 and BALB/c wildtype mice and corresponding knockout mice were bred at the Institute of Medical Microbiology, Immunology and Parasitology (IMMIP) or the House for Experimental Therapy (HET). Mice were kept with access to food and water *ad libitum* under specific-pathogen-free (SPF) conditions in accordance with

German animal protection laws and EU guidelines 2010/63/E4. The animal studies were conducted in accordance with an application to perform *in-vivo* experiments (license number TVA: 84-02.04.2014.A131 and TVA: 84-02.04.2011.A326) and were approved by the local government authorities: Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany.

2.2.2 Infections of mice with Litomosoides sigmodontis

Experiments were performed in accordance with local ethical regulations. Mice were infected with *L. sigmodontis* using a natural infection method. In brief, mites (*O. bacoti*) were allowed to feed from *L. sigmodontis* infected microfilaremic cotton rats. The following day, the infected mites were separated from a blood source and kept for 10-14 days in restricted conditions, allowing the L1 larvae to develop into the infective L3 larvae stage. Subsequently, on the day of infection, mites were allowed to feed on 6-8 week-old mice that were to be infected, thereby transmitting L3 larvae. After 70-21 days of infection for BALB/c mice or 30 days for C57BL/6 mice, mice were sacrificed and their cells prepared according to each experiment (section 2.2.4). For each infected mouse, the microfilarial (BALB/c strain) and adult worm burden (both strains) was determined (sections 2.2.2.1 and 2.2.2.2).

2.2.2.1 Determination of microfilarial burden

Microfilarial burden was determined in blood samples taken from the cheek or tail of the infected BALB/c mice with the help of a lancet and capillary. Thereafter, 10 µl of blood was diluted in 300 µl of Hinkelmann solution, vortexed and incubated for 20-30 minutes at room temperature. Afterwards samples were centrifuged at 1800 rpm for 5 minutes and the resulting supernatant, with the exception of 10 µl, was discarded. The remaining samples were re-suspended, applied onto a microscopic slide with cover glass and the number of microfilaria in the sample counted.

2.2.2.2 Counting and morphological analysis of worms

Adult worms were removed from the thoracic cavity of infected mice post mortem and rinsed in PBS solution. Worms were applied onto microscopic slides for counting,

analysis of sex and size. For use of the worms in the worm stimulation assays (section 2.2.7.1), all work was done under sterile conditions.

2.2.3 Preparation of cell stimulation extracts

For use in stimulation assays (section 2.2.7), stimulation extracts of *L. sigmodontis* as well as *M. ulcerans* were obtained and prepared as described below.

2.2.3.1 Litomosoides sigmodontis extract

Litomosoides sigmodontis extract (LsAg) was prepared using worms isolated from the thoracic cavity of jirds (*Meriones unguiculatus*) three months post infection. Worms were rinsed in sterile PBS and subsequently mechanically minced. Insoluble material was removed by centrifugation at 4000 rpm for 10 minutes at 4°C. Protein concentrations of soluble crude extracts were determined using the Bradford protein assay (Advanced Protein Assay) as a standard method for spectroscopical analysis of protein concentration following the manufacturer´s protocol. The LsAg was diluted with PBS to concentrations of 1 mg/ml and stored at -80°C until use. All procedures were conducted under sterile conditions.

2.2.3.2 Mycobacterium ulcerans extract

The *M. ulcerans* extract (BuAg) was provided by our collaborative partner Prof. Dr. Richard Odame Phillips from the KCCR in Ghana (Phillips et al., 2006a). The powdery crude extract was diluted in PBS to a concentration of 10 mg/ml and stored at -20°C until required. After the first experiments, variable results began to appear because of unsatisfactory solubility and a loss of function through repeated thawing and freezing. Thus, in subsequent experiments, BuAg was used as a solution prepared in single-use tubes and repeatedly vortexed before each application. With this method satisfactory homogeneous concentrations and constant stimulation strength was obtained.

2.2.4 Preparation and cultivation of immune cells from mice

For the preparation of all cells, mice were sacrificed by inhaling Isoflurane, pinned on a dissection board, and subsequently the fur disinfected with ethanol. All preparation was done under sterile conditions.

2.2.4.1 Preparation and cultivation of dendritic cells from bone marrow

Skin and muscle tissue from the hind legs were removed with scissors and tweezers to obtain bare bones. These were cut at the hip joint and ankle and divided by removing the knee. Bone marrow was flushed out of the bone channels into a 50 ml Falcon tube with cold PBS, using a hypodermic needle and a 5 ml syringe. The resulting bone marrow was broken up by pipetting up and down through a Pasteur pipette, and then centrifuged at 1200 rpm for 5 minutes at 4°C. To lyse the erythrocytes, the supernatant was discarded and the cells were re-suspended in 3 ml of ACT buffer and incubated for 3 minutes at room temperature. Cells were filtered through a gauze into a new 15ml Falcon tube, washed with 10 ml of cold PBS, and centrifuged once more at 1200 rpm for 5 minutes at 4°C. After discarding the supernatant, the cells were re-suspended in 2 ml of BMDC medium and counted (section 2.2.5).

To obtain bone marrow-derived dendritic-cells (BMDC), $1x10^7$ cells gained from the bone marrow were seeded onto culture plates with a total of 10 ml BMDC medium and 20 µl GM-CSF per plate and the cells were incubated at 37°C and 5 % CO_2. After three days, another 10 ml BMDC medium and 20 µl GM-CSF were added to each BMDC culture. On day 6, 10 ml of supernatant was collected from every petri dish, kept separately to avoid contamination, and centrifuged at 1200 rpm for 5 minutes at 4°C. Afterwards the cell-pellet was re-suspended in 10 ml of BMDC medium and 20 µl of GM-CSF and added again to the culture plates. On day 7, the BMDCs were detached from the culture plate with a cell scraper and transferred with the complete supernatant into a 50 ml Falcon tube. The plate was flushed several times with PBS and the washing fluid was added to the Falcon tube. After centrifugation at 1200 rpm for 5 minutes at 4°C the cells were re-suspended in the appropriate medium for cell stimulation assays (sections 2.2.7.1, 2.2.7.2 and 2.2.7.4) and counted (section 2.2.5). At each point in time, plated cells were microscopically controlled for viability and differentiation.

2.2.4.2 Preparation of spleen cells

The abdomen was carefully opened with scissors and the spleen dissected and transferred into a petri dish filled with cold PBS. The spleen was squashed with the piston of a 5 ml syringe through a sieve into another petri dish. The sieve was rinsed several times with PBS and cells were disrupted by pipetting through a Pasteur pipette. The resulting solution was transferred into 15 ml Falcon tubes and centrifuged for 8 minutes at 1200 rpm and 4°C. After decanting the supernatant, the spleen cells were re-suspended in 5 ml of ACT buffer and incubated for 6 minutes under gentle shaking at room temperature to lyse the erythrocytes. After washing with PBS, the cells were again centrifuged as described above, the supernatant was discarded, and the cell-pellet was re-suspended in 1 ml of the appropriate medium for the cell stimulation assays. The cells were then filtered through gauze into a new 15 ml Falcon tube which was filled up with an additional 9 ml of medium. Finally, cells were counted (section 2.2.5) and used for MACS-separation (sections 2.2.6.1 and 2.2.6.2) and/or cell stimulation assays (sections 2.2.7.3).

2.2.4.3 Preparation of cells from the pleural cavity

The abdomen was carefully opened as to give a clear view on the diaphragm from below. Upholding the thorax with tweezers at the sternum, a small hole was cut into the upper part of the diaphragm. The pleural cavity was then repeatedly washed with approximately 10ml PBS using a Pasteur pipette, and the cell suspension was transferred into a 15 ml Falcon tube, separating them from the residing worms. After centrifugation at 1200 rpm for 5 minutes at 4°C, the cells were re-suspended in the appropriate medium and counted (section 2.2.5) to use either for MACS-separation (section 2.2.6.2) and/or cell stimulation assays (section 2.2.7.3).

2.2.4.4 Preparation of mediastinal lymph node cells

Mediastinal draining lymph nodes were removed from the thoracic cavity and transferred into a 96-well plate (U-bottom) filled with PBS. The lymph nodes were crushed with tweezers and transferred with 3 ml PBS into 15 ml Falcon tubes. After centrifugation at 1200 rpm for 5 minutes at 4°C, the supernatant was discarded and the cell-pellet was re-suspended in 1 ml of the appropriate medium for the cell stimulation assays. Afterwards,

the cells were filtered through gauze into a new 15 ml Falcon tube and counted (section 2.2.5) to use for MACS-separation (section 2.2.6.2) and/or cell stimulation assays (section 2.2.7.3).

2.2.4.5 Preparation of cells from peritoneal lavage

The fur of the mice was carefully removed from the abdomen to provide a clear view onto the peritoneum. A hypodermic needle attached to a 5 ml syringe was inserted through the peritoneum, the peritoneal cavity rinsed with 2x 5 ml of PBS and the washing fluid transferred into a 15 ml Falcon tube. After centrifuging at 1200 rpm for 5 minutes at 4°C, the cells were re-suspended in 1 ml of MACS® buffer and counted (section 2.2.5) to use for MACS-separation (section 2.2.6.2).

2.2.5 Cell viability and counting

The number of living cells was determined using the trypan blue exclusion method. Trypan blue is a vital stain, meaning that only dead or damaged cells take up the dye, thus appearing blue. In brief, small amounts of cells were diluted 1:10 with 0.4 % trypan blue and mixed. Thereafter, 10 µl were applied onto a Neubauer counting chamber and the living cells (non-coloured) were counted in 16 quadrants of the grid. Finally, the cell titre of the original suspension was expressed as cell number per ml using the following formula:

$$\text{Cells / ml} = \text{counted cells} \times 10 \text{ (Trypan blue dilution factor)} \times 10^4 \times \text{ml}^{-1}$$

2.2.6 Magnetic cell sorting

For analysis of specific cell types in this study, cells were magnetically sorted using the Miltenyi Biotec MACS® separation system.

2.2.6.1 Isolation of CD4+ T cells

$CD4^+$ T cells were isolated from spleen cells (section 2.2.4.2) using the Miltenyi Biotec MACS® separation system with mouse CD4 (L3T4) MicroBeads according to the manufacturer's protocol. In brief, spleen cells were incubated for 15 minutes at 4-8°C with 90 µl of MACS buffer and 10 µl of CD4 Micro Beads per $1x10^7$ total cells. After washing with 1-2 ml of buffer per $1x10^7$ cells and centrifugation at 300 g for 10 minutes,

up to $1x10^8$ cells were re-suspended in 500 µl of buffer. Depending on the maximal number of total cells, either MS MACS Columns with the OctoMACS separator (up to $2x10^8$ total cells) or LS MACS Columns with the QuadroMACS separator (up to $2x10^9$ total cells) were used. The columns were always pre-rinsed with the appropriate amount of buffer (MS: 500 µl, LS: 3 mL). Afterwards, the labelled cells were added onto the column, which was then washed three times with buffer (MS: 3x500 µl, LS: 3x3 mL). The resulting collected effluent contained the unlabelled cell fraction. For the magnetically labelled $CD4^+$ T cells, the column was removed from the separator and the cells flushed out with the appropriate amount of buffer (MS: 1 ml, LS: 5 mL) with the help of a plunger into a fresh tube. Finally, the cells were centrifuged and counted (section 2.2.5) to use for cell stimulation assays (section 2.2.7.4). Analysis of purity was conducted using flow cytometry (section 2.2.9).

2.2.6.2 Isolation of monocytes

Monocytes were isolated from spleen, mediastinal lymph nodes (mLN), pleural wash (PW) and peritoneal lavage (PL) (sections 2.2.4.2, 2.2.4.3, 2.2.4.4 and 2.2.4.5) using the Miltenyi Biotec MACS® separation system with the mouse Monocyte isolation kit, according to the manufacturer's protocol. The kit isolates monocytes by depletion of non-target cells. In brief, cells were incubated with 175 µl of MACS buffer, 25 µl of FcR blocking reagent, and 50 µl of Monocyte Biotin-Antibody Cocktail per $5x10^7$ total cells for 5 minutes at 2-8°C. After washing with 10 ml of buffer per $5x10^7$ cells and centrifugation at 300 g for 10 minutes, the cell pellet was re-suspended in 400 µl of MACS buffer and 100 µl of Anti-Biotin MicroBeads per $5x10^7$ total cells and incubated for 10 minutes at 2-8°C. For magnetic isolation MS MACS Columns with the OctoMACS separator were used. As the kit sorts out all non-target cells, the negative fraction was collected and the purity analysed using flow cytometry (section 2.2.9).

2.2.7 Stimulation of immune cells

The different isolated cell types were stimulated under sterile conditions as described in the following chapter.

2.2.7.1 Stimulation of bone-marrow derived dendritic cells for ELISA

In all BMDC stimulation assays, the cells were seeded after seven days of cultivation (section 2.2.4.1) onto flat bottom 12-well or 96-well plates and stimulated with different stimuli for 24 hours (or as otherwise indicated) at 37°C and 5 % CO_2. After incubation, the cells were centrifuged, the supernatant removed, and frozen at -20°C to measure cytokine responses via ELISA (section 2.2.8).

For the *M. ulcerans* stimulation studies $2x10^5$ cells/well were stimulated with different known TLR-agonists such as P_3Cys (100 ng/ml), HKLM (10^{10} c/ml) and LPS (titrated concentrations ranging from 100 to 0.1 ng/ml) as well as with *M. ulcerans* extract (BuAg) (section 2.2.3.2) (titrated concentrations ranging from 50 µg/ml to 5 ng/ml) in 96-well plates with and end volume of 200 µl/well. For the TLR knockout studies, $1x10^5$ cells/well were stimulated with BuAg (1, 2, 3 and 5 µg/ml) and P_3Cys (100 ng/ml) and LPS (5 ng/ml) as TLR controls in 96-well plates with and end volume of 200 µl/well. For the *L. sigmodontis* extract modulation studies, in some wells, cells were pre-stimulated with *. sigmodontis* extract (LsAg) (section 2.2.3.1) for 2 hours before adding the BuAg (1, 2, 3 and 5 µg/ml). LPS (5 ng/ml) was used as positive control. The stimulation was performed in 96-well plates with $1x10^5$ cells/ well and an end volume of 200 µl/well, with the exception of the titrated modulation experiment, which was conducted with $5x10^4$ cells/well and an end volume of 250 µl/well. For the worm stimulation assays $2.25x10^5$ cells/well were seeded into 12-well plates, and in half of the wells, cells were pre-stimulated with one live male and one live female worm/well for 2 hours before adding the BuAg (1, 2, 3 and 5 µg/ml) and LPS (5 ng/ml) as positive control adding up to an end volume of 750 µl/well. The cells were stimulated for 48 hours and the viability of the worms verified microscopically after 24 and 48 hours.

2.2.7.2 Stimulation of bone-marrow derived dendritic cells for western blot

For western blot analysis, BMDC were seeded in 24-well plates, with $1x10^6$ cells/well and 1 ml/well end-volume. Cells were stimulated with BuAg (5 µg/ml), LPS (5 ng/ml) or P_3Cys (0.5 µg/ml). In some wells, cells were pre-stimulated with LsAg (50 µg/ml) for two hours. After 15, 30, 60, or 180 minutes of stimulation at 37°C and 5 % CO_2, cells were centrifuged at 2500 g for 5 min, the supernatant discarded, and cells were washed with 500 µl PBS per well at 2500 g for 5 min to stop the stimulation. After discarding the

supernatant, cells were lysed with 150 µl of cOmplete™ Lysis-M reagent for 5min at room temperature under gentle shaking. The resulting cell-lysate was centrifuged at 14000 g for 10 minutes and the supernatant was mixed with the soluble protein in a 1:5 concentration with 5x SDS sample buffer. Samples were frozen at -20°C until use for SDS-PAGE (2.2.10.1).

2.2.7.3 Stimulation of spleen, mediastinal lymph node and pleural wash bulk-cell-assays

For the bulk-cell assay stimulation, $5x10^5$ cells/well, gained from spleen, mediastinal lymph nodes (mLN) and pleural wash (PW) (sections 2.2.4.2, 2.2.4.3 and 2.2.4.4) were independently seeded and then stimulated in round bottom 96-well plates with a total end-volume of 250 µl/well. All cells were incubated for 48 to 72 hours at 37°C and 5 % CO_2. After incubation, the cells were centrifuged at 1200rpm for 5min at 4°C, the supernatant removed and frozen at -20°C to measure cytokine responses via ELISA (section 2.2.8).

In some assays, spleen and mLN cells were pre-stimulated with LsAg (50µg/ml) for 2 hours before adding the BuAg (1, 2, 3 and 5µg/ml). As positive control, LPS (5ng/ml) and αCD3/CD28 (5/1.25 µg/ml) were used in all assays. After 48 hours, the cells were centrifuged and 70 µl of supernatant, removed, and frozen at -20°C. Subsequently, the plate was incubated and after 72 hours of total stimulation, cells were again centrifuged at 1200 rpm for 5 minutes at 4°C, and the remaining supernatant was removed and frozen at -20°C. The PW cells were stimulated with BuAg (1, 2, 3 and 5 µg/ml) and LPS (5 ng/ml) as described above. After 48 hours of incubation and following centrifugation, all supernatant was removed and frozen at -20°C.

2.2.7.4 Stimulation of T-cell assays

For the T-cell assays BMDCs ($5x10^4$/well) (section 2.2.4.1) were plated onto a round bottom 96-well plate and allowed to settle in the incubator at 37°C and 5 % CO_2 for at least two hours. Afterwards, MACS® sorted $CD4^+$ T cells ($2x10^5$/well) (section 2.2.6.1) were added together with LsAg (50 µg/ml) to the BMDCs, with appropriate controls and further incubated for at least two hours to allow the uptake and presentation of the LsAg by the BMDC to $CD4^+$ T-cells. As positive control, BuAg (1, 2, 3 and 5 µg/ml) as well as

LPS (5 ng/ml) or αCD3/CD28 (5/1.25 µg/ml) were then added to the plates, resulting in a total end volume of 250 µl/well. Cells were incubated for 48 hours, centrifuged at 1200 rpm for 5 minutes at 4°C, and 70 µl of supernatant was collected and frozen at -20°C. The plate was further incubated and after 72 hours of total stimulation the cells were again centrifuged, the remaining supernatant was removed and it was again frozen at -20°C to measure cytokine responses via ELISA (section 2.2.8).

2.2.8 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) were performed to measure the cytokines in the supernatant of the cell cultures. ELISA is an immune-assay based on an enzyme reaction as detection method. In the present study, a Sandwich-ELISA method was used, as it is highly sensitive to detect and quantify antigens, such as cytokines. In brief, a 96-well assay plate is coated with an antibody (capture antibody). This antibody is specific for the antigen to be detected. Secondly, the plate is saturated with a protein solution to prevent unspecific binding. Then, the samples are applied to the plate and incubated, allowing the coated antibodies to bind the epitope of the antigen, and thus bind it to the plate. In case the concentration of antigen is very high in the sample, the sample is diluted to optimize the result. For detection, a second antibody (detection antibody) is applied onto the plate, to also bind the antigen. This antibody is biotinylated and can thus be bound by a conjugate of streptavidin and horse radish peroxidase (HRP) in a second step. When 3,3',5,5'-tetramethylbenzidine (TMB) is added onto the plate, the enzyme HRP reacts with it, leading to a blue colouring. Sulphuric acid stops the reaction and leads to colour change from blue to yellow, which can be detected spectrophotometrically. Results are quantified by adding a standard dilution row with known concentrations of the antigen, while applying the sample onto the plate. In the present study, Duo Set® ELISA kits from R&D and OptEIA™ ELISA sets from BD were used.

2.2.8.1 Duo Set® ELISA kits (R&D)

The Duo Set® ELISA kits from R&D were used to measure IL-6 and TNF-α, according to the manufacturer's protocol. In brief, the capture antibody was diluted with PBS to the recommended working concentration of 50 µl/ well added onto the 96-well ELISA plates,

and incubated at room temperature overnight. Plates were washed three times with washing buffer and blocked with blocking buffer for at least one hour at room temperature to prevent unspecific binding. After another three washing steps, the samples were diluted in blocking buffer (1:2 to 1:10) and 50 µl/well were added to the antibody-coated plates. Two standard serial dilution rows with 50 µl/well were added onto each plate with varying maximal concentrations between 1-2 ng/ml, depending on the cytokine and assay, always leaving a few blank wells to subtract the background at the end. Samples and standard were incubated for 2 hours at room temperature or at 4°C overnight. The plates were again washed five times, and 50 µl/well of the biotinylated detection antibody, diluted with blocking buffer, were applied. The plates were then further incubated for 2 hours and washed another 5 times. Afterwards, 50 µl/well of the streptavidin-horseradish peroxidase (HRP) conjugate, diluted 1:40 with blocking buffer, was applied and incubated for at least 45 minutes. Finally, after 5 additional washing steps 50 µl/well of developing solution were added and the plates were incubated in the dark. As soon as the first five wells of the standard serial dilution turned blue, the reaction was stopped by adding 50µl/well of stopping solution. Without delay, optical density was measured with wavelength correction (450 nm and 570 nm) using the Spectra Max microplate reader and analysed using the SoftMax Pro 7 software.

2.2.8.2 OptEIA™ ELISA sets (BD)

For the measurement of IL-5, IL-10 and IFN-γ the Duo Set® ELISA kits from R&D were used according to the manufacturer's protocol. In brief, the capture antibody was diluted in coating buffer to the recommended working concentration of 50 µl/ well, added onto 96-well ELISA plates, and incubated at 4°C overnight. Plates were washed four times with washing buffer and blocked with blocking buffer for at least one hour at room temperature to prevent unspecific binding. After another four washing steps, the samples were diluted in blocking buffer (1:2 to 1:10) and 50 µl/well added to the antibody-coated plates. Two standard serial dilution rows with 50µl/well were added onto each plate with varying maximal concentrations between 1 and 3 ng/ml, depending on the cytokine and essay, always leaving a few blank wells to subtract the background at the end. Samples and standard were incubated at 4°C overnight. The plates were

washed again five times, and 50µl/well of the biotinylated detection antibody, diluted with blocking buffer, applied. The plates were then further incubated for 1 hour and washed another 5 times. Afterwards, 50µl/well of the streptavidin-horseradish peroxidase (HRP) conjugate, diluted 1:5000 with blocking buffer, was applied and incubated for at least 45 minutes. Finally, after 5 additional washing steps 50 µl/well of developing solution were added and the plates were incubated in the dark. As soon as the first five wells of the standard serial dilution turned blue, the reaction was stopped by adding 50 µl/well of stopping solution. Without delay, optical density was measured with wavelength correction (450 nm and 570 nm) using Spectra Max microplate reader and analysed using the SoftMax Pro 7 software.

2.2.9 Flow cytometry

FACS analysis with different fluorophore-conjugated FACS antibodies was performed to verify the purity of MACS-sorted cells (section 2.2.6), (section 2.1.5). Flow cytometry is a technique for analysing phenotypical characteristics of microscopic particles such as cells suspended in liquid. FACS is an abbreviation for Fluorescence-activated cell sorting or scanning (FACS) and is often used as a synonym for Flow Cytometry.

A beam of light of a single wavelength is directed into a focused stream of fluid containing the target cells. The light is scattered in a specific way by the cells and picked up by detectors. The forward scatter (FSC) is used as a parameter for cell size, while the side scatter (SSC) indicates granularity or inner complexity of the cell. For more detailed analysis, cells are stained with antibodies, specific for a certain characteristic of the cell, such as CD molecules expressed on immune cells, conjugated with a fluorophore. In some cases, fluorescence originates from natural material of the cell itself. Fluorescent particles can be excited onto emitting light of a specific wavelength that is again picked up by the detectors. Through electronic subsystems, light signals are converted into equivalent electronic signals.

2.2.9.1 Cell surface staining

Cells were applied onto a 96-well plate and washed with 200 µl of FACS buffer per well at 1200rpm for 5 minutes at 4°C. The supernatant was discarded and the cells resuspended in FACS buffer with Fc block diluted 1:1000 to block unspecific binding of

antibodies. Cells were incubated for 20 minutes at 4°C and washed as before. After discarding the supernatant, the cells were re-suspended in FACS buffer containing FACS antibodies specific for different cell surface marker combinations at a dilution of 1:100 (section 2.1.5). For each sample, additional unstained and compensation samples (stained with only one of the fluorophore-conjugated antibody) were prepared in the same manner. All samples were incubated for 30 minutes at 4°C in the dark. After incubation, the cells were washed with FACS buffer and centrifuged as previously described. Finally, the cells were re-suspended in 200 µl of FACS buffer and transferred into appropriate FACS tubes for the FACS scan. Additionally, cells were sometimes fixed with 200 µl of fixation buffer for 15 minutes at room temperature to allow storage at 4°C for a few days.

2.2.9.2 FACS acquisition and analysis

Cells were analysed using a FACSCanto II flow cytometer and evaluated with the FACSDiva software. For each experiment, unstained and compensation samples were used to tune voltage of the laser for optimal position of the target cell population in the FSC-SSC dot plot as well as to compensate cell background and overlapping emission of fluorophore colours. At least 10.000 events were acquired for each sample. The FlowJo software was used to depict the gating strategy employed.

2.2.10 Methods of protein purification and analysis

To analyse proteins of the down-stream signalling of stimulated immune cells in this study, SDS-Polyacrylamide gel electrophoresis followed by western-blot analysis were conducted as described in this chapter.

2.2.10.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method used to separate proteins according to their molecular mass in an electric field using the anionic detergent sodium dodecyl sulfate (SDS).

The present study used discontinuous polyacrylamide gels, consisting of 12.5 % SDS-separating and a SDS-stacking gels. Gels were prepared a day prior to electrophoresis and kept in running buffer. For preparation, components (2.1.6.3) were mixed, adding

TEMED last, and solutions added to prepared glass plates. The separating gel was prepared first and allowed to polymerize for 30-45 minutes. While it was setting, to ensure an even interface, a thin layer of isopropanol was applied on top. The isopropanol was removed before adding the stacking gel that was allowed to polymerize for 20-30 minutes with an inserted comb, to form pockets for sample application. On the day of electrophoresis, the gels were placed into the chambers filled with running buffer. Samples (section 2.2.7.2) were denaturized at 95°C for 5 minutes and loaded together with the protein ladder into the pockets of the gel. Gels were run at 200 V and 300 mA for 45 minutes.

2.2.10.2 Western blot

Western Blot is a technique used to transfer protein separated by SDS-PAGE (section 2.2.10.1) onto a membrane, allowing the detection of specific target proteins using antibodies. For the present study, a semi-dry transfer system was used. In brief, a cellulose membrane and blotting paper were pre-soaked in transfer buffer and the gel applied onto the membrane with a blotting paper on top and below the stack. Blotting was performed using the Trans-Blot Turbo Transfer System at 15 V and 1 A for 80 minutes.

For detection, the membrane was washed with TBS and incubated with blocking buffer for 1 hour at room temperature to block unspecific binding. Afterwards the membrane was washed 3 times with 15 ml of TBST before being incubated with the primary antibody diluted 1:1000 or 1:2000, depending on the antibody in 5ml of blocking buffer with gentle agitation over night at 4°C. After 3 more washing steps, the membrane was incubated with the secondary antibody and anti-biotin HRP-linked antibody, both diluted 1:2000 in 5 ml of blocking buffer, with gentle agitation for 1 hour at room temperature.The membrane was washed again 3 times before starting detection using the C-DiGit® Blot Scanner. For this, 2 ml of freshly mixed WesternSure ® PREMIUM Chemiluminescent Substrate was applied onto the reader before placing the membrane onto the scanner. The membrane was incubated for 5 minutes in the dark before being scanned. Scan results were analysed using the Image Studio™ software.

2.2.11 Statistical analysis

Statistical analysis of acquired data was performed using the GraphPad Prism 5 software. Groups were tested for Gaussian distribution (using Kolmogorov-Smirnov test, D'Agastino and Pearson omnibus normality test, and Shapiro-Wilk normality test) and the test for statistical significance chosen accordingly. Parametric data were analysed by unpaired t-test, when comparing two groups, or one-way ANOVA, followed by Bonferroni's post hoc test, when comparing more than two groups. Non-parametric data were analysed with Mann-Whiney test, when comparing two groups, or with Kruskal-Wallis followed by Dunn's post hoc test, when comparing more than two groups. Data were presented as mean + standard error of mean (SEM). P values of 0.05 or less were considered significant.

3. Results

This chapter is divided into three main sections. The first section presents immune responses upon direct stimulation with the *Mycobacterium ulcerans* stimulation extract (BuAg) as well as its signalling pathway. In the second section, the immune-modulation of the established immune responses by the *Litomosoides sigmodontis* stimulation extract (LsAg) is analysed. Finally, the third section addresses the effect of live infection with *L. sigmodontis* on BuAg-elicited responses in immune cells *ex-vivo*.

3.1 BuAg response and signalling

Infections with *M. ulcerans* are known to elicit a TH1-based pro-inflammatory response in patients and in immune cells *in-vitro* (Röltgen and Pluschke, 2020). For use in further experiments in this thesis, there was a need to establish the optimal handling and the concentration of *M. ulcerans* extract (BuAg) for stimulation of different immune cells *in-vitro,* as well as to establish cytokine profiles upon cell stimulation with BuAg. Therefore, bone marrow-derived dendritic cells (BMDC) as well as bulk spleen cells from BALB/c and C57BL/6 mice were used for this analysis. This section also analyses the role of Toll-like receptors (TLRs) during BuAg stimulation.

3.1.1 Establishment of immune profiles in immune cells upon exposure to BuAg

In the first experiments of this thesis bone marrow-derived dendritic cells (BMDC) from BALB/c as well as C57BL/6 mice were generated and stimulated for 24 hours with BuAg and known TLR agonists as described in section 2.2.4.1 and 2.2.7.1. The resulting pro-inflammatory cytokines (IL-6 and TNF-α) were measured in the supernatant via ELISA (section 2.2.8) and the results are presented in Fig**.** 4. Cytokine levels between the different stimuli as well as between to two mouse strains were depicted in comparison to each other, and BuAg proved to be a strong inducer of pro-inflammatory cytokines in BMDC from both mouse strains.

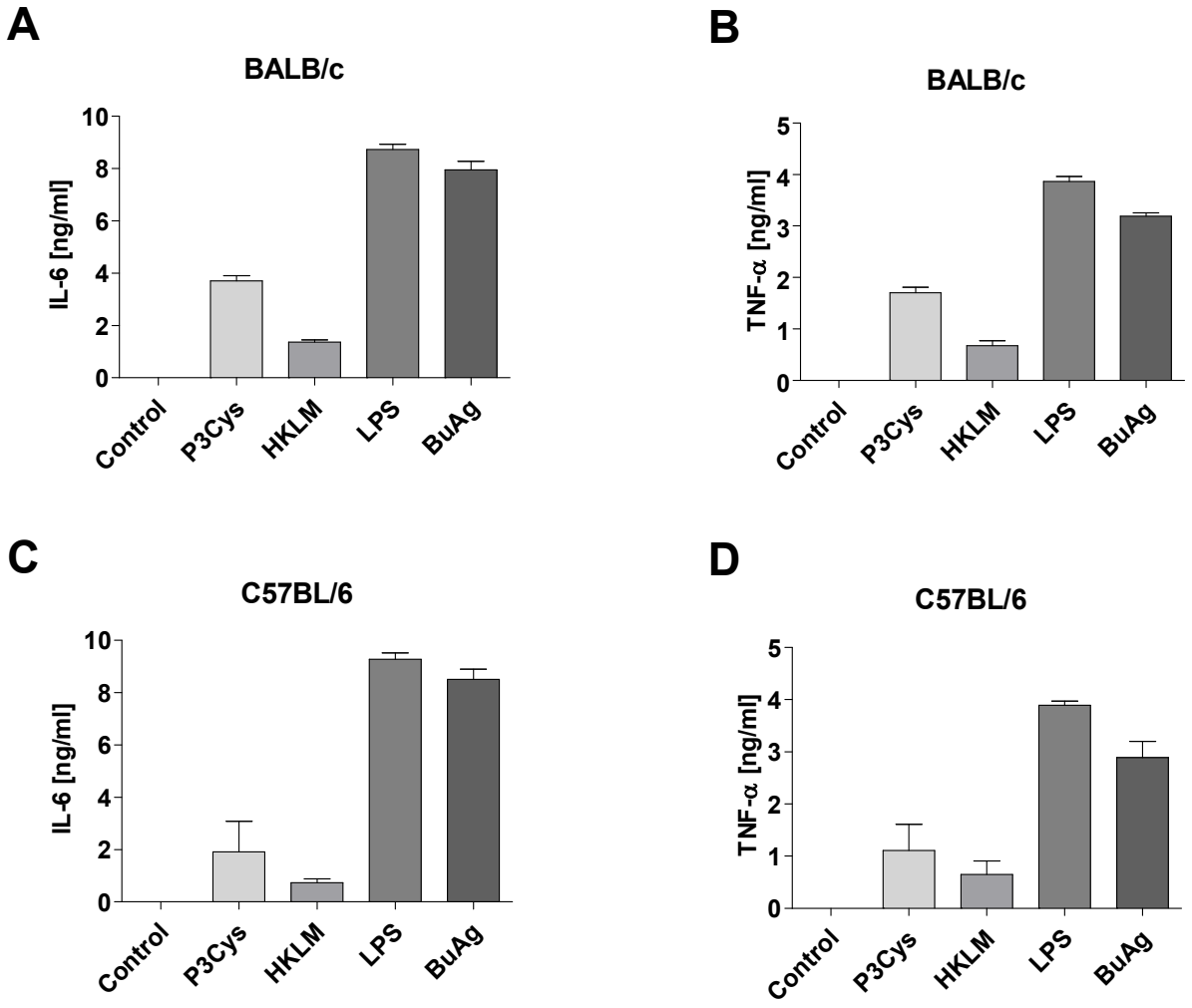

Fig. 4: Comparison of responses by BMDC from BALB/c and C57BL/6 to *M. ulcerans* stimulation extract (BuAg) response with known TLR-agonists.

BMDC ($2x10^5$ cells/ well) from BALB/c (A, B) and C57BL/6 (C, D) mice were left unstimulated (control) or stimulated with the known TLR-agonists P_3Cys (100 ng/ml) and HKLM (10^{10} c/ml) for TLR-2 and LPS (100ng/ml) for TLR-4 as well as BuAg (50µg/ml) for 24h. Thereafter, levels of IL-6 (A, C) and TNF-α (B, D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed BMDC cultures from 2 independent experiments per mouse strain (n=2 BALB/c mice, and n=2 C57BL/6 mice).

As the elicited cytokine release upon stimulation with BuAg was very strong, titration assays with varying concentrations of BuAg were performed to determine the appropriate dose for cell culturing (Fig. 5).

For both mouse strains, the release of IL-6 and TNF-α was observed following stimulation with BuAg at concentrations between 500 ng and 50 µg/ml. Cytokine

releases from BMDC of BALB/c as well as C57BL/6 mice showed similar patterns. Release of both cytokines could be induced starting from concentrations between 500 ng and 1 µg/ml in a dose-dependent manner in BMDCs from both mouse strains. Accordingly, BuAg concentrations of between 1 and 5µg/ml were used in all further experiments of this study for both mouse strains.

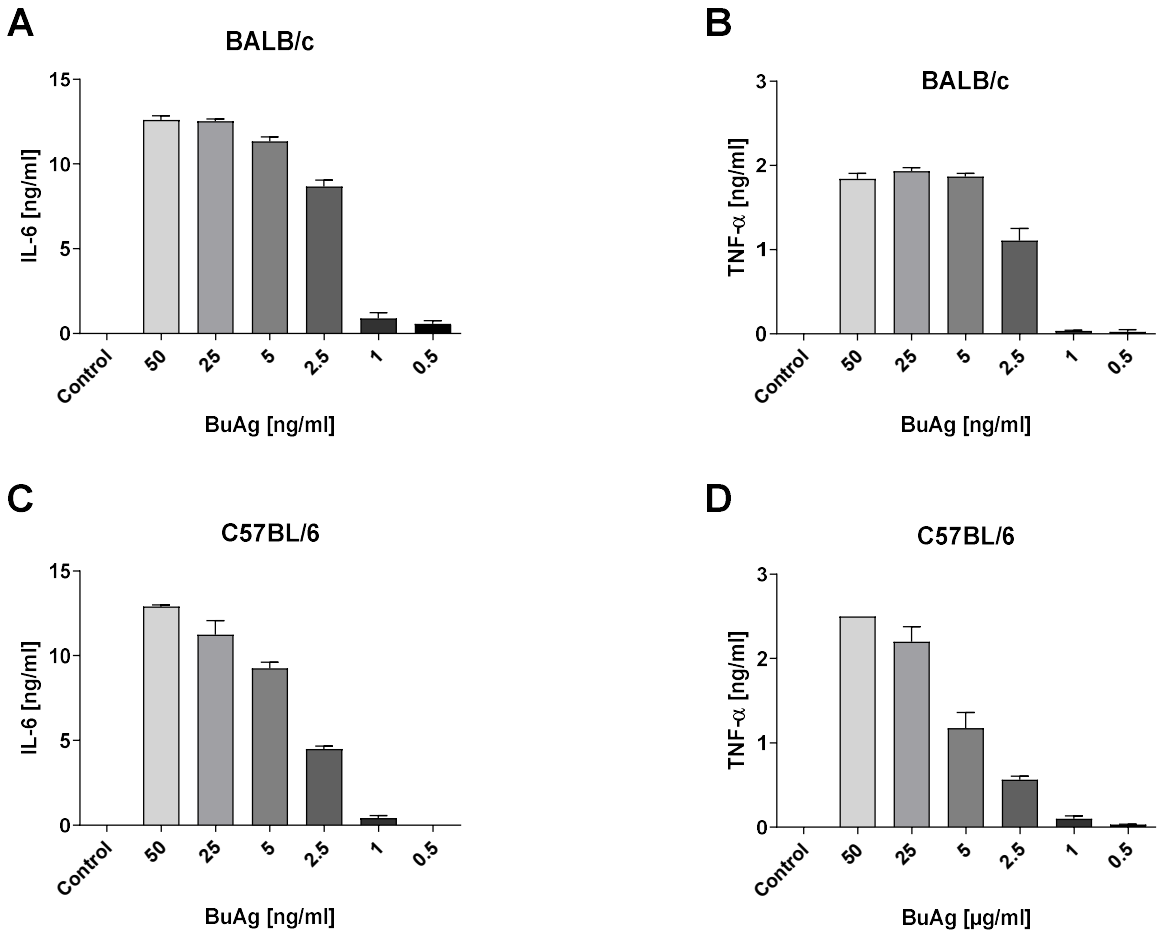

Fig. 5: Titration of BuAg.

BMDC (2x105 cells/ well) from BALB/c (A, B) and C57BL/6 (C, D) mice were left unstimulated (control) or stimulated with different concentrations of BuAg for 24h. Thereafter, levels of IL-6 (A, C) and TNF-α (B, D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed BMDC cultures from 1 experiment per mouse strain (n=1 BALB/c mouse and n=1 C57BL/6 mouse).

In addition, to be able to better compare the cytokine responses between stimuli, as cytokine production of the different TLR-stimuli controls showed strong variations between the different stimuli, the concentration of the TLR-stimuli controls was also

titrated (data not shown). Adapted concentrations of 5 ng/ml LPS were used for further experiments.

Further experiments used bulk spleen cells from BALB/c and C57BL/6 mice (section 2.2.4.2). Bulk spleen cell cultures consist of different immune and allow a broader approach on deciphering elicited responses. Furthermore, spleen cells are relatively easy to harvest and, contrary to BMDC, they can also be gained from mice infected with *L. sigmodontis* as needed for the infection assays in section 3.3. For establishment of cytokine release of spleen cells after stimulation with BuAg, cells were stimulated for periods of 48 and 72h as described in section 2.2.7.3, before measuring cytokine profiles in the supernatant via ELISA. LPS was used as before as a control for innate cell activation (48h stimulation) and antiCD3/CD28 was added as a control for T-cell-activation (72h stimulation). The study measured IL-6 and TNF-α (48h) as well as IFN-γ, IL-5 and IL-10 (72h). With cell cultures from BALB/c mice, LPS and antiCD3/CD28 induced the production of all measured cytokines (Figure 6).

Interestingly, levels after LPS-stimulation were not as strong in cultures from C57BL/6 mice (Figure 7). Fig. 6 and Fig. 7 show a release of IL-6, TNF-α and IL-10 upon stimulation with BuAg in a concentration-dependant manner in cell cultures from both strains. While the release of IFN-γ was also concentration-dependent in cultures with spleen cells of BALB/c mice (Fig. 6D), it was mostly absent from cultures with spleen cells from C57BL/6 mice (Fig. 7D). IL-5 could not be induced in spleen cells of either mouse strain (data not shown).

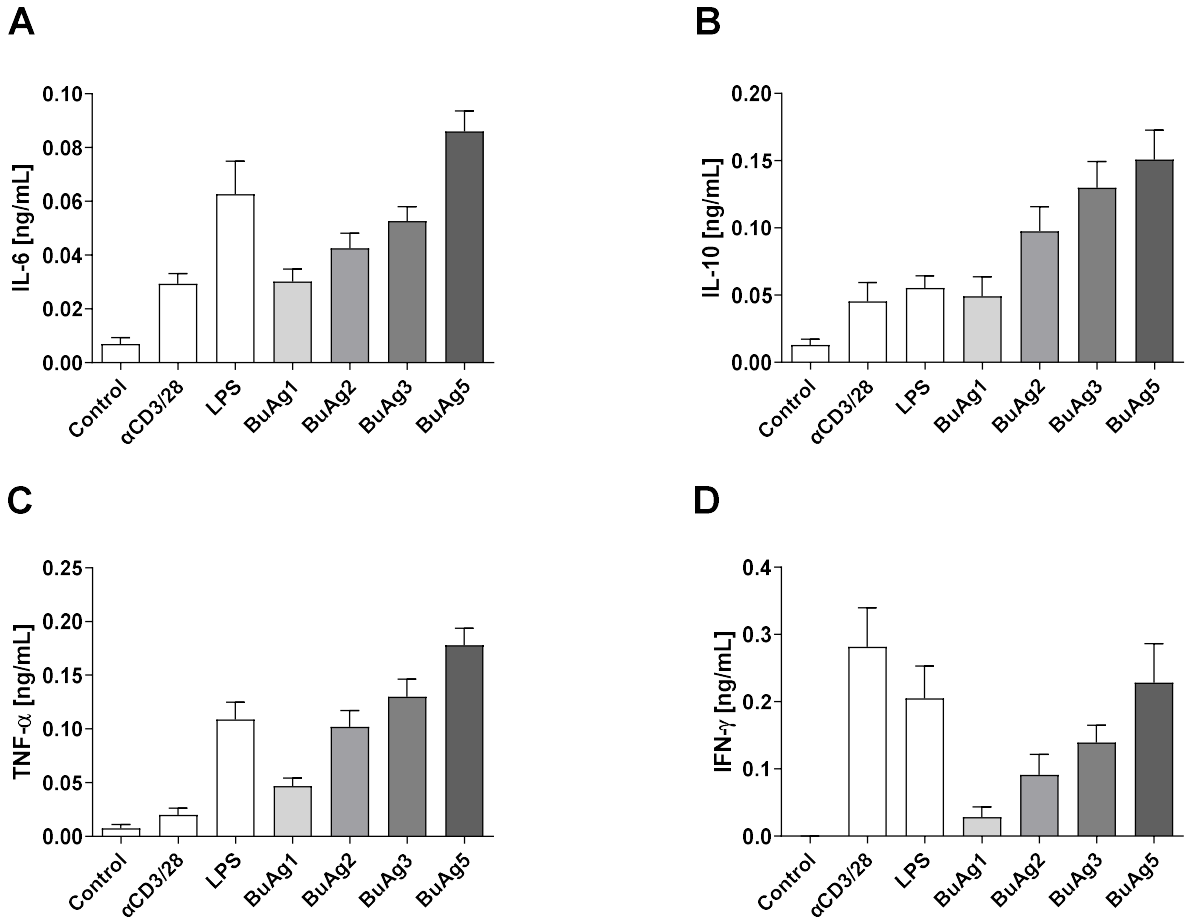

Fig. 6: BuAg-induced cytokine production in a concentration-dependent manner in spleen cells from BALB/c.

Erythrocyte-depleted spleen cells ($5x10^5$ cells/ well) from BALB/c mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml) and different concentrations of BuAg1-5 (1-5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 3 independent experiments (n = 9 mice).

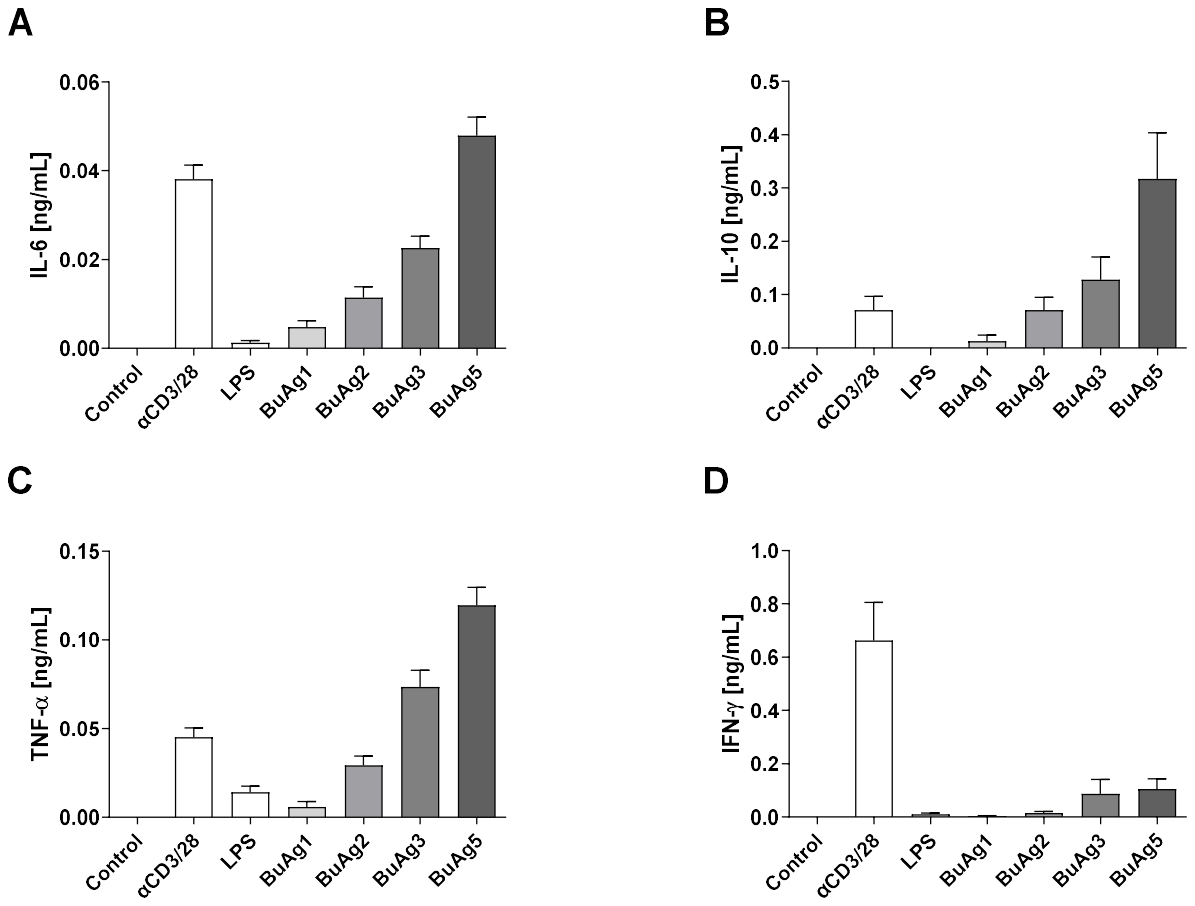

Fig. 7: BuAg-induced cytokine production in a concentration-dependent manner in spleen cells from C57BL/6 mice.

Erythrocyte-depleted spleen cells ($5x10^5$ cells/ well) from C57BL/6 mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml) and different concentrations of BuAg1-5 (1-5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 2 independent experiments (n=6 mice).

3.1.2 Role of TLR in activation by *Mycobacterium ulcerans*

It is known that Mycobacteria interact with TLR2 and that *M. ulcerans* activates keratinocytes in a TLR2-dependent manner (Lee et al., 2009; Underhill et al., 1999). To confirm TLR2-dependant signalling of *M. ulcerans* extract in BMDC, the TLR-dependent BuAg response was assessed using BMDCs derived from wild type (WT), TLR2-, TLR4- and MyD88-deficient BALB/c and C57BL/6 mice. Pro-inflammatory cytokine release

upon stimulation with known TLR agonists and BuAg were measured in the supernatant of the cell cultures after 24 hours via ELISA (Fig. 8).

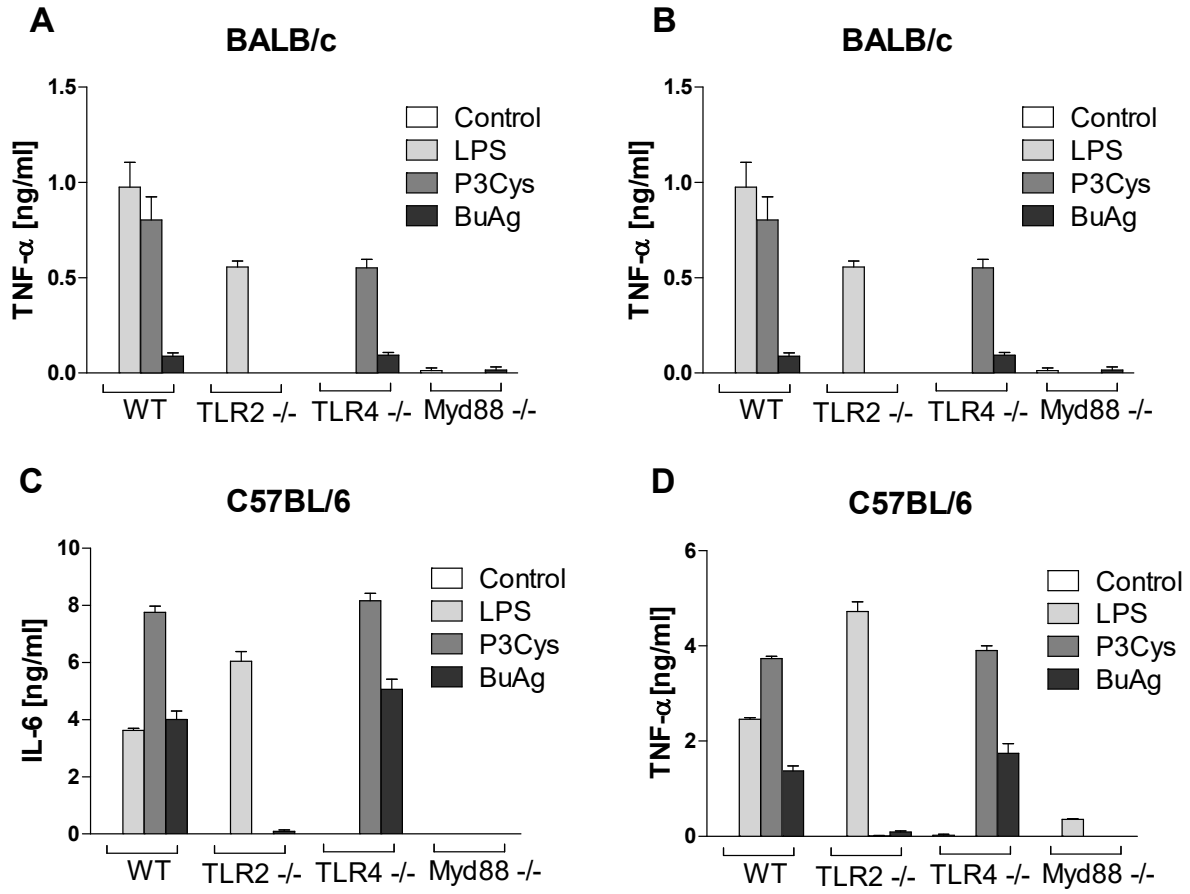

Fig. 8: BuAg signals via TLR2.

BMDC (10^5 cells/ well) from WT, MyD88-/-, TLR2-/- and TLR4-/- BALB/c (A, B) and C57BL/6 (C, D) mice were left unstimulated (control) or stimulated with BuAg (3µg/ml), LPS (5ng/ml) and P_3Cys (100ng/ml) for 24h. Thereafter, levels of IL-6 (A, C) and TNF-α (B, D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) from 1 experiment per mouse strain (n=1 BMDC culture from 1 BALB/c mouse per group and n=1 BMDC assay pooled from 2 C57BL/6 mice per group).

As expected, no cytokine production was observed in non-stimulated control cultures, while WT BMDC showed proinflammatory cytokine responses to all stimulants. In the BMDC cultures from knock-out strains, BuAg stimulation showed similar profiles to stimulation with the known TLR2 agonist P_3Cys, with maintained responses in BMDC from $TLR4^{-/-}$ mice and absent responses in the $TLR2^{-/-}$ and $MyD88^{-/-}$ groups. Stimulations with the TLR4 agonist LPS, mirrored these results as expected, maintaining responses

in cultures from TLR2$^{-/-}$ cells without responses in the TLR4$^{-/-}$ and MyD88$^{-/-}$ groups. These findings were observed in both BALB/c and C57BL/6 mouse strains, suggesting that BuAg signals in a TLR2- but not in a TLR4-dependent manner leading to the release of pro-inflammatory cytokines in BMDC. Further down-stream signalling analysis was performed stimulating BMDCs as described in section 2.2.7.2 and using SDS-PAGE for protein purification and western blot for analysis as described in section 2.2.10. western blot analysis of phosphorylated and non-phosphorylated MAPK did not show any conclusive results (data not shown) and further analysis was abandoned for the context of this thesis.

3.2 Modulation of BuAg response by filarial antigen

As helminths are known to polarize different immune responses, *in-vitro,* modulation experiments with BuAg in the presence of filarial-derived antigen (LsAg) from whole worms or live worms on different immune cells were performed.

3.2.1 Effects of LsAg or live worms on BuAg response in BMDC

To investigate whether filarial antigen (LsAg) could polarize the BuAg-induced pro-inflammatory cytokine release in BMDC (established in section 3.1.1), BMDCs of BALB/c and C57BL/6 mice were cultured with LsAg for two hours, before being stimulated with BuAg for a further 24 hours. The release of IL-6 and TNF-α was measured in the supernatant via ELISA as depicted in Fig. 9. As expected, the positive control, LPS, released a strong cytokine release in all measured cultures. Both BuAg and LsAg lead to the release of the pro-inflammatory cytokines on their own, albeit BuAg stronger, especially in cell cultures from C57BL/6 mice. In the co-stimulated group, the amount of released Il-6 as well as TNF-α from C57BL/6 cell cultures appeared equivalent to those from BuAg alone (Figure 9 C, D). Within the BALB/c BMDC cultures,

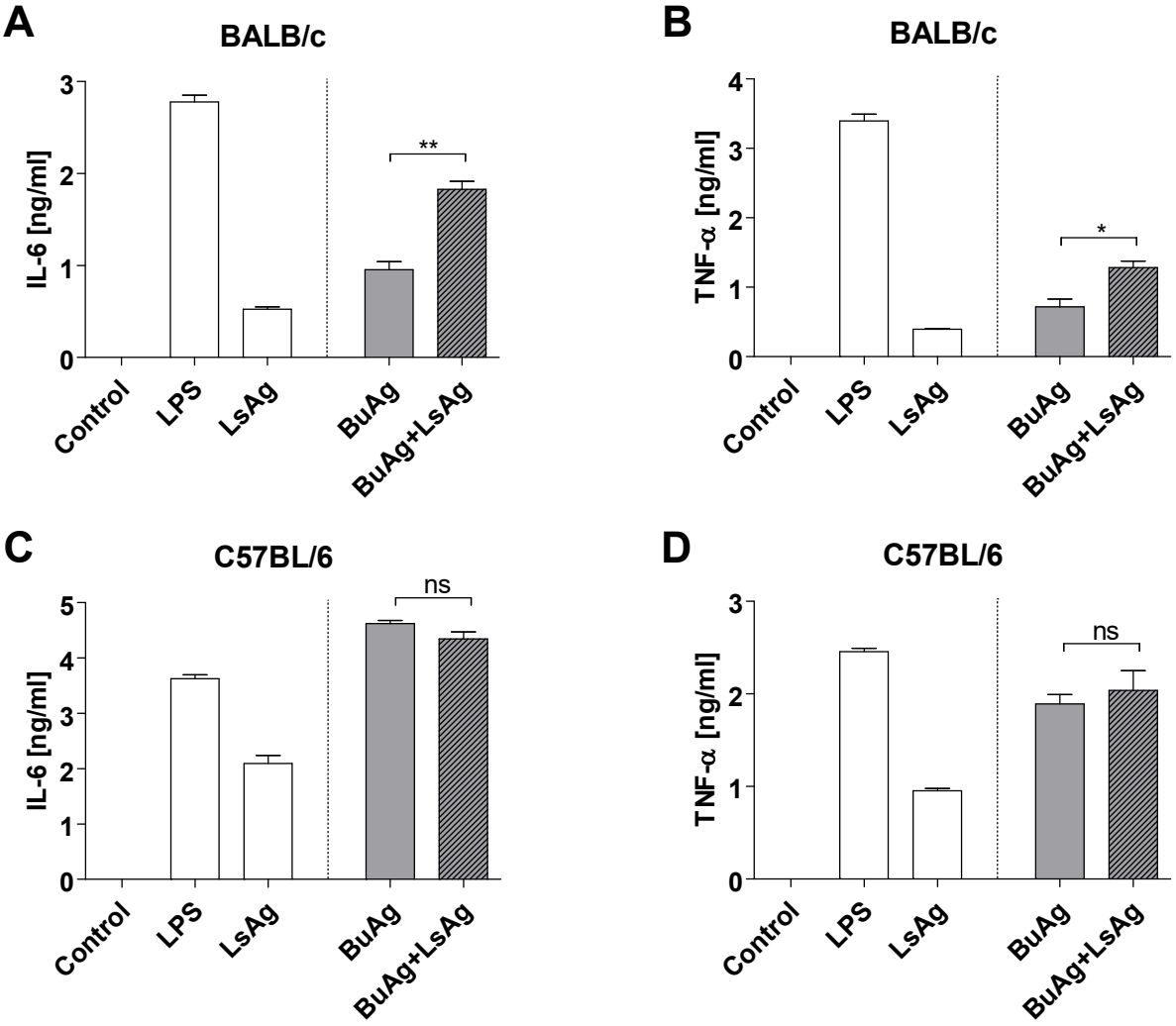

Fig. 9: BuAg response in BMDC appears not to be modulated by *Litomosoides sigmodontis* extract (LsAg).

BMDC ($1x10^5$ cells/ well) from BALB/c (A, B) and C57BL/6 (C, D) mice were left unstimulated (control) or stimulated with LPS (5ng/ml), LsAg (Ls, 50µg/ml) and/or BuAg (5µg/ml) for 24h. LsAg was always added 2h before the other stimuli. Thereafter, levels of IL-6 (A, C) and TNF-α (B, D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) from 1 experiment per mouse strain (n= 1 BMDC assay from 1 BALB/c mouse and n=1 BMDC assay pooled from 2 C57BL/6 mice). Statistical significances between the groups indicated by the brackets were obtained after unpaired t-test (ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$).

there appeared to be significantly elevated cytokine release most prominently seen with IL-6 (Figure 9A). Titration experiments with varying concentrations of BuAg and LsAg investigated the concentration dependency of such effects (Fig. 10).

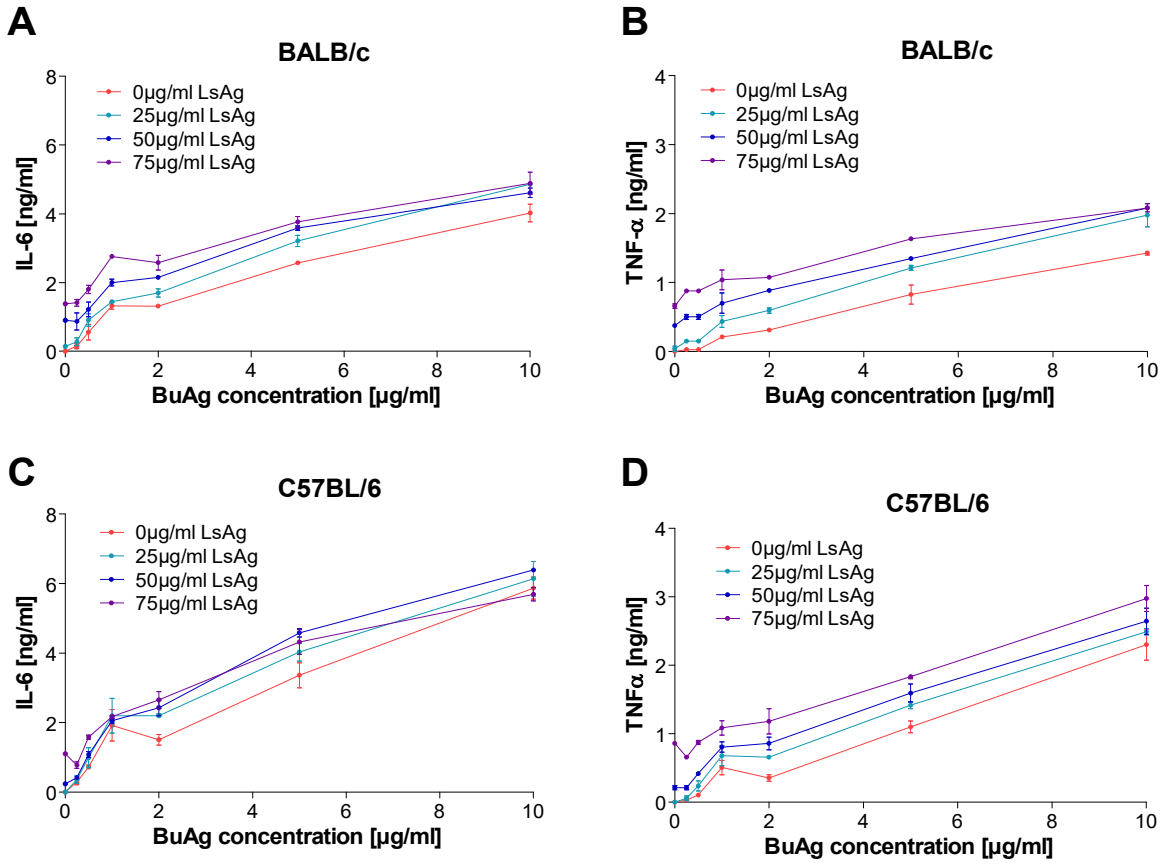

Fig. 10: Box-titration of BuAg and LsAg in BMDC: Concentration-independent additive effects.

BMDC ($5x10^4$ cells/ well) from BALB/c (A, B) and C57BL/6 (C, D) mice were stimulated with different concentrations of BuAg and LsAg for 24h. LsAg was always added 2h before the other stimuli. Thereafter, levels of IL-6 (A, C) and TNF-α (B, D) were measured in the culture supernatants via ELISA. Graphs show cytokine release (mean + SEM) from 1 experiment per mouse strain (n=1 BMDC assay per mouse strain, each pooled from 2 mice per group).

BMDCs of BALB/c and C57BL/6 mice were cultured with varying concentrations of LsAg for two hours, before being stimulated with varying concentrations of BuAg for a further 24 hours and the release of IL-6 and TNF-α was measured in the supernatant via ELISA. The release of proinflammatory cytokines increased steadily with increasing amounts of LsAg and BuAg, most clearly seen with the TNF-α production in BMDCs of C57BL/6 mice (Figure 10 D). The release of IL-6 in BMDCs of BALB/c and C57BL/6 mice (Figure 10 A, C) showed some variations with the higher LsAg amounts of 50 and 75µg/ml leading to a crossing of the graphs, nevertheless even then, the release of IL-6 increased with increasing concentrations of BuAg. The findings underline that an

additive effect was preserved, independent of the concentration. Further experiments were conducted using adult live worms as filarial stimulant instead of the antigen derived thereof. This is essential for understanding how host immunity is manipulated during infection, since excretory-secretory material and cloaking effects that are present *in-vivo* are potentially different than the composition of crushed worm extracts. For these experiments, similar to the ones described above (Fig. 9 and Fig. 10), additional cultures of BMDCs were pre-stimulated for two hours with one male and one female live worm, taken directly from the thoracic cavity of *L. sigmodontis*-infected BALB/c mice before adding the other stimuli. The proinflammatory cytokine release of IL-6 and TNF-α was measured in the supernatant of the cultures after 48 hours (Figure 11).

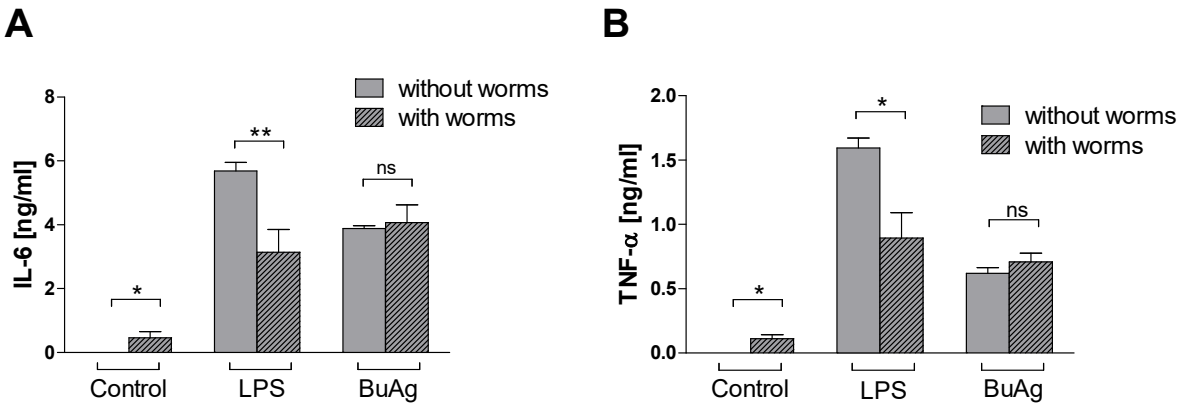

Fig. 11: BuAg response from BMDC is not modulated by the presence of live worms while LPS is.

BMDC (3x10^5 cells/ ml) from BALB/c mice were left unstimulated (control) or co-cultured for 2 hours with one male and one female worm gained from the pleural cavity of *Litomosoides sigmodontis* infected BALB/c mice. The cells were then stimulated with BuAg (5µg/ml) and LPS (5ng/ml) for 48h. Thereafter, levels of IL-6 (A) and TNF-α (B) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed BMDC preparations from within 2 independent experiments (n=3 mice). Statistical significances between the groups indicated by the brackets were obtained after Mann-Whitney test (ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$).

Similar to stimulation of BMDC with LsAg, stimulation with live worms also lead to a release of IL-6 and TNF-α. While ELISA results show that cytokine release of BMDC could not be modulated by live worms, showing a similar additive effect as with LsAg with a non-significant difference between the groups, cytokine responses to the positive control LPS of both IL-6 and TNF-α were significantly reduced by the presence of live worms.

3.2.2 Effects of LsAg on BuAg response in spleen cells

The modulatory effects of LsAg on cytokine responses released by spleen cells activated with BuAg (section 3.1.1) was also investigated. As reported before, additional cultures of spleen cells from BALB/c (Fig. 12) and C57BL/6 mice (Fig. 13) were pre-stimulated with LsAg for two hours, and the cytokine release upon exposure to BuAg was compared with the cytokine releases of cultures without LsAg after 48 to 72 hours.

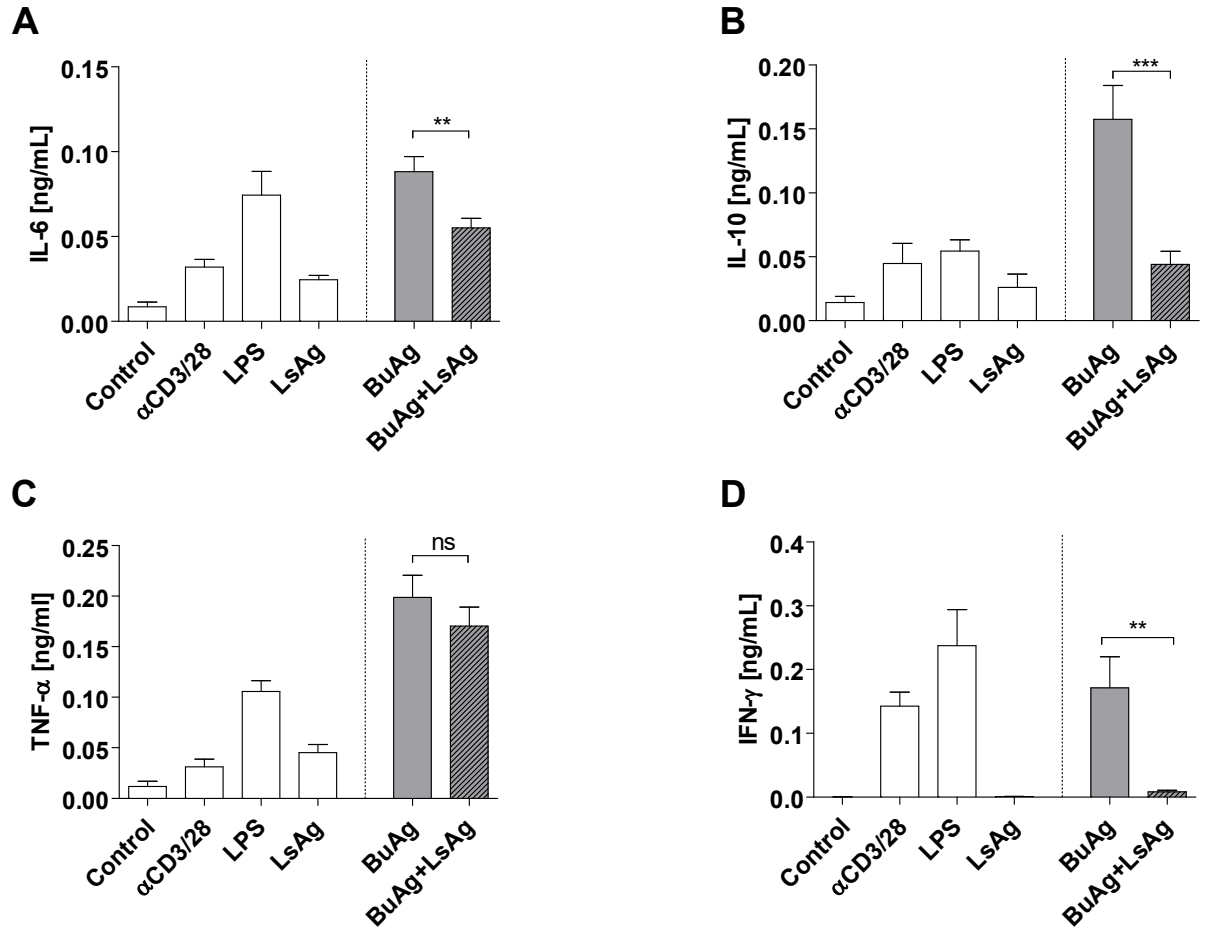

Fig. 12: BuAg response from spleen cells of BALB/c mice is reduced by LsAg.

Erythrocyte-depleted spleen cells ($5x10^5$ cells/ well) of BALB/c mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml), LsAg (50µg/ml) and/or BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). LsAg was always added 2h before the other stimuli. Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 3 independent experiments (n=9 mice). Statistical significances between the groups indicated by the brackets were obtained after Mann-Whitney test (ns= $p>0.05$, **= $p<0.01$, ***= $p<0.001$).

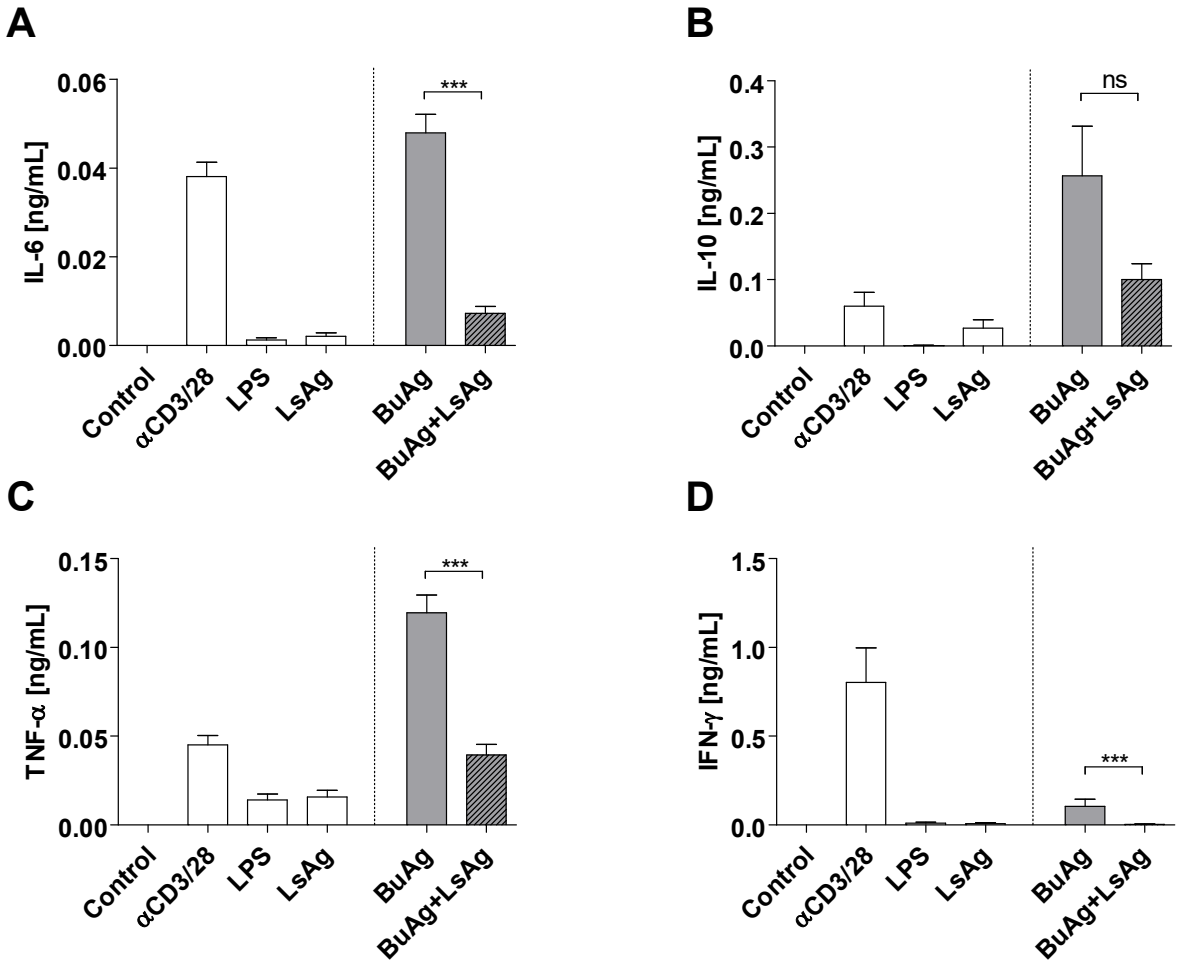

Fig. 13: BuAg response from spleen cells of C57BL/6 mice is reduced by LsAg.

Erythrocyte-depleted spleen cells ($5x10^5$ cells/ well) from C57BL/6 mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml), LsAg (50µg/ml) and/or BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). LsAg was always added 2h before the other stimuli. Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 2 independent experiments (n=6 mice). Statistical significances between the groups indicated by the brackets were obtained after Mann Whitney test (ns= $p>0.05$, ***= $p<0.001$).

As seen before in the experiments performed with BMDCs (Figure 9 and Figure 10), LsAg also lead to the release of IL-6 and TNFα (Figure 12 A, B), as well as the additionally measures IL-10 (Figure 12 C) in spleen cells of BALB/c mice, and to a lesser extend in spleen cells of C57BL/6 mice (Figure 13 A-C). The production of IFN-γ could not be induced by LsAg in spleen-cells of both mouse strains (Figure 12 D and Figure 13 D). As shown before (Figure 6 and Figure 7) BuAg was able to induce all measured

cytokines in BALB/c as wells as C57BL/6 mice. Interestingly in both mouse strains, the release of cytokines IL-6, IL-10, TNF-α and IFN-γ following stimulation with BuAg, was significantly lower in the presence of LsAg. Although following the same general trend, the release of TNF-α in spleen cell cultures of BALB/c mice (Fig. 12 C) and the release of IL-10 from cultures with C57BL/6 mice (Fig. 13B) were not significant.

3.2.3 Effects of LsAg on BuAg response elicited by CD4⁺ T-cells

As LsAg was shown to dampen cytokine release in bulk spleen cell cultures following exposure to BuAg, further experiments were conducted to investigate whether this modulation could also be observed in CD4⁺ T-cells. MACS separation isolated the CD4⁺ T-cells from the spleens of BALB/c mice (section 2.2.6.1). The purity of the MACS-sorted cells was assessed by staining of the isolated cells with the fluorochrome-conjugated antibody αCD4-APC and analysed using flow cytometry (section 2.2.9). The gating strategy depicted in Fig. 14 was employed, resulting in a purity of MACS-sorted CD4⁺ T cells of 93 %.

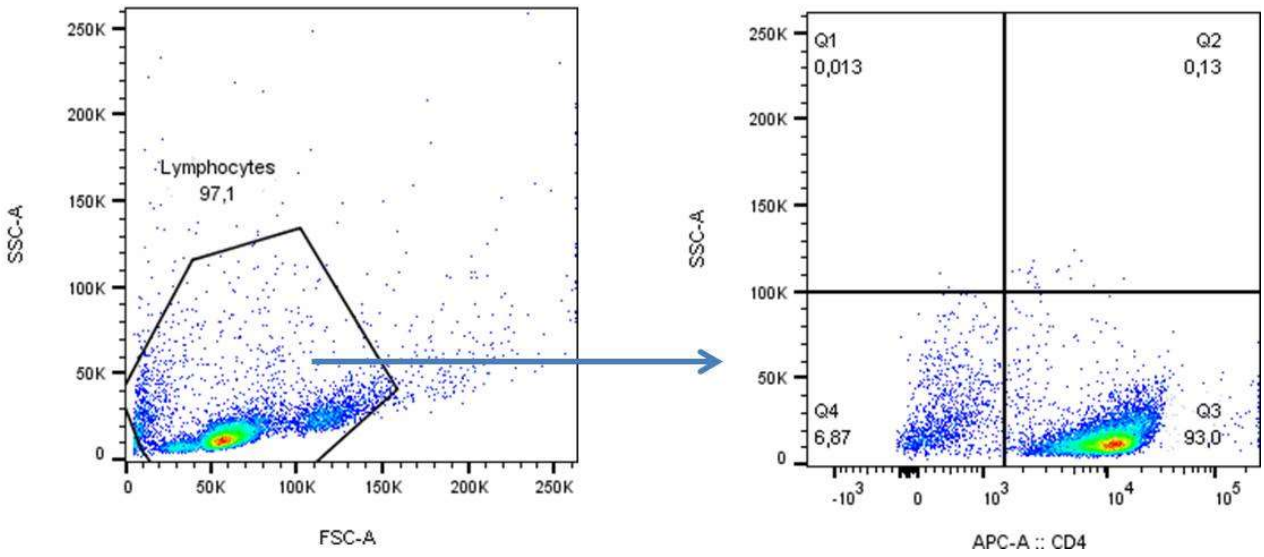

Fig. 14: Gating strategy for purity analysis of CD4⁺ T-cells

CD4⁺ T-cells, isolated by MACS separation and fluorescently stained with αCD4-APC antibodies, were analysed using flow cytometry and the gating strategy is depicted with the FlowJo software. Cell debris and dead cells were excluded from the analysis based on scatter signal. The resulting living lymphocyte population was gated for positive APC signal (Q3).

Isolated T cells were co-cultured with BMDC generated from BALB/c mice in the presence of αCD3/CD28, BuAg with and without pre-stimulation for 2 hours with LsAg as described in section 2.2.7.4. The resulting supernatant was monitored for production of IL-6 and TNF-α (48 hours) or IL-10 and IFN-γ (72 hours) and profiles were compared between cultures with and without LsAg to determine its immunomodulatory capacity on BuAg-activated T-cell responses. As shown in Fig. 15, immune responses upon

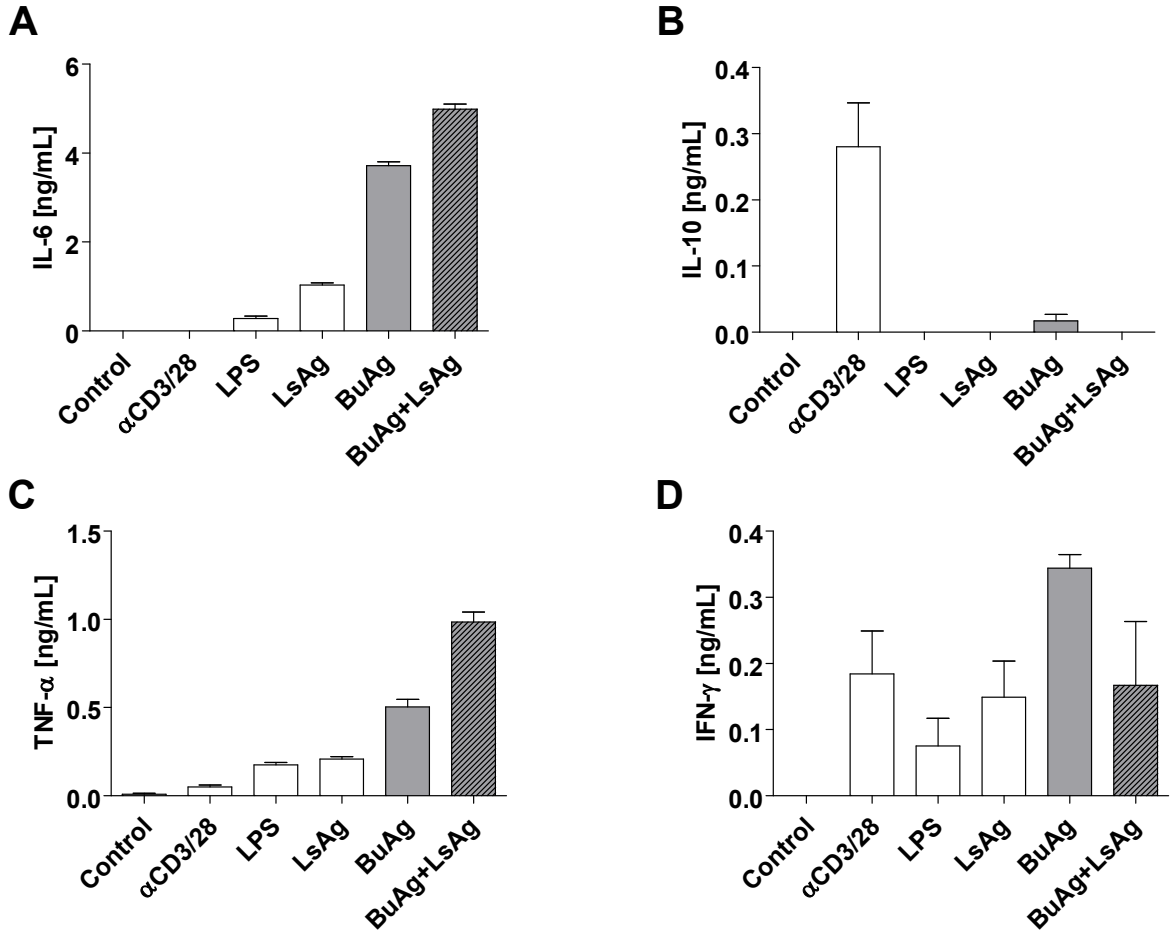

Fig. 15: In T-cells of BALB/c mice only IFN-γ response to BuAg appears to be reduced by LsAg.

BMDCs ($5x10^4$ cells/ well) from BALB/c mice were co-cultured with MACS-sorted $CD4^+$ T cells (2x10 cells/ well) from BALB/c mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml), LsAg (50µg/ml) and/or BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). LsAg was always added 2h before the other stimuli. Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of one experiment using isolated $CD4^+$ T-cells pooled from three mice.

stimulation with BuAg were not modulated by LsAg, although this finding cannot be statistically supported due to the small sample size. Nevertheless, IL-6 and TNF-α production was high upon stimulation of BuAg and showed the same additive effect of BuAg and LsAg as described above in BMDCs (section 3.2.1). The production of IL-10 was very low, while that of IFN-γ was highly variable. This data, even though preliminary, indicates that the immunomodulatory capacity of LsAg on BuAg-elicited responses in spleen cells does not appear to be T-cell-derived.

3.2.4 Isolation of monocytes

Monocytes of *L. sigmodontis*-infected BALB/c mice were isolated from spleen, mediastinal lymphnodes (mLN), pleura wash (PW) and peritoneal lavage (PL), prepared as described in section 2.2.4.2, 2.2.4.3, 2.2.4.4 and 2.2.4.5 respectively, using the Miltenyi Biotec MACS® separation system with the mouse Monocyte isolation kit (section 2.2.6.2). Isolated cells were counted and tested for purity with flow cytometry (section 2.2.9). Isolation from spleen and mLN bulk cells resulted in too small amounts to count. Isolation of monocytes from PW and PL was successful but resulted in purity of only 65-75 % (gating strategy not shown) due to small cell numbers. Therefore, no functional experiments could be performed using monocytes in this setting.

3.3 Modulation of BuAg responses by infection with *Litomosoides sigmodontis ex-vivo*

As shown in section 3.2.2, immune responses to BuAg in spleen cells could be modulated by LsAg. To investigate whether this effect can be replicated in immune cells of mice infected with *L. sigmodontis ex-vivo*, further experiments compared immune responses of cells from naïve and infected mice in different cell populations.

3.3.1 Effects of *L. sigmodontis* infection on BuAg response in spleen cells

Since there was a clear modulatory effect of LsAg on BuAg provoked responses in spleen cells could be demonstrated, the effect of *L. sigmodontis*-infection on these cells *ex-vivo* was investigated. This effect was also analysed depending on the microfilarial status of the infected mice. Furthermore, immune responses from the spleen cells of infected BALB/c as well as infected C57BL/6 mice were compared. This is because

BALB/c mice are known to be a TH2-dominant mouse strain and present a chronic infection with *L. sigmodontis*, whilst C57BL/6 mice, are a TH1-dominant mouse strain, and can only be used as a model for early-stage Ls infection since worms are eliminated shortly after on the development of the adult stage (section 1.2.2).

Bulk spleen cells from *L. sigmodontis* infected mice were prepared and stimulated as described in section 2.2.4.2 and 2.2.7.3 and elicited immune responses were compared to naïve controls as depicted in Figure 16.

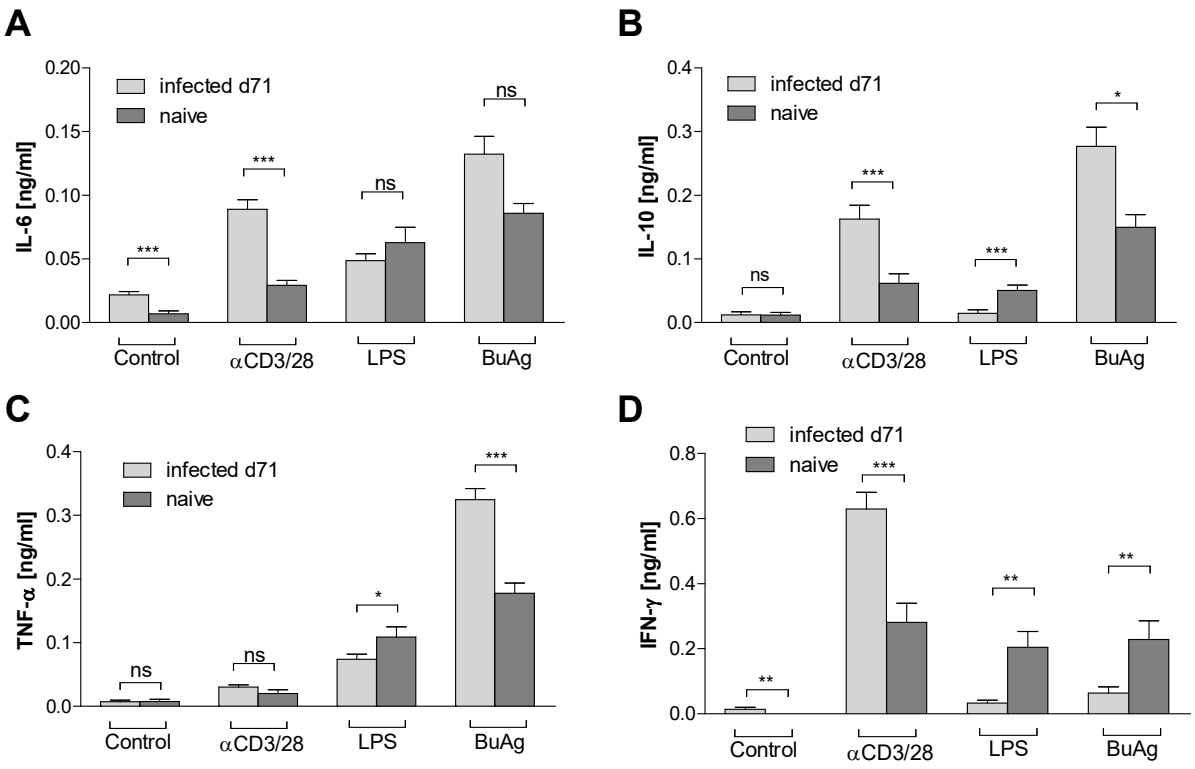

Fig. 16: Reduced IFN-γ response from spleen cells of *L. sigmodontis* -infected compared to naïve BALB/c mice to stimulation with BuAg.

Wild-type BALB/c mice were naturally infected with *Litomosoides sigmodontis*. On day 71 post-infection, erythrocyte-depleted spleen cells of infected mice, as well as those from naïve mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml) and BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 4 independent experiments (n=18 infected mice and n=9 naïve mice). Statistical significances between the groups indicated by the brackets were obtained after Mann-Whitney test (ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$).

In assays from BALB/c mice, IL-6, IL-10 and TNF-α production upon exposure to BuAg was higher in *L. sigmodontis*-infected mice when compared to naïve mice, but this effect was only significant for IL-10 and TNF-α (Fig. 16 A-C). Levels of IFN-γ however, were significantly lower in the infected group (Fig. 16 D). All unstimulated controls showed a low-level cytokine release with higher IL-6 and IFN-γ levels in cells from infected compared to naïve mice (Figure 16 A, D). Interestingly, stimulation with αCD3/CD28 showed higher release of all cytokines in infected mice compared to naïve, while LPS showed the contrary effect with higher release of all cytokines in the naïve group. These effects were significant except for IL-6 release upon LPS-stimulation (Fig. 16 A) and TNF-α upon αCD3/CD28 stimulation (Fig. 16 C). Patency of worms during filarial infection is known to influence immune responses in infected patients, with stronger immune-suppression in Mf+ individuals compared to Mf- individuals (Arndts et al., 2012). To investigate whether patency of the worms during a filarial infection had an impact on its effect on BuAg responses in spleen cells, mice were screened for micro-filarial (Mf) status before preparation of the spleen cells. The peak of Mf in peripheral blood in infected BALB/c mice is between days 70-72 post-infection (Rodrigo et al., 2016). Dividing up the spleen-cell responses of microfilaremic (Mf+) and non-microfilaremic BALB/c mice as depicted in Fig. 17, appears to lead to a graduation of the cytokine responses upon BuAg stimulation. Indeed, comparing the results from cells of Mf+ and naïve mice upon stimulation with BuAg, the release of IL-6, IL-10 and TNF-α was significantly higher from cells of Mf+ mice (Figure 17 A-C) whereas the reverse was seen for IFN-γ (Figure 17 D). The stimulation of spleen cells of Mf- mice showed that cytokine profiles were comparable to the naïve group (Figure 17 A, B) or diverging from these to a lesser extent than the cytokine profiles of spleen cells of Mf+ mice (Figure 17 C, D). No clear differences in cytokine responses upon stimulation with the control stimuli LPS and αCD3/CD28 were apparent for differentiating between Mf+ and Mf- mice (data not shown).

Stimulation of spleen cells from C57BL/6 mice with BuAg as depicted in Figure 18 resulted in significantly more IL-6 and IL-10 released from cells of infected mice when compared to cultures from naïve mice (Fig. 18 A, B).

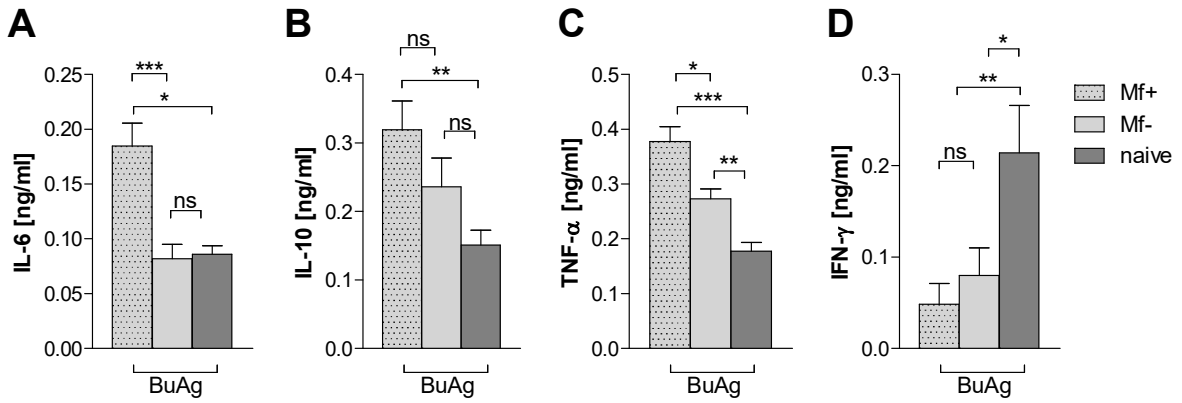

Fig. 17: Modulation of BuAg-elicited responses from spleen cells of BALB/c mice following *L. sigmodontis* infection is associated with the presence of peripheral microfilaria.

Wild-type BALB/c mice were naturally infected with *Litomosoides sigmodontis*. On day 71 post-infection, mice were screened for the presence of peripheral microfilaria (Mf) and erythrocyte-depleted spleen cells from Mf+ and Mf- infected, as well as naïve mice were left either unstimulated or stimulated with BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 4 independent experiments (n=18 infected mice with n=9 Mf+ and n=9 Mf- mice, and n=9 naïve mice). Statistical significances between the groups indicated by the brackets were obtained after Kruskal-Wallis followed by Dunn's post hoc test (ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$).

However, the TNF-α and IFN-γ levels were significantly lower in the infected group when compared to cultures from naïve mice (Fig. 18 C, D). As in cells of BALB/c mice, all unstimulated controls of cells from infected mice showed a low-level cytokine release with a significantly higher release of IL-6 and IL-10 compared to cells from naïve mice (Figure 18 A, B). The above-described effect of elevated cytokine levels after stimulation with αCD3/CD28 and lower cytokine levels after Here, stimulation with αCD3/CD28 as well as with LPS showed significantly higher IL-6, and IL10 (Figure 18 A, B) levels in the infected group. The results for TNF-α and IFN-γ were non-significant (Figure 18 C, D). In cultures from both BALB/c and C57BL/6 mice, re-stimulation of the spleen cells with LsAg, lead to the release of high levels of all measured cytokines (data not shown).

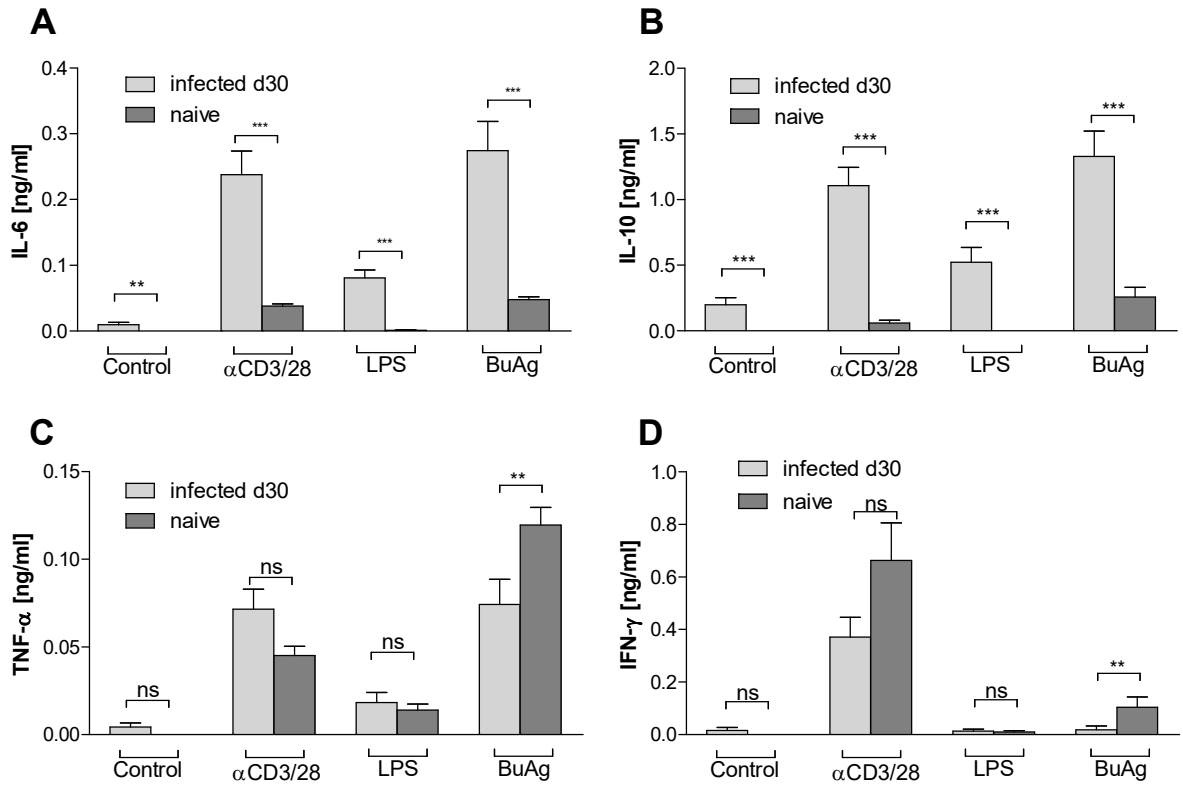

Fig. 18: Reduced IFN-γ and TNF-α response from spleen cells of *L. sigmodontis* - infected compared to naïve C57BL/6 mice to stimulation with BuAg.

Wild-type C57BL/6 mice were naturally infected with *Litomosoides sigmodontis*. On day 30 post-infection, erythrocyte-depleted spleen cells of infected, as well as naïve C57BL/6 mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml) and BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) of individually assessed mice from 2 independent infection experiments (n=6 infected and n=6 naïve mice). Statistical significances between the groups indicated by the brackets were obtained after Mann-Whitney test (ns= $p>0.05$, **= $p<0.01$, ***= $p<0.001$).

3.3.2 Effects of *L. sigmodontis* infection on BuAg response in cells of mediastinal lymph nodes

Mediastinal lymph nodes (mLN) are the draining lymph nodes of the thoracic cavity which is site of infection for *L. sigmodontis* in rodents, and thus a directly involved in developing adaptive immune responses. To investigate the effect of *L. sigmodontis* infection on BuAg induced responses in bulk mLN-cells, mLN from naïve as well as

infected BALB/c mice were prepared and stimulated as described in section 2.2.4.4 and 2.2.7.3 and the elicited immune responses compared (Fig. 19 and Figure 20).

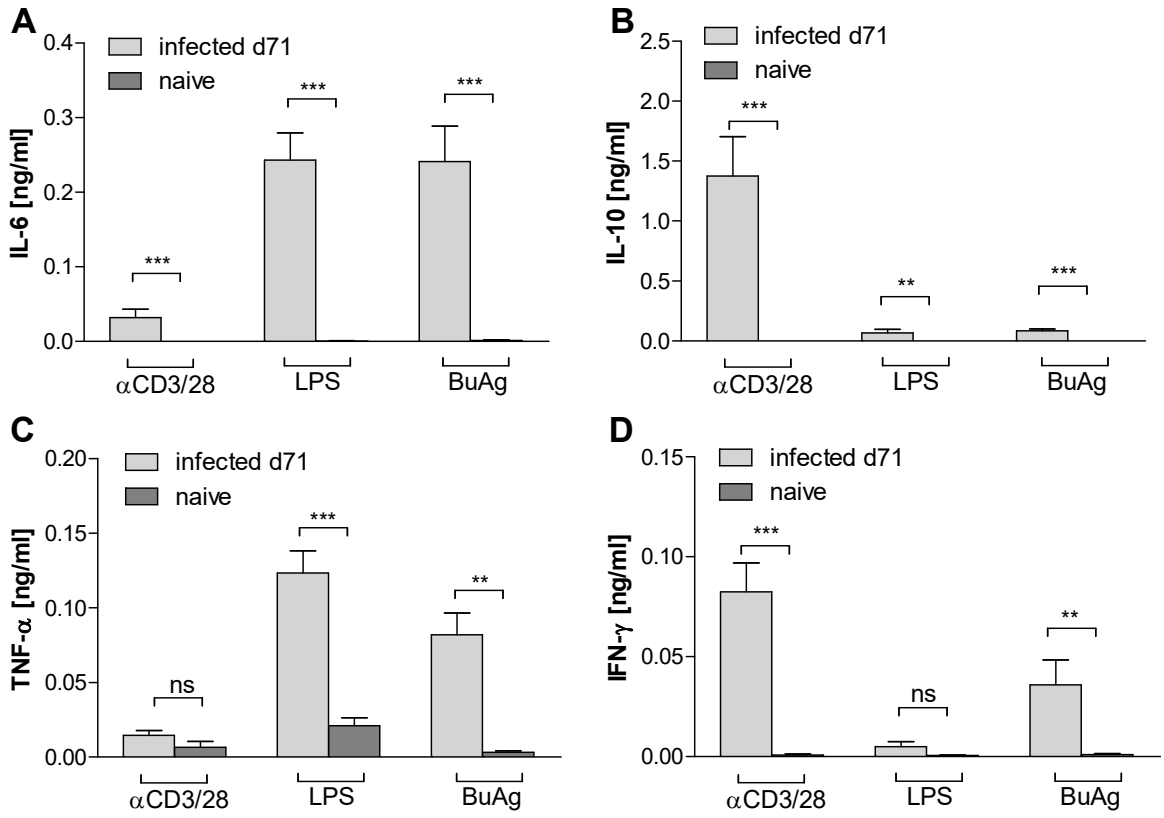

Fig. 19: Increased BuAg-induced cytokine responses in mediastinal lymph node cells of *L. sigmodontis*-infected mice.

Wild-type BALB/c mice were naturally infected with *Litomosoides sigmodontis*. On day 71 post-infection, erythrocyte-depleted cells from mediastinal lymph nodes of infected, as well as naïve BALB/c mice were left unstimulated (control) or stimulated with antiCD3/CD28 (5/1.25µg/ml), LPS (5ng/ml) or BuAg (5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM), following subtraction of levels in unstimulated controls, from pooled cells of infected mice (n=6 cultures with 2-5 mice per culture) from three independent infection experiments. Pooled cells from naïve mice (6-8 mice per culture) were used as controls. Statistical significances between the groups indicated by the brackets were obtained after Mann-Whitney test (ns= $p>0.05$, **= $p<0.01$, ***= $p<0.001$)

No response could be detected in cells from naïve mice by any of the applied stimuli except for TNF-α, and these were extremely low (Figure 19 C). In cultures using mLN-derived cells from *L. sigmodontis*-infected mice however, stimulation with BuAg elicited strong immune responses, with significantly higher levels released from all measured

parameters when compared to cultures from naïve mice. However, IL-10 release in mLN cultures from infected mice was exceptionally low compared to control stimulation with αCD3/CD28 (Fig. 19 B). To investigate the effect of LsAg on BuAg-induced responses in mLN-derived cells of *L. sigmodontis*-infected mice, mLN cultures were not only stimulated with BuAg but also re-stimulated with LsAg. As shown in Figure 20, both LsAg and BuAg were able to induce release of all measured cytokines. High levels of IL-10 could be induced after re-stimulation with LsAg (Fig. 20 B). Interestingly, re-stimulation of mLN-cells of *L. sigmodontis*-infected mice with LsAg as well as BuAg, lead to a decrease of BuAg-induced IL-6, TNF-α and IFN-γ production, even though this effect was not significant (Fig. 20 A, C, D).

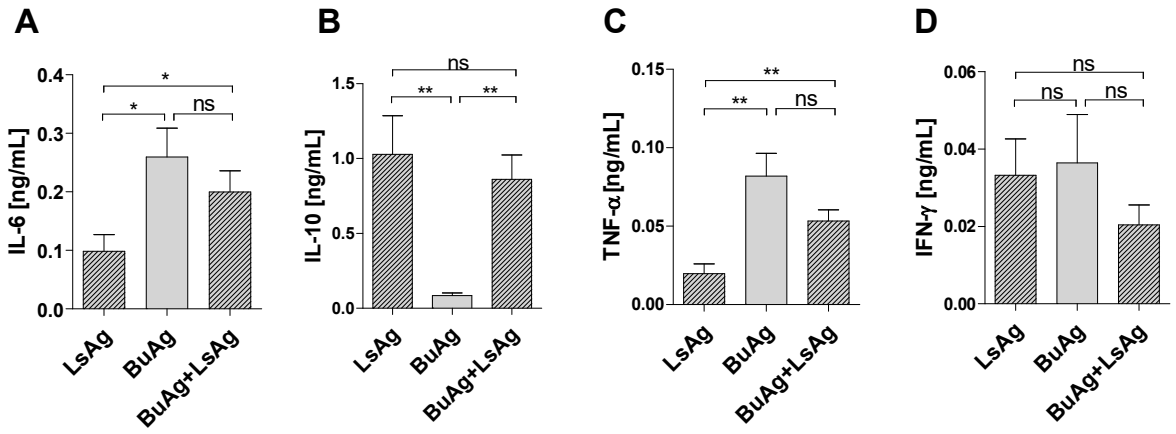

Fig. 20: Modulatory effects of LsAg on BuAg response in mediastinal lymph node cells following *L. sigmodontis* infection.

Wild-type BALB/c mice were naturally infected with *Litomosoides sigmodontis*. On day 71 post-infection, erythrocyte-depleted cells from mediastinal lymph nodes of infected BALB/c mice were left unstimulated (control) or stimulated with either LsAg (Ls, 50µg/ml) and/or BuAg (Bu5, 5µg/ml) for 48h (IL-6 and TNF-α) and 72h (IL-10 and IFN-γ). LsAg was added 2h before the BuAg. Thereafter, levels of IL-6 (A), IL-10 (B), TNF-α (C) and IFN-γ (D) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM), following subtraction of levels in unstimulated controls, from pooled cells of infected mice (n=6 cultures with 2-5 mice per culture) from three independent infection experiments. Statistical significances between the groups indicated by the brackets were obtained after Kruskal-Wallis followed by Dunn's post hoc test (ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$).

3.3.3 Effects of *L. sigmodontis* infection on BuAg response in pleura wash cells

Additional bulk cell assays used cells taken directly from the thoracic cavity, which is the site of filarial infection, and where cells had been in direct contact with worms and filarial

products. Cells were collected from the pleural wash (PW) of naïve and *L. sigmodontis*-infected mice, prepared and stimulated as described in sections 2.2.4.3 and 2.2.7.3 and elicited immune responses were compared (Fig. 21).

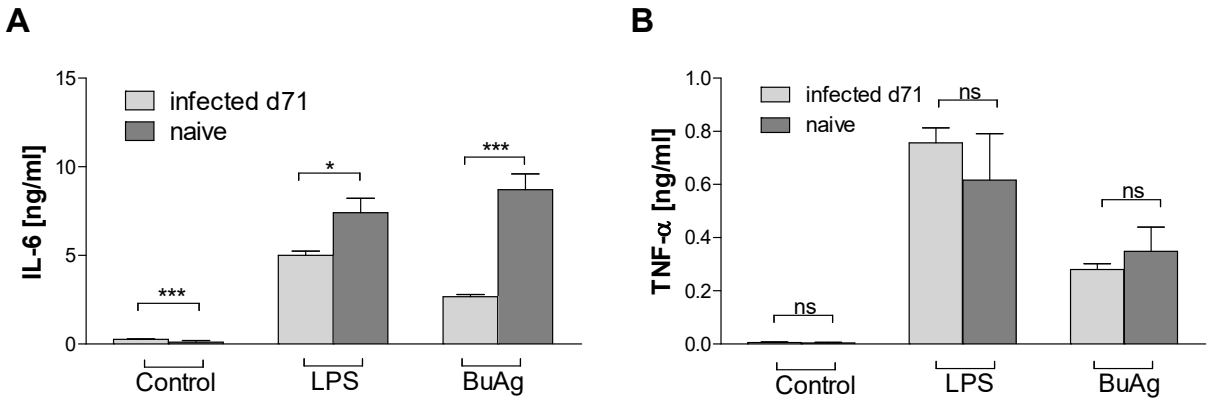

Fig. 21: Reduced IL-6 response from pleura wash cells of *L. sigmodontis*-infected compared to naïve mice to stimulation with BuAg.

Wild-type BALB/c mice were naturally infected with *Litomosoides sigmodontis*. On day 71 post-infection, cells from the pleural cavity of infected, as well as naïve BALB/c mice were left unstimulated (control) or activated with either LPS (5ng/ml) and BuAg (5µg/ml) for 48h. Thereafter, levels of IL-6 (A) and TNF-α (B) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) from individually infected mice (n=28) from five independent infection experiments. Pooled cells from naïve mice (3-6 mice per culture) were used as controls. Statistical significances between the groups indicated by the brackets were obtained after Mann-Whitney test (ns= $p>0.05$, *= $p<0.05$, ***= $p<0.001$).

The release of IL-6 upon stimulation with both LPS and BuAg was significantly lower in pleura wash cells of infected than in that of naïve BALB/c mice (Fig. 21 A). The effects on TNF-α production were non-significant (Fig. 21 B). As shown is section 3.3.1, patency of the worms during *L. sigmodontis* infection has a significant impact on how spleen cells from infected mice respond to BuAg. Moreover, it was revealed that the effects of *L. sigmodontis* infection on BuAg responses were strongest when comparing mice with patent (Mf+) infection and those uninfected. Therefore, data sets were further subdivided to show cytokine responses between PW cells from infected mice (Mf+ and Mf-) and naïve, which are depicted in Fig. 22.

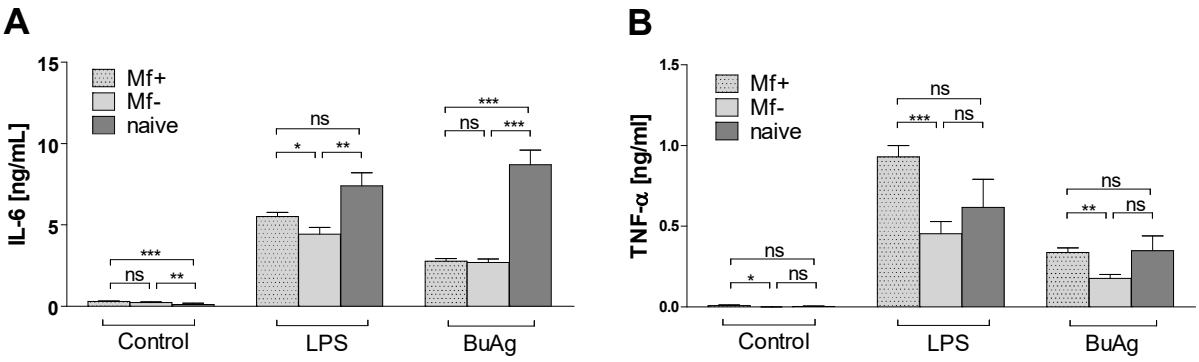

Fig. 22: Absence of peripheral microfilaria is associated with a reduced TNF-α responses by pleural wash cells from *L. sigmodontis*-infected mice upon stimulation with BuAg.

Wild-type BALB/c mice were naturally infected with *Litomosoides sigmodontis*. On day 71 post-infection, mice were screened for the presence of peripheral microfilaria (Mf) and cells from the pleural cavity of Mf+ and Mf- infected, as well as naïve BALB/c mice were left unstimulated (control) or activated with LPS (5ng/ml) or BuAg (5µg/ml) for 48h. Thereafter, levels of IL-6 (A) and TNF-α (B) were measured in the culture supernatants via ELISA. Bars show cytokine release (mean + SEM) from individually infected mice (n=19 Mf+ mice and n=9 Mf- mice) from five independent infection experiments. Pooled cells from naïve mice (3-6 mice per culture) were used as controls. Statistical significances between the groups indicated by the brackets were obtained after Kruskal-Wallis followed by Dunn's post hoc test (ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$).

IL-6 responses upon stimulation with BuAg showed no difference between Mf+ and Mf- mice, while stimulation with LPS elicited significantly lower cytokine release in Mf- compared to naive mice (Fig. 22 A). TNF-α release upon stimulation with both LPS and BuAg was also significantly lower in cells from Mf- compared to Mf+ mice (Fig. 22 B). These findings are comparable to the ones observed in spleen cells, where cells from Mf+ mice also showed a higher release of IL-6 and TNF-α upon stimulation with BuAg compared to cells from Mf- mice. However, in comparison to cytokine responses of pleura wash cells from naïve mice, cytokine release to BuAg was lower in cells from infected mice. This stands in contrast to the results reported from spleen cells, and the immune-modulatory effect of *L. sigmodontis* infection was increased in the absence instead of the presence of Mf.

4. Discussion

Buruli ulcer disease is a neglected tropical disease that affects at least 33 countries and can lead to disfigurement and disability in humans (World Health Organization, 2019c). Until now it remains unclear why some people develop the disease, while others appear to be immune, and why in some patients the disease heals spontaneously, while it progresses to severe disfiguring forms in others (Yotsu et al., 2018). Helminth infections are co-endemic in areas of *M. ulcerans* and the former are renowned for their ability to modulate the host's immune system (Maizels and McSorley, 2016). Data obtained in the last few years show an impact of helminth infections on infection with other pathogens, including other mycobacteria such as *Mycobacterium tuberculosis* (Mabbott, 2018).

So far, very few studies have been conducted on co-infections with helminths and *M. ulcerans*. Similar to infections with *M. tuberculosis*, effective clearance of *M. ulcerans* is also attributed to a strong TH1 response, which is hindered by helminth infection (Gooding et al., 2002; Rajamanickam et al., 2017). However, so far epidemiological studies have remained inconclusive as to the enhanced susceptibility to *M. ulcerans* infections by helminth co-infection (Phillips et al., 2014; Stienstra et al., 2004).

The results of this thesis present the first systematic analysis of the immunomodulatory capacity of filaria on *M. ulcerans*-induced immune responses.

In the first part of this thesis immune responses of different immune cells upon direct stimulation with the *M. ulcerans* stimulation extract (BuAg) were established and its signalling pathway analysed. In the second part, the immune-modulation of the established immune responses by the *L. sigmodontis* stimulation extract (LsAg) was investigated. Finally, the third part analysed the effect of live infection with *L. sigmodontis* on BuAg-elicited responses in different immune cells *ex-vivo*.

4.1 Immune responses and signalling of BuAg-activated cells

Deciphering immune responses elicited by Buruli ulcers disease is a key step in understanding the disease. Data of previous studies, acquired of both patients as well as murine models, show activation of the innate immune system at the site of infection

(Peduzzi et al., 2007; Phillips et al., 2006b), followed by a mixed TH1 and TH2 immune response with a later dominance of TH1 response (Phillips et al., 2006a).

A TH1-based immune response (and especially the release of IFN-γ) appears to be crucial in preventing and eliminating the disease, and has been shown to be down-regulated by the bacterium in early stages allowing it to spread (Bieri et al., 2016; Torrado et al., 2010; Zavattaro et al., 2010). Histological, microbiological and immunological differences in different stages of Buruli ulcers disease pose a challenge in understanding elicited immune responses. Murine models and the use of cell-stimulation extracts derived of *M. ulcerans* provide an experimental platform to further investigate under controlled conditions the cytokine profiles and the signalling elicited by Buruli ulcers disease. For the experiments performed in this thesis, cytokine responses upon stimulation with *M. ulcerans*-derived cell stimulation extract (BuAg) were measured in BALB/c and C57BL/6 mice, as the development of the parasite and associated immune responses are quite distinct in these two mouse strains: BALB/c allow full infection and elicit strong TH2-dominant response whereas TH1 responses occur in C57BL/6 mice which eliminate worms following adulthood (Watanabe et al., 2004). Both mouse strains have been shown to be susceptible to infection with *M. ulcerans* (Marion et al., 2016). Furthermore BALB/c and C57BlL6 mice present two different murine infection models of filarial infection with *L. sigmodontis* (Hoffmann et al., 2000), required for the experiments conducted in the frame of this thesis.

4.1.1 BuAg elicits concentration-dependent cytokine responses

As mentioned above, to date no in-depth analysis exists of immune responses upon stimulation with BuAg in a murine model. DCs are key initiators and regulators of immune responses, and they have been shown to be activated by infection with *M. ulcerans* in tissue samples, leading to production of pro-inflammatory cytokines such as IL-6 and TNF-α (Peduzzi et al., 2007). This cytokine production in dendritic cells has been shown to be only marginally affected by mycolactone (Coutanceau et al., 2007). In the present study the effect of mycolactone has not been specifically analysed. However, mycolactone is known modulator of immune responses (Adusumilli et al., 2005; Demangel and High, 2018) and should be considered for follow up studies. The results presented in this thesis, analysing cytokine responses in BMDC also showed the

release of pro-inflammatory cytokines. It was shown, that stimulation of BMDC of BALB/c and C57BL/6 mice with BuAg lead to a concentration-dependent release of IL-6 and TNF-α (section 3.1.1).

Further experiments in this thesis analysed cytokine responses in bulk spleen cells, that provide a broader impression of elicited immune responses by BuAg in mixed cell population. *M. ulcerans* is known to induce both TH1 and TH2 responses (Gooding et al., 2002; Phillips et al., 2006a; Zavattaro et al., 2010) and indeed, stimulation of bulk spleen cells with BuAg elicited the release of both TH1 (IFN-γ and TNF-α) and TH2 cytokines (IL-6 and IL.10) in a concentration-dependent manner (section 3.1.1). Interestingly, IFN-γ production was relatively low in bulk spleen cells of C57BL6 mice following BuAg stimulation. However, also in the literature, IFN-γ production during infection with *M. ulcerans* in patients showed strong variations depending on the stage or form of the disease (Kiszewski et al., 2006; Torrado et al., 2010; Zavattaro et al., 2010). Similar mechanisms could be responsible for the difference in BuAg-induced IFN-γ production in BALB/c and C57BL/6 mice and present an interesting avenue for further investigation.

IL-5 production upon stimulation with BuAg could not be detected in experiments conducted for this thesis (data not shown), nor in patients infected with *M. ulcerans* in the literature (Phillips et al., 2006b; Zavattaro et al., 2010). These findings suggest that *in-vitro* stimulation of murine BALB/c and C67BL/6 mouse cells with BuAg is a suitable model for analysing immune responses in Buruli ulcers disease.

4.1.2 BuAg cytokine responses are TLR2-dependent

Several studies investigated the role of TLR activation by mycobacteria, concentrating mostly on *M. tuberculosis* (Stenger and Modlin, 2002). It was shown that mycobacterial antigens such as the glycopeptide lipoarabinomannan, the dominant components of the cell wall of *M. tuberculosis*, interact with TLR2 (Means et al., 1999; Moreno et al., 1989; Underhill et al., 1999). Little is known on TLR-activation through *M. ulcerans* specifically, but Lee et al. (2009) showed that *M. ulcerans* activates keratinocytes in a TLR2-dependent manner. However, activation of innate pathways in murine BMDC upon exposure to *M. ulcerans* extract has not been shown to date.

The results presented in this thesis demonstrate that pro-inflammatory cytokine responses in BMDC of BALB/c and C57BL/6 mice upon stimulation with *M. ulcerans* are TLR2-dependent (section 3.1.2). In light of these findings, further down-stream signalling experiments via western blot analysis were conducted. Experiments could not be concluded due to time restrictions and thus no results are presented in this thesis. However, analysing downstream-signalling is likely to help understand immune responses in Buruli ulcers disease and should be further investigated. TLRs are a key component of the initiation of the immune system. They are pattern-recognition receptors and regulate activation of both innate and adaptive immunity (Takeda et al., 2003). Because of their central role in the activation of the immune system, modulation of TLRs is a way for pathogens to evade the immune system. In *M. ulcerans,* its immune-suppressive properties have mostly been attributed to mycolactone (Adusumilli et al., 2005; Coutanceau et al., 2007). However, studies so far could find no effect of mycolactone on the activation of signalling pathways, including the MAPK and NF-κB pathways, suggesting a posttranscriptional mechanism (Simmonds et al., 2009). Nevertheless, filarial parasites have been shown to modulate TLR expression, including TLR2, (Babu et al., 2005; Rodrigo et al., 2016; Venugopal et al., 2009), and could thus also have an impact on Buruli ulcers disease.

4.2 Modulation of BuAg response by filarial antigen

Filarial parasites are known to polarize the immune system of their host to survive. Infections with filaria are highly co-endemic with Buruli ulcers disease and co-infections have been reported (Phillips et al., 2014). However, very little is known about the effect of a co-infection with these two diseases. Immune-modulation by filaria has been shown to influence different arms of the immune system including APCs and lymphocytes (Ludwig-Portugall and Layland, 2012; Maizels and McSorley, 2016) that were chosen as the main cell population to investigate in this thesis. Filarial infections are known to show different courses of illness, from chronic stages with little or strong pathology to effective elimination of the disease. In the murine model for filarial infection with *L. sigmodontis*, BALB/c mice display a chronic stage of the infection with patency (Mf+) while C57BL/6 mice are more resistant to infection and capable of eliminating the worms (Hoffmann et al., 2000). Thus, in the experiments presented in this thesis, immune responses of cells

from both mouse strains, that had been shown to display different immune responses to stimulation with BuAg as described above, were analysed.

4.2.1 Filarial antigen does not modulate BuAg responses in bone-marrow-derived dendritic cells

Previous studies have shown that filarial parasites are able to dampen pro-inflammatory innate immune responses. Several studies have shown dampened LPS induced immune responses through filarial infection or filarial antigen in patients and murine models (Arndts et al., 2012; Gondorf et al., 2015; Panda et al., 2012). Experiments conducted in this thesis showed live worms were able to dampen LPS-induced pro-inflammatory cytokine release in BMDC, but neither LsAg nor live worms had an influence on immune responses elicited by BuAg-stimulation in BMDC from either mouse strain irrespective of the concentration (section 3.2.1). These findings indicate that the immunomodulatory capacity of filarial antigens influence pathways in BMDC activated by LPS but not by BuAg.

TLR2- and TLR4-dependent pathways appear to play a central role in immune-modulation of innate immune responses by filarial parasites. Both the TLR2 and the TLR4 activation status has been shown to be reduced in monocytes from filaria-infected patients compared to uninfected controls (Babu et al., 2005). Pro-inflammatory LPS-induced activation of murine macrophages and/or human monocytes is reportedly dampened by LsAg via TLR2 (Gondorf et al., 2015) and by filarial glycoprotein via TLR4 (Panda et al., 2012). Also in patients co-infected with filaria and *Mycobacterium tuberculosis*, the filarial infection diminished TLR2 (and TLR9) expression and pro-inflammatory cytokine responses to TLR2 ligands in PBMCs (Babu et al., 2009). TLR-signalling also plays a central role for the development of patent filarial infection (Rodrigo et al., 2016; Wiszniewsky et al., 2019) and could thus also influence immune responses. Further investigation of TLR signalling upon stimulation of BMDC with LPS and BuAg in the presence of filarial antigen could help understanding the discrepancy in innate immune-modulation.

4.2.2 LsAg dampens of BuAg-induced responses in bulk spleen cells

As described above, neither filarial antigen nor live worms were able to influence BuAg responses in BMDCs. However, further experiments conducted in this thesis revealed that LsAg was able to dampen cytokine responses of all measured cytokines elicited by BuAg in bulk spleen cells (section 3.2.2). This immune-modulation was seen in both TH1 (TNF-α and IFN-γ) and TH2 (IL-6, IL-10) cytokines from spleen cells of both mouse strains.

A low and non-significant modulation of the release of the TH1 cytokine TNF-α in TH2-dominant BALB/c mice and of the TH2 cytokine IL-10 in TH1-dominant C57BL/6 mice might be explained by the TH1/2-bias of these two mouse strains (Watanabe et al., 2004).

It is quite interesting that not only the production of pro-inflammatory cytokines was down-regulated by co-stimulation with LsAg, but also of the anti-inflammatory IL-10. Indeed levels of IL-10 together with TGF beta are usually high in chronic filarial infection (Doetze et al., 2000; Mahanty and Nutman, 1995). This indicates that the dampening effect of LsAg on BuAg-induced immune responses might not be IL-10 dependent but might rely on another mechanism.

These results conclusively demonstrate, that filarial antigens appear to have a broad dampening effect on immune responses elicited by *M. ulcerans* and could thus be of relevance for co-infections. The spleen, being a lymphoid organ combining innate and adaptive immune systems, comprises different kinds of immune cells, including lymphocytes and monocytes (Mebius and Kraal, 2005). Thus, bulk spleen cells are an ideal model to study immune responses in a broader cell population. However, it remains unclear which cell population within the bulk cell assay is responsible for the above shown effect since further studies with isolated $CD4^+$ T cells did not confirm the results from bulk cells assays as discussed in the next section.

4.2.3 Which cell type is responsible for LsAg-induced immune-modulation of BuAg- activated spleen cells?

The experiments presented here showed that LsAg but not BMDC affected BuAg responses in bulk spleen cells. Further experiments investigated the cell-populations within the bulk spleen cells leading to above-described effect.

The down-regulation of T-cell function is crucial for the immune suppression seen in chronic helminth infections. Especially $CD4^+$ T-cells play a vital role, including induction of Tregs and development of hypo-responsive effector T-cells (Babu et al., 2006; Gillan and Devaney, 2005; Taylor et al., 2005). Experiments with $CD4^+$ T-cells isolated from the spleen (section 3.2.3) did not show the same clear-cut effects as in spleen cells. Indeed, IL-6 and TNF-α production were higher when co-stimulated with LsAg and BuAg compared to BuAg alone. However, the presence of BMDC in the co-culture, needed to activate the $CD4^+$ T-cells in this experiment, could bias the revealed pro-inflammatory cytokine release, as IL-6 and TNF-α cytokine responses to BuAg have been shown to be strong and not modulated by LsAg in BMDC (section 3.2.1). Interestingly. IL-10 could not be induced in $CD4^+$ T-cells by stimulation with BuAg and/or LsAg. IL-10 plays a crucial role in preventing inflammatory and autoimmune pathologies and has been demonstrated to function at different stages of an immune response, including during helminth infection (Moore et al., 2001). It is widely known as a TH2-cytokine as well as the main cytokine produced by Treg cells (Fiorentino et al., 1989; Grazia Roncarolo et al., 2006). However, it was also shown to be produced by many other cells of the innate (including dendritic cells, macrophages, mast cells, natural killer cells, eosinophils and neutrophils) as well as adaptive immunity (including Th1, Th17, $CD8^+$ T-cells and B cells) (Saraiva and O'Garra, 2010). The fact that IL-10 could not be induced by neither LsAg nor BuAg in the $CD4^+$ T-cell assay of this study but very well in the bulk spleen cell assay, suggests that the cells responsible for secretion of IL-10 are either missing in this assay but are naturally present in bulk spleen cell assays, or that they were not sufficiently activated here. These cells might play a role in the demonstrated immune-suppression in bulk spleen cells. However, BuAg responses in the spleen appeared to be independent of IL-10 (section 4.2.2). IFN-γ release had a high variability but it appeared to show an Ls-mediated immunomodulatory tendency comparable to that seen in spleen cells and should be further investigated. Overall, data obtained in this study suggest that the observed Ls-mediated immune-modulatory effects were not on $CD4^+$ T-cells, but other cells such as monocytes and B-cells or a combination of different immune cells. Nevertheless, only data from one experiment could be included in this thesis and the experiment needs to be repeated to confirm these findings. These data are preliminary and remain partially inconclusive as to whether the immune-modulatory

capacity of LsAg on Bu responses in spleen cells stems from $CD4^+$ T-cells derived thereof.

Monocytes have been shown to play a role in coinfections with helminth and *M. tuberculosis* (Rajamanickam et al., 2020). In infections with *M. ulcerans,* high pathogenicity is associated with the production of mycolactone which is known to dampen monocyte functions (Pahlevan et al., 1999; Simmonds et al., 2009), indicating that monocytes might play a crucial role in Buruli ulcers disease. In further preliminary experiments conducted within this study monocytes were isolated from different tissues, including spleen. However, isolated cell numbers were very low (section 3.2.4) and thus may be of little relevance for Ls-mediated immune-modulatory effects in spleen cells. Nevertheless, results presented within this study are preliminary and further experiments with monocytes isolated from spleen cells pooled from different mice or using another method of monocyte isolation to reap higher cell numbers could clarify these findings and open further interesting studying opportunities.

The role of regulatory B-cells might also present an interesting research avenue as their immune-modulatory capacity during helminth infection has been shown to be both IL-10 dependent and independent (Ritter et al., 2018; Smits et al., 2010; Wilson et al., 2010).

4.3 Modulation of BuAg response by infection with *Litomosoides sigmodontis ex-vivo*

Following the above-described effects of co-stimulating naïve cells with BuAg and LsAg, additional experiments were conducted using cells derived from *L. sigmodontis* infected mice and were compared to naive controls. Infection with *L. sigmodontis* is an established murine model to study filariasis (Fulton et al., 2018; Hoffmann et al., 2000) and offers the opportunity to study the more complex effects of live infection, compared to the effect of a mere antigen extract on BuAg-induced immune cell responses.

A live infection model allows different compartments of immunity and immune cells to interact unrestricted with each other and the worms, secreted products and microfilariae for a longer period of time than is possible in *in-vitro* experiments. Furthermore, it allows for a more adequate study of adaptive immune responses in the delicate equilibrium of immune-modulation typical for chronic filarial infection. However, multiple factors often lead to less clear-cut results and it is to note that immune responses in the infection

model with *L. sigmodontis* are known to be susceptible to alteration such as the amount of transferred L3 larvae during infection (Babayan et al., 2005).

4.3.1 Infection with *L. sigmodontis* dampens BuAg-induced TH1 but not TH2 responses in spleen cells

Previous experiments in spleen cells showed dampened BuAg responses by co-stimulation with LsAg for all measured cytokines. Interestingly, comparing spleen cell cytokine responses of infected and naïve BALB/c mice revealed a significant down-modulation of BuAg induced cytokine responses by infection with *L. sigmodontis* only for IFN-γ. Indeed, IL-6, IL-10 and TNF-α production was higher in cultures from the infected group than the ones from naïve mice. The same experiments with C57BL/6 mice showed similar results with the exception of TNF-α which was down-modulated as well as IFN-γ (section 3.3.1).

Compromised T-cell responses, especially on IFN-γ responses to antigen challenge have also been shown to be typical for chronic filarial infections (Maizels and McSorley, 2016). Dampened IFN-γ production by filarial infection could have a huge impact on co-infections with Buruli ulcers disease as an appropriate TH1 response, and especially IFN-γ-response, has been shown to be crucial for early host immune defence against *M. ulcer*ans (Bieri et al., 2016; Schipper et al., 2007).

Why the BuAg induced TNF-α production was down-modulated only in cells derived from infected C57BL/6 mice and not BALB/c mice remains unclear and should be further investigated. Especially the investigation of the role of adaptive immunity might present an interesting study opportunity as it is crucial for the elimination of worms typical for infection of C57BL/6 mice (Layland et al., 2015).

It is also unclear why LsAg would down-modulate BuAg responses of all cytokines in naïve spleen cells while *L. sigmodontis*-infection only dampened IFN-γ and partially TNF-α responses of the cells. This effect might be specific to LsAg. However, the higher levels of IL-6, IL-10 (and TNF-α in cells derived of BALB/c mice) upon stimulation with BuAg could also be explained by an already activated immune system, primed by the infection itself. Induction of a TH2 polarized immune response by filarial infection is well established, and especially high levels of IL-10 are typical for chronic infection with

filarial parasites (Doetze et al., 2000; S Mahanty et al., 1996). Adaptive immune responses are slower and more complex than innate immune responses and short-time *in-vitro* stimulation with LsAg might not be sufficient to achieve the same complex equilibrium of adaptive immune responses seen in chronic infections. Control stimulation with TCR activating αCD3/CD28 antibodies in experiments in this thesis, revealed higher release of all measured cytokines in cells from infected compared to naïve mice, suggesting a higher number of activated T-cells. $CD4^+$T-cells have been shown to play a central role in parasite containment and release of TH2 cytokines during infection (Al-Qaoud et al., 1997) and Tregs that are the main source of IL-10 are responsible for maintaining tolerance and preventing severe pathology (Maizels and McSorley, 2016). A difference in T-cell activation by LsAg *in-vitro* or *L. sigmodontis* infection *ex-vivo* might well explain the difference of their immune-modulatory capacity on BuAg-induced responses. Whether this effect is IL-10 dependent remains unclear. Nevertheless, also additional presence of secreted products or microfilariae in *L. sigmodontis* infection could influence BuAg-induced responses.

Interestingly, LPS-induced cytokine release in BALB/c was dampened for all cytokines in cells from *L. sigmodontis* infected compared to naïve mice. However, the same effect could not be replicated in cells from C57BL/6 mice. Dampened LPS-induced responses by helminth infections has been shown several times in previous studies (Han et al., 2019; Kane et al., 2004; Summan et al., 2018). The fact that all measured LPS- but not all BuAg- induced responses were dampened by *L. sigmodontis* infection, suggests a difference in immunomodulatory pathways. Helminth infections have been shown to interfere both with expression and function of TLRs (Venugopal et al., 2009). In BALB/c mice the patency of *L. sigmodontis* during infection has been shown to lead to different immune-responses and to be TLR4-dependent and filarial-specific $CD4^+$ T-cell responses to be influenced by TLR2-signalling (Rodrigo et al., 2016). As LPS is a known agonist of TLR4 and BuAg was shown to signal via TLR2 (section 3.1.2), the different TLR activation might be a key difference to explain this discrepancy and should be further investigated.

4.3.2 IL-10 dependent down-modulation of BuAg-induced responses in mediastinal lymph nodes by filarial infection

Lymph nodes are central organs for the induction of adaptive immunity and the major sites of lymphocytes (Gasteiger et al., 2016). Analysis of draining lymph nodes of the thoracic cavity (mLN) presents an opportunity to investigate adaptive immune responses primed by a filarial infection.

In the mLN-experiments conducted within this study (section 3.3.2), very few cells could be gained from naive mLN. After pooling of mLN from several mice to achieve sufficient cell numbers no relevant cytokine production could be measured after stimulation with different stimuli including BuAg. This was to be expected, as in naïve mice neither had APCs been activated and then migrated to the lymph nodes, nor had the residing cells of adaptive immunity been activated. Cells gained from mLN of *L. sigmodontis*-infected mice could be stimulated into producing pro-inflammatory cytokines IL-6, TNF-α and IFN-γ but not IL-10 by BuAg.

Release of IL-6, TNF-α and IFN-γ upon stimulation of cells of *L. sigmodontis*-infected mice with BuAg was seen in both spleen as well as mLN cells. Interestingly, the IL-10 production seen in spleen cells could not be shown in mLN. This suggests that the cells responsible for BuAg-induced IL-10 production in the spleen-cell assays were not present in the mLN-cell assays. However, re-stimulation of mLN of *L. sigmodontis*-infected mice with LsAg induced strong IL-10 production, while, even though this effect was not significant, re-stimulation of mLN of *L. sigmodontis*-infected mice with LsAg lead to decreased levels of Bu-Ag induced IL-6, TNF-α and IFN-γ production. Filarial induced IL-10 production and its hypo-responsive effect during chronic filarial infection is well known (S Mahanty et al., 1996; Specht et al., 2004). This data suggests an IL-10 dependent down-regulation of BuAg-induced immune responses in mLN by filarial antigen. Even though several cell types have the ability to produce IL-10, regulatory T-cells have been shown to be the predominant source during filarial infection (Metenou and Nutman, 2013; Mitre et al., 2008) and thus present the most likely source of IL-10 production in this assay.

4.3.3 Infection with *L. sigmodontis* dampens pro-inflammatory BuAg-induced responses in cells from the pleural cavity

Infection with *L. sigmodontis* occurs through the skin, however the main site of infection is the pleural cavity in which the larvae moult, develop into adult worms and can persist over extended periods of time during hosts that allow chronic infection. Thus, the pleural cavity represents the main site of interaction between the host´s immune system and the filarial infection. Previous studies have shown that infection with *L. sigmodontis* leads to local inflammation with an increase of macrophages, eosinophils, neutrophils and lymphocytes in the pleural cavity (Fercoq et al., 2019; Ritter et al., 2017). These cells have been shown to form granulomas adherent to the worms (Attout et al., 2008).

Experiments conducted within this study compared cytokine profiles of cells gained from the pleural cavity of *L. sigmodontis*-infected and naïve mice upon stimulation with BuAg. Interestingly results showed significantly lower BuAg- (and LPS) induced IL-6 production in Ls-infected compared to naïve mice. Results were not significant for TNF-α. However, they suggest a dampening of pro-inflammatory immune responses by filarial infection. Previous studies have shown that in late stages of filarial infection especially the expansion of $CD4^+$ T-cells with strong TH2 responses as well as expansion of Tregs occurs (Rodrigo et al., 2016; Taylor et al., 2012). Down regulation of pro-inflammatory responses could thus be explained either by a TH2-shift of immune responses, or by down-modulation by Treg cells. Comparing data obtained from cells of the pleural cavity with data from spleens suggests that this effect is strictly local and not systemic. In the last few years, innate lymphoid cells (ILC), and especially the role of ILC2 cells during filarial infection, has moved into the centre of attention (Herbert et al., 2019). ILC2 cells have been shown to be an important source of TH2 responses during helminth infection, and to produce not only IL-5, IL-9 and IL-13 but also IL-6 (Spits et al., 2013; Wilhelm et al., 2011). Furthermore, they have been shown to play an important role in local reaction during *L. sigmodontis* infection, while their contribution to systemic immune responses (in mLN or spleen) was shown to be limited (Boyd et al., 2015). Thus, their role in the above-described effect on BuAg-induced responses should be further investigated as these results can only be considered as being preliminary and no control stimulations with αCD3/CD28 could be done because of the low cell yield obtained from the pleural

cavity of naïve mice. Furthermore, other cytokines such as IFN-γ and IL-10 should be measured as well.

4.3.4 The role of patency of *L. sigmodontis* infected mice on BuAg-induced responses

Patency of the worms has previously been shown to play a role in immune responses. Indeed the presence of microfilaria was shown to result in elevated IL-10 and reduced IFN-γ production (Doetze et al., 2000; Sartono et al., 1997; Semnani et al., 2003).

Also, in experiments conducted within this thesis the above-described effects, including elevated IL-10 and decreased IFN-γ production in spleen cells from infected mice, were strongest comparing cell responses from patently infected (Mf+) mice with naïve, even though also latently infected (Mf-) mice showed the same tendency. These findings indicate that on a systemic level in spleen cells, microfilaria might not be responsible for the above described effects on BuAg induced immune responses but may be able to reinforce it. TNF-α and IL-6 production was shown to be elevated in spleen cells of Mf+ compared to Mf- mice.

Also, analysis of cell-responses of cells from the pleural cavity that were in direct contact with the microfilaria showed higher TNF-α production in cells of Mf+ compared to Mf- mice upon stimulation with LPS and BuAg. IL-6 was only elevated in Mf+ cells upon LPS challenge, the BuAg induced IL-6 production was independent of the Mf status. Indeed these findings are reflected in previous studies, where higher plasma levels IL-6 and TNF-α (as well as IFN-γ and IL-12) upon LPS challenge were shown in the presence of microfilaria as well (Hübner et al., 2008). Why only TNF-α but not IL-6 production upon BuAg stimulation was influenced by the presence of microfilaria remains unclear. However, previous data showed that the presence of microfilaria leads to TNF-α-induced apoptosis in monocyte-derived dendritic cells (Semnani et al., 2008b), possibly explaining these findings.

4.4 Conclusions

The results generated in this work provide the first proof of a possible immune-modulatory effect of filarial infection on co-infection with *M. ulcerans.*

The *in-vitro* model system employed using antigen extracts generated from *M. ulcerans* (BuAg) and *L. sigmodontis (LsAg)* as well as using the murine filarial infection model with *L. sigmodontis* provided conclusive results, reflecting typical immune responses observed in patients infected with Buruli ulcers disease or filarial infection (Fevereiro et al., 2019; Maizels and McSorley, 2016). Thus, the applied model appears to be suitable to study co-infection with these diseases in a regulated setting.

The reported findings also show that *M. ulcerans* activates immune cells in a TLR2-dependent manner. These immune responses are down-modulated by the presence of filarial antigen in spleen cells but not in dendritic cells. Especially TH1 immune responses to *M. ulcerans,* and most importantly IFN-γ production, appear to be down-modulated by a filarial infection. This effect was reinforced but not dependent on patency of the filarial infection. This immune-modulation appeared to be IL-10-independent, while preliminary results in lymph node cells implicate an IL-10-dependent immune-modulation. All-in all the results presented in this thesis suggest a clear impact of filarial infection on *M. ulcerans*-induced IFN-γ production (Fig. 23).

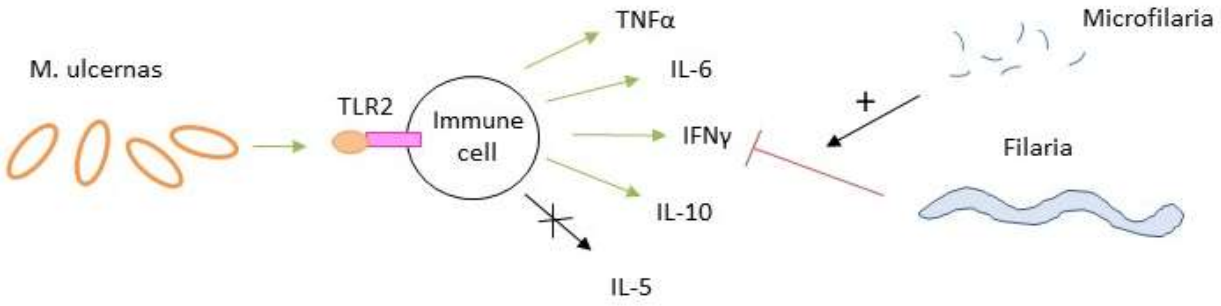

Fig. 23: Filarial infection dampens *M. ulcerans*-induced IFN-γ production.

Mycobacterium ulcerans activates immune cells in a TLR-2 dependent manner leading to the release of various cytokines. Filarial infection and the presence of filarial antigens dampens especially IFN-γ-production. This effect is reinforced by the presence of microfilaria.

The effects of other cytokines vary dependent on the cells stimulated and the filarial stimulant and require further studies. Since strong TH1 responses and especially the

release of IFN-γ were shown to be crucial immune responses in Buruli ulcers disease (Röltgen and Pluschke, 2020; Schipper et al., 2007), these results indicate that infection with filaria might increase susceptibility to Buruli ulcers disease and aggravate the outcome of the disease. Thus, filarial co-infections should be considered for prevention and treatment of Buruli ulcers disease. The results of this thesis present a good basis for further studies of this neglected disease.

5. Summary

Buruli ulcers disease is a neglected tropical disease, caused by *Mycobacterium ulcerans*, that affects skin and sometimes bones and can lead to large, disfiguring skin ulcers and sometimes long-term disability. So far, it remains unclear why some people develop the disease and what factors influence its outcome, but previous studies suggest that a strong TH1-based immune response has a protective effect. Buruli ulcers disease is co-endemic with filarial infections which are often asymptomatic and can persist for many years. Very little is known about the effect of a co-infection with these two infections, but helminths are known to be able to polarize different immune responses.

Therefore, the present thesis aimed at analysing the consequences of such co-infections by studying the effects of filarial antigen on *M. ulcerans*-activated cell populations *ex-vivo*. Within this thesis immune profiles of different immune cells upon stimulation with antigen extract generated from *M. ulcerans* (BuAg) were established and it was shown that the BuAg-induced pro-inflammatory immune response in bone-marrow derived dendritic cells (BMDC) was TLR2-dependent. The murine filarial infection model with *L. sigmodontis* and an antigen extract generated thereof (LsAg) were employed to study the potential effect of filarial infection on infection with *M. ulcerans*. Results from co-stimulation with filarial antigen showed that Bu-Ag induced responses in BMDC were unaltered in the presence of filarial-antigens or live worms while immune responses in spleen cells were dampened. Further work described in this thesis analysed the effect of filarial infection on BuAg induced responses *ex-vivo* and revealed dampened TH1, and most importantly IFN-γ responses, which appeared to be reinforced by but not dependent of patency of the filarial infection. Overall, these data substantiate the hypothesis that filarial infection may influence the development and outcome of Buruli ulcers disease. Further research into these aspects might broaden the understanding of this neglected disease and help with its prevention and treatment.

6. List of Figures

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